Purification and Properties of Spherical Virus Particles Associated with Grapevine Ajinashika Disease

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ABSTRACT

Namba, S., Boscia, D., Yamashita, S., Tsuchizaki, T., and Gonsalves, D. 1991. Purification and properties of spherical virus particles associated with grapevine ajinashika disease. Plant Dis. 75:1249-1253.

Grapevine ajinashika associated virus (GAaV) was successfully purified from ajinashika-affected berry pulps by an enzyme treatment purification procedure. Virions were spherical (approximately 25 nm in diameter), sedimented as a single component of approximately 110S, had a buoyant density in CsCl of approximately 1.38 g/cm^3, and had an estimated nucleic acid content of 30%. GAaV contained a single species of single-stranded RNA of an estimated 6.8 kb and a coat protein of 23,000 Da. GAaV was effectively detected in extracts of shoots and mature berries by immunosorbent electron microscopy (IEM) and enzyme-linked immunosorbent assay (ELISA) using specific antiserum. Biological and physicochemical properties of GAaV were similar to luteoviruses. However, GAaV shared no serological relationships with barley yellow dwarf and potato leafroll viruses. Antisera of grapevine fanleaf virus and grapevine phloem-limited isometric virus (GPLIV) did not react with GAaV in IEM and double-diffusion tests. However, anti-GPLIV antiserum reacted with GAaV in protein A-gold labelling immunosorbent electron microscopy.

In Japan, grapevine ajinashika disease has been one of the most important graft-transmissible diseases of grapevines for the last 20 yr. Ajinashika, a Japanese word, is defined as unpalatable berries of low sugar content. Diseased vines show no visible symptoms and can be recognized only by a marked decline of sugar content of their fruits, which makes them unusable as table grapes or wine (10).

In 1979, we reported on the association of a small, spherical phloem-limited virus with the disease (10). At that time, it was designated as grapevine ajinashika virus. Because the pathogenicity of GAaV according to Koch's postulates has not been determined, we now refer to this virus as grapevine ajinashika associated virus (GAaV).

In this paper, we report on the further purification and properties of the virus. The results indicate that GAaV is a newly recognized virus of grapevine with properties similar to those of the luteoviruses (14). Parts of this work were previously reported (8,9).

Accepted for publication 29 May 1991 (submitted for electronic processing).

MATERIALS AND METHODS

Viruses and plant material. The initial vine that served as the source of GAaV was obtained from a commercial vineyard in Yamanashi prefecture. In 1980, five healthy vines (8- to 10-yr-old *Vitis vinifera* L. 'Koshu') were graft-inoculated by budding shoots from one Koshu vine with typical ajinashika symptoms. Two years later, these inoculated vines showed typical ajinashika symptoms. The berries from these vines were used extensively in our experiments. Also, grapevine phloem-limited isometric virus (GPLIV) was purified from infected LN-33 (Couderc 1613 × Thompson seedless) roots as described by Boulila et al (2). Another disease examined was grapevine fleck, vines of which were supplied by Y. Terai at the Yamanashi Fruit Tree Experiment Station, Yamanashi, Japan, and D. Golino at the USDA-ARS, University of California, Davis. Treminer (*V. vinifera*) and St. George (*V. rupestris* Scheele) affected with grapevine fleck were tested in immunosorbent electron microscopy (IEM) or enzyme-linked immunosorbent assay (ELISA). Tissues of *V. vinifera* 'Campbell Early' affected with grapevine stunt supplied by M. Hatamoto at the Okayama Agricultural Experiment Station, Okayama, Japan, were also tested by ELISA.

Virus purification. Symptomatic mature berries were harvested and were either used fresh or stored frozen at −80 C. Virus purification was accomplished by the Driselase/polyethylene glycol procedure described below. Ber-

ries were homogenized in a Waring blender in two volumes of 0.1 M sodium citrate buffer, pH 6.0, containing 0.01 M EDTA, 1.5% Driselase (Kyowa Hakko Kogyo Ltd., Tokyo, Japan), 0.02% sodium azide, and 0.03% (w/v) cephaloridine. The homogenate was incubated at 28 C for 13 hr on a shaker, macerated again with a glass homogenizer, and then emulsified with 1% (v/v) Triton X-100 and a half-volume of chloroform. After shaking for 30 min, the emulsion was broken by centrifugation at 3,000 *g* for 15 min. Solid polyethylene glycol, MW 6,000, and sodium chloride were added in the aqueous phase to final concentrations of 8% (w/v) and 0.4 M, respectively.

