

Grapevine Leafroll-Associated Virus 2 (GLRaV-2) - Mechanical Transmission, Purification, Production and Properties of Antisera, Detection by ELISA

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Submitted for publication: February 1996

Accepted for publication: July 1996

Key words: GLRaV-2, mechanical transmission, antisera, ELISA

Grapevine leafroll-associated virus 2 (GLRaV-2) was transmitted from *Vitis vinifera* cv. Muscat d'Alexandrie to *Nicotiana benthamiana* following inoculation with grapevine leaf petioles extract concentrated by ultracentrifugation. Virus-infected *N. benthamiana* showed symptoms of chlorotic local lesions, systemic vein clearing followed by yellowing, stem necrosis and death of the plant. The plants were shown to be infected only with GLRaV-2. Antisera produced to GLRaV-2 strongly decorated particles of the virus in immunoelectron microscopy (IEM) and clearly and specifically detected GLRaV-2 in concentrated extracts of infected grapevines in Western Blots. The antisera were used with success for the specific and sensitive detection of GLRaV-2 by ELISA. Treatment of purified GLRaV-2 preparations with glutaraldehyde before immunisation markedly improved the quality of the antisera.

Grapevine leafroll (GLR) is one of the most important diseases of grapevine but its etiology is still uncertain (Bovey & Martelli, 1992). Six closteroviruses (grapevine leafroll-associated viruses, GLRaV-1 to -6) were found to be associated with this disease (Gugerli *et al.*, 1984; Hu *et al.*, 1990; Zimmermann *et al.*, 1990; Gugerli & Ramel, 1993; Boscia *et al.*, 1995).

Leafroll-affected grapevines with mixed infections of GLRaV are common (Hu *et al.*, 1990; Zimmermann *et al.*, 1990). This, along with limited availability of specific antisera, low concentration of the viruses extracted from infected grapevine plants and the lack of herbaceous species for propagation of GLRaV, considerably hamper studies of these viruses.

In this study we report the successful mechanical transmission of GLRaV-2 from infected grapevine to *Nicotiana benthamiana*. High-quality antisera to GLRaV-2 were produced which allowed specific and sensitive detection of the virus by IEM, Western Blot and ELISA techniques.

MATERIALS AND METHODS

Source of viruses: Virus-infected *Vitis vinifera* cultivars primarily used in this study are as follows. Muscat d'Alexandrie (P113): obtained from Plant Quarantine Station, Stellenbosch, South Africa. This grapevine was indexed positive for leafroll, corky bark and fleck and negative for stem grooving diseases. Canes of this grapevine were rooted in pots and after four months petioles of developed leaves were collected and used directly or after storing at -75°C for extraction of viruses. Chasselas clone 8/22 (Gugerli & Ramel, 1993) was obtained from Dr P. Gugerli (Federal Agricultural Research Station of Changins, Nyon, Switzerland) whilst Pinot noir was obtained from Dr H. Kassemeyer (Staatliches Weinbauinstitut, Freiburg, Germany). Cultivars Chasselas

and Pinot noir, described above, were maintained on their own roots to prevent contamination by grafting. Local cultivars namely Tinta Barocca (5/2/7, Fisantekraal, Durbanville) and Barlinka (24/7/3, Hex River Valley) were from our collection. Cultivars Shiraz (Groenhof, SFW, Stellenbosch) and Black Spanish (VOPI, Roodeplaat, Pretoria) (Pietersen & Kasdorf, 1993) were field collected. Other virus-infected grapevine cultivars included were as follows: Black Seedless, Barlinka and Ohanex (Plant Quarantine Station, Stellenbosch, South Africa) and hybrid LN33 (our collection). *Nicotiana benthamiana* plants infected with grapevine virus A (GVA) (Engelbrecht & Kasdorf, 1990) or grapevine virus B (GVB) (D.E. Goszczynski 1995, unpublished) were also used.

GLRaV-2-free grapevines: Cultivars Shiraz (I327), Merlot (I460), Mission (I822) and Gamay (FVC) were from Plant Quarantine Station, Stellenbosch, South Africa; Tinta Barocca (P129), Cabernet franc (P10), Pinot noir (I1030) and