After stirring for 30 min, the precipitate was collected by centrifugation at 16,000 *g* for 15 min, the pellet was resuspended in one-tenth volume of 0.01 M phosphate buffer containing 0.01 M EDTA, pH 7.6, and clarified by centrifugation at 8,800 *g* for 15 min. The resulting fluid was subjected to two cycles of differential centrifugation (97,000 *g* for 1.5 hr, 8,800 *g* for 15 min). The final pellets were resuspended in a small amount of 0.01 M phosphate buffer, pH 7.6, and subjected to a sucrose density gradient centrifugation at 74,000 *g* for 230 min. Virus-containing fractions were collected with an ISCO model 640 density gradient fractionator and UA-5 UV analyzer. The virus particles were then concentrated by centrifugation at 97,000 *g* for 2.0 hr.

Buoyant density of virions in CsCl. The buoyant density of the virus was determined by equilibrium banding in a Hitachi RPS50 rotor. The purified preparation was centrifuged to equilibrium in a CsCl solution with a mean density of 1.40 g/ml in 0.001 M potassium phosphate buffer, pH 7.6, using a Hitachi RPS40 rotor for 30 hr at 85,000 *g* at 20 C. The gradients were scanned and fractionated as described earlier. Each 0.25-ml fraction was collected and read at 25 C in an Atago Abbe 1 refractometer to determine the density of the cesium chloride fraction containing the highest virus concentration. The percentage of nucleic acid was calculated according to the formula developed by Gibbs et al (5).

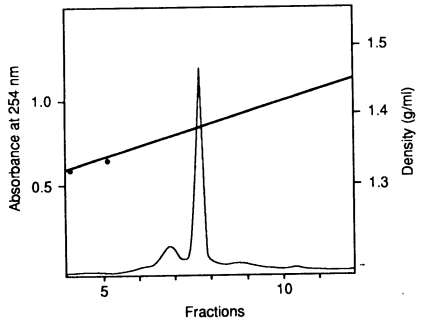

Fig. 1. Distribution curve of GAaV after centrifugation in CsCl for 30 hr at 36,000 rpm in a Hitachi RPS40 rotor. The straight line indicates the relative densities of the CsCl gradient. Calculated density of GAaV was 1.38 g/cm^3.

Analytical ultracentrifugation. Purified preparations were examined in a Hitachi Model 282 analytical ultracentrifuge equipped with an RA72TC rotor and absorption scanning recorder. Sedimentation coefficients were determined by a Hitachi ultracentrifuge processor.

Electron microscopy. Purified virus was placed on an electron microscope grid and stained with an equal volume of 2% PTA (phosphotungstic acid), adjusted to pH 7.0 with KOH, and examined in a Hitachi H-800 electron microscope.

Nucleic acid. Nucleic acid was extracted from virions by heating for 5 min at 60 C in 1% SDS, 0.5% mercaptoethanol, 0.05 M $NaHPO_4$, 0.01 M EDTA, and 10% sucrose, at pH 7.4. It was then precipitated with ethanol and suspended in the desired amount of disruption buffer. Alternatively, virions were disrupted by heating for 5 min at 60 C in 200 µl of 2× SSC containing 1% sodium dodecyl sulfate (SDS) and 0.5% mercaptoethanol. The SDS was precipitated by adding KCl to 0.05 M and incubating for 4 hr at 2 C. After centrifugation to eliminate precipitated SDS, 0.02 µg of Type IA RNase (0.1 µg/ml) or 2 µg of DN-25 DNase I was added to the supernatant. Sufficient $MgCl_2$ to make 0.04 M was added before the DNase. Samples were incubated for 1 hr at 25 C after the addition of an enzyme. Nucleic acid was precipitated with two volumes of 95% ethanol and bentonite (1.5 mg/ml) and then resuspended in 100 µl of 1% SDS in 0.05 M phosphate buffer, pH 7.4, with 0.5% mercaptoethanol and 1 mM EDTA. Preparations were heated for 5 min at 60 C and analyzed by gel electrophoresis.

Polyacrylamide gel electrophoresis was carried out in 2.5% gels using buffer containing 0.02 M Tris, 0.02 M NaH_2O_4, and 0.001 M EDTA, pH 7.6. Diethylpyro carbonate (0.02% final concentration) was added to the buffer immediately before it was placed in the apparatus for pre-electrophoresis. Buffer was not changed between pre-electrophoresis and sample electrophoresis. Tobacco mosaic virus RNA (6.0 kb), and *Escherichia coli* (Migula) Castellani and Chalmers 16S and 23S RNAs (1.6 and 3.2 kb, respectively) were used as size standards. Gels were usually visualized by silver stain.

Coat protein. The molecular weight of the virus coat protein was determined by SDS-polyacrylamide gel electrophoresis. Purified virus preparation and protein standards were boiled for 2 min in a equal volume of dissociation buffer containing 40% glycerol, 2% SDS, 2% 2-mercaptoethanol, and 0.28 M Tris-HCl buffer, pH 6.8. The protein molecular weight markers were lactalbumin (14,200), trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), egg albumin (45,000), and bovine serum albumin (66,000). The markers and the dissociated virus were applied to a 12% gel and electrophoresed at 30 mA per gel. Gels were visualized by silver stain.

Antiserum production. Purified virus preparations (approximately 0.5 mg in total) of GAaV were injected subcutaneously at multiple sites on the legs of a New Zealand white rabbit at weekly intervals for three consecutive weeks. The first immunization consisted of 1 ml of purified virus preparation mixed with 1 ml of Freund's complete adjuvant. The two subsequent injections were with 1 ml of purified virus preparations mixed with 1 ml of Freund's incomplete adjuvant. The rabbit was bled 2 wk after the third injection. Anti-GAaV antiserum was preabsorbed with healthy antigens prepared from virus-free Koshu grapevine seedlings. Immunoglobulin was purified from the antiserum with a DEAE cellulose column (4).

Immunosorbent electron microscopy. Antisera to GAaV and that of GPLIV produced in Italy (2) were used. Diluted antisera (1:100) in 0.1 M phosphate buffer (PB), pH 7.0, were incubated on grids for 1 hr. After washing with PB, drops of virus suspensions were incubated for 15 min on grids and then washed with PB. The trapped viruses were incubated with more antiserum diluted 1:1,000 for 15 min, washed with PB, then water, and finally stained with a solution of 2% uranyl acetate. Grids were examined in a Hitachi H-800 electron microscope.

In protein A-gold labelling IEM assays, purified virus preparations were directly absorbed to the grids. The immobilized virions were rinsed with 0.1 M sodium acetate solution (pH 5.0) containing 1% (w/v) bovine serum albumin (BSA), decorated with antiserum (1:1,000 for GAaV antiserum, 1:10 for GPLIV antiserum) in PBS for 10 min, rinsed with sodium acetate solution, labelled with the protein A-gold complexes particle suspension (diluted to $A_{520} = 0.5$ with sodium acetate solution) for 10 min, rinsed, and stained with 2% uranyl acetate solution.

Double-diffusion tests. Micro-agar gel double-diffusion tests were carried out in a thin layer of agar on small glass plates (70 × 90 × 1.4 mm). A film of agar, approximately 1 mm thickness, was poured over the glass plates. The agar gel consisted of 1% agar and 0.95% NaCl prepared in 0.1 M potassium phosphate buffer, pH 7.0, and 0.05–0.1% sodium azide. In addition to homologous reactants of GAaV, we compared antisera of barley yellow dwarf virus

(BYDV), GPLIV (prepared in the Dipartimento di Patologia vegetale, Università degli Studi, Italy), and potato leafroll virus (PLRV) with purified samples of BYDV (prepared in the Laboratory of Plant Pathology, University of Tokyo) and GPLIV. Antiserum to grapevine fanleaf virus (GFV) was supplied by T. Nishio, Yokohama Plant Protection Station, Japan.

ELISA. Grapevine tissues were screened by DAS-ELISA (double antibody sandwich direct enzyme-linked immunosorbent assay) as described by Clark and Adams (4). Plates were coated with immunoglobulin at 2.5 μg/ml and conjugated alkaline phosphatase was used at a 1:400 dilution. Tissue extracts included those of leaf veins, petioles, shoots, bark, young fruit, and matured fruit cores. To compare GAaV and GPLIV, DAS-ELISA was done by coating plates with crude GAaV-AS (diluted 1:250, 1:500, 1:1,000, and 1:2,000) or GPLIV-IgG at 4, 2, 1, and 0.5 μg/ml, plates were rinsed and loaded with extracts of GPLIV. After rinsing, the plates were incubated with GPLIV-IgG conjugate (1:250, 1:500, 1:1,000, and 1:2,000 dilutions) for both plates.

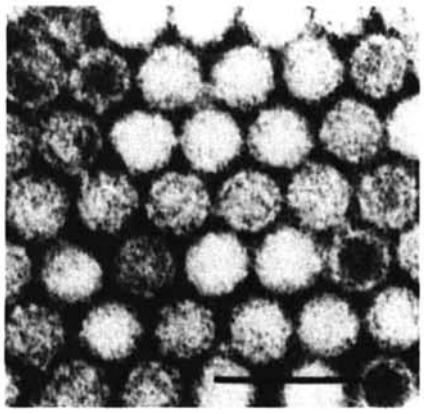

Fig. 2. Electron micrograph of purified GAaV particles negatively stained with 2% PTA. Bar = 50 nm.

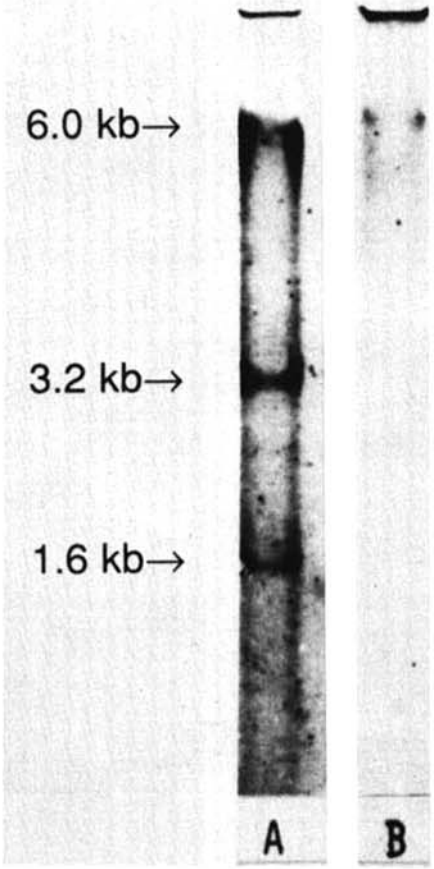

Fig. 3. Polyacrylamide gel electrophoretic analyses of RNA preparations. **(A)** 23S RNA (3.2 kb) and 16S RNA (1.6 kb) of *Escherichia coli* ribosome and RNA isolated from purified tobacco mosaic virus (6.0 kb); **(B)** RNA (6.8 kb) isolated from purified GAaV.

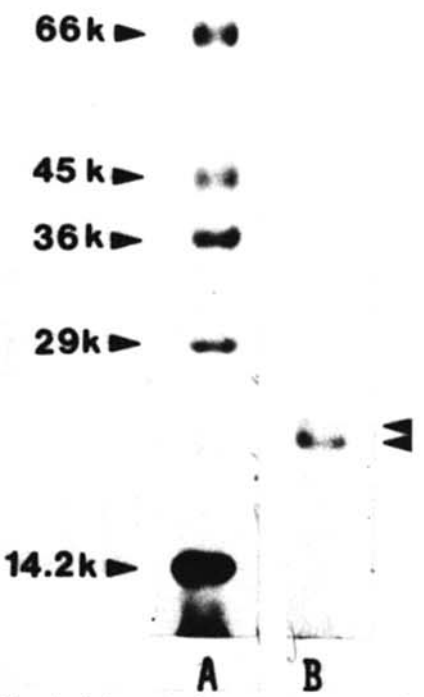

Fig. 4. Polyacrylamide gel electrophoretic analyses of protein preparations. **(A)** Marker proteins; **(B)** dissociated GAaV coat protein. Major band = 23,000; minor band = 24,000.

RESULTS

Virus purification. Initially, several purification schemes were tested. Extraction buffers made up of phosphate, Tris, borate, or citrate were used at different pHs (6.0, 6.5, 7.0, 7.5, and 8.0) and ionic strengths (0.05, 0.1, 0.2, 0.5, and 1.0 M). These buffers were tried with several additives, i.e., EDTA, Triton X-100, DIECA, thioglycolic acid, thioglycerol, nicotinic acid, or enzymes with antibiotic. Also, various maceration apparati, including French press, mortar and pestle, glass homogenizer and cooking blender, and centrifugation methods, including sucrose cushion and sucrose-CsCl step gradients were tested. Resuspension buffers of low ionic strength (0.001, 0.005, 0.01, and 0.05 M) containing additional chemicals as above were systematically compared. The enzyme extraction procedure (as described in Materials and Methods) produced the highest and cleanest yield of virus particles. Nevertheless, the yield was very low. Other extraction procedures gave negligible amounts of purified virus.

Physical properties of the virus. Isopycnic centrifugation in CsCl yielded a highly purified monodispersed virus preparation with a buoyant density of 1.38 g/cm^3 (Fig. 1). Virus preparations

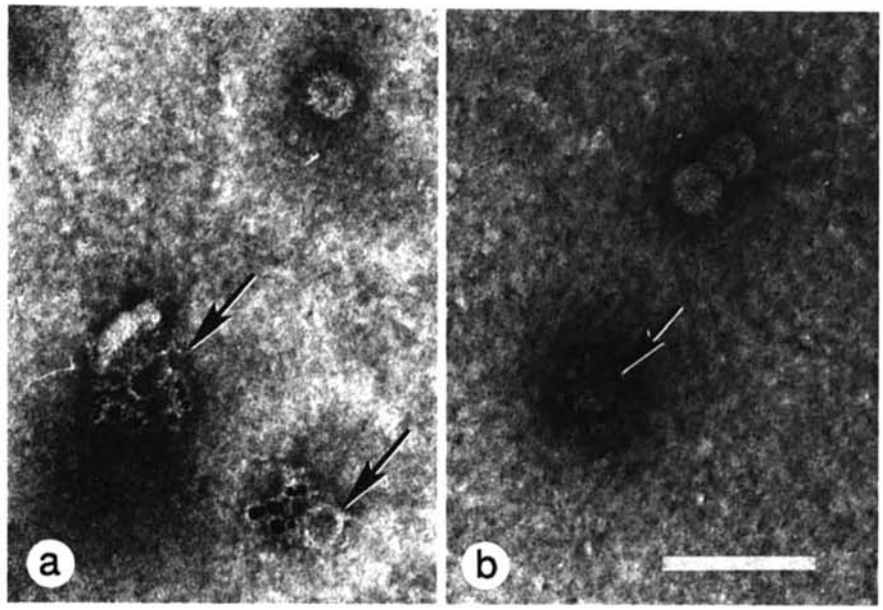

Fig. 5. Electron micrographs of GAaV and cucumber mosaic virus (control) particles labelled by protein A-gold particles and negatively stained with 2% uranyl acetate solution. Bar represents 100 nm. The immobilized virions (with arrows) were decorated with **(A)** GAaV antiserum and **(B)** with GPLIV antiserum. The unlabelled virions were cucumber mosaic virus particles.

sedimented as a single component of approximately 110S with a $A_{260/280}$ ratio of 1.76. Virions were isometric with a diameter of 25 nm (Fig. 2).

Nondenatured nucleic acid of the virus gave a single band of 6.8 kb when electrophoresed on 2.5% polyacrylamide gels (Fig. 3). Nucleic acid of GAaV was susceptible to digestion by RNase but not DNase.

Electrophoresis of the virus coat protein in SDS-polyacrylamide gels revealed a major band and a minor band (Fig. 4) of approximately 23,000 and 24,000 Da, respectively.

Serology. Antiserum to GAaV had titers of 1/1,024 when tested against the purified virus as the antigen in double-diffusion tests in agarose gel. In tests with the partially purified virus preparation from 125 g of affected berries, the virus antiserum (diluted 1/16) reacted with preparations diluted up to 1/4.

In IEM, large numbers of virus particles were trapped on grids, and all of them were decorated with homologous antibodies. In IEM and double-diffusion tests, serological relationships were not detected between GAaV and other viruses when GAaV and BYDV were used as antigens against antisera to GAaV, GFV, BYDV, and PLRV. With GPLIV antiserum and GAaV, the virus particles were positively labelled with gold (Fig. 5). However, GPLIV antiserum did not react with GAaV in double diffusion tests (Fig. 6).

In ELISA tests, purified GAaV was detected to a concentration of 5 ng/ml, and the absorbance values showed a linear relationship to virus concentration. When extracts of leaf veins, petioles, current shoots, bark, and young berry and matured berry pulps from the cultivar Koshu were ELISA tested at different times of the growing season, matured berry pulps, current shoots, and stems were the most reliable for diagnosis (Fig. 7). GAaV could be detected in extracts of mature berry pulps diluted 10^{-2} to 10^{-3}. Leaf veins, petioles, and young berries were not suitable for detection (*data not shown*). GAaV was also detected in rootstock clones, Teleki 8B and Teleki 5BB, which have been used in Japan for a long time. Campbell Early infected with GSV and St. George infected with fleck were tested by ELISA. There were no clear differences in the absorbance values between these samples and the healthy ones. In DAS-ELISA with GAaV-IgG coated plates and GPLIV-IgG conjugate as the probe, extracts of GPLIV-infected tissues were negative. The reciprocal control using homologous GPLIV reactants were positive (*data not shown*).

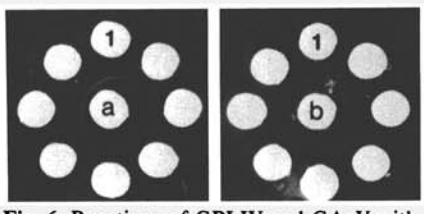

Fig. 6. Reactions of GPLIV and GAaV with antiserum to GPLIV in agar gel double-diffusion tests. Antigens of **(A)** GPLIV and **(B)** GAaV are in the center wells and GPLIV-antiserum diluted 1:1 (1), 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 counterclockwise are in the outer wells.

DISCUSSION

In this work, we have characterized in more detail the isometric virus that we previously found associated with ajinashika-affected grapevines (10). Because the disease has not yet been reproduced by inoculations with purified virus preparations, we have elected to call this virus grapevine ajinashika associated virus. Our data does not exclude the possibility that ajinashika disease may be caused by another agent(s). In fact, many grapevines that tested positive for GAaV were also affected with grapevine leafroll.

Terai et al (12) reproduced ajinashika symptoms on Koshu vine by bud inoculation with leafroll-affected Koshu and fleck-affected Kyoho. These vines were not available for our use. However, extracts of fleck-affected tissues were negative by ELISA using GAaV antisera. More recently, we tried the trap-decoration tests with grapevine fleck-affected Treminer. Here, we found a small number of spherical viruslike particles trapped and decorated with anti-GAaV antiserum (S. Namba et al, *unpublished*), suggesting a relationship between fleck and GAaV. Grapevine leafroll is widespread in Japan, and critical experiments involving leafroll vines free of GAaV and fleck and only fleck-affected vines for graft inoculations must be done to determine the possible role(s) of leafroll (15) and fleck with ajinashika disease. The availability of antisera to leafroll and now to GAaV should provide invaluable assistance in future investigations.

GAaV was purified from berries by enzyme treatments. This purification procedure is also effective for other fruit tree viruses that do not have available herbaceous host plants (S. Namba et al, *unpublished*). Tsuchizaki et al (13) reported that pericarps of citrus fruits from which the flavedo has been removed are useful for purification of citrus tristeza virus. Boulila et al (2) reported that GPLIV can be purified by enzyme treatment from roots or the main veins and petioles of affected vines. Our report is the first to use both fruit and the enzyme treatment for the purification of a phloem-limited virus.

Biological and physicochemical properties of GAaV are similar to that of luteoviruses, although there were no serological relationships with BYDV and PLRV. Of the spherical viruses associated with grapevines, GAaV has most similarities to grapevine stunt virus (GSV) (7,8,11) and GPLIV (2) (Table 1), although there are some differences. GSV is phloem-limited but causes different symptoms, i.e., stunting of the vine and no decline in sugar content of the berries (6). Interestingly, GSV is

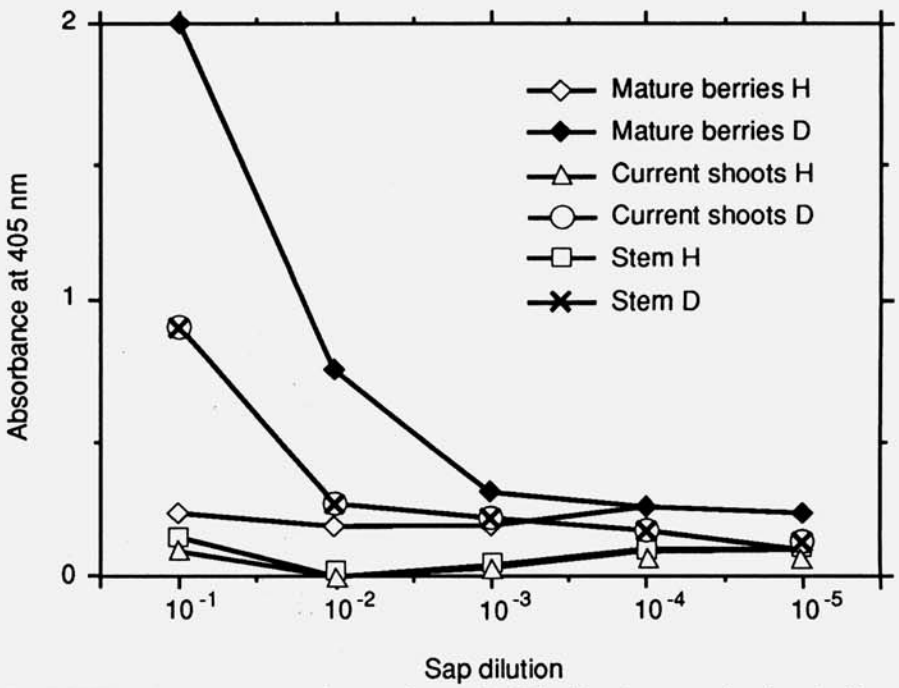

Fig. 7. ELISA absorbance values for detection of GAaV in diluted extracts of various healthy (H) and ajinashika-affected (D) grapevine crude tissue extracts. Precoated IgG was used at 2.5 μg/ml, and alkaline phosphatase conjugated immunoglobulin were used at a 1:400 dilution. Mature berries from ajinashika affected vines gave highest ELISA reaction followed by (equally) diseased stems and current shoots.

Table 1. Comparative properties of grapevine ajinashika associated virus (GAaV), grapevine phloem-limited isometric virus (GPLIV), and grapevine stunt virus (GSV)

Property	Virus		
	GAaV	GPLIV	GSV
Symptoms	Unpalatable fruits	Leaf fleck	Shoot stunt
Vector	...	...	Leafhopper
Particle size (nm)	25	30	25
RNA (%)	30	35	...
$S_{20,w}$	110	...	...
Buoyant density (CsCl)	1.38	1.45	...
MW coat protein	23,000	28,000	...
RNA size (kb)	6.8	7.4	...

transmitted by the grapevine leafhopper, *Arboridia apicalis* Nawa (6).

GPLIV and GAaV have similar physicochemical properties (8) and both are phloem-limited. We also showed that GPLIV-AS reacted with GAV in a protein-gold labelling IEM (Fig. 5), but no reactions were observed in reciprocal tests. However, there are a number of differences between these viruses. GAaV was effectively purified from berries, whereas GPLIV was purified in large quantities from young, fast-growing roots or, with less efficiency, from main veins and petioles. Leaf veins and petioles are good tissues for detection of GPLIV (2) but are not very suitable for that of GAaV (Fig. 7). Also, GPLIV is not associated with grapevines that have low sugar content nor unpalatable berries (2); however, it is closely associated with fleck on St. George (1). The association of GAaV with fleck is not clear.

Cytological studies also suggested differences between these two viruses. In tissue infected with GAaV, there were typical vesicular bodies with single or double membranes approximately 70–90 nm in diameter which contained nucleic acid-like fibrous materials originating from mitochondria (8–10). In tissue infected with GPLIV, vesicular bodies bound by a double membrane measuring approximately 80–100 nm in diameter were found to originate from chloroplasts, making it similar to the tymovirus group (3) (G. P. Martelli, *personal communication*).

In conclusion, critical information on the specific etiological agents are not available for many grapevine virus diseases, including ajinashika and fleck diseases. A major obstacle has been the lack of susceptible herbaceous hosts. In this work, we have purified, characterized, and produced antisera to virus particles isolated from grapevine affected with ajinashika disease. The information and reagents we developed will help to advance our understanding of this important disease.

ACKNOWLEDGMENTS

We thank T. Nishio, Yokohama Plant Protection Station, Japan, for technical assistance and discussion, and V. Butler, USDA-ARS, University of California, Davis, for her assistance. This study was supported in part by grant 63560040 from the Ministry of Education, Science and Culture of Japan.

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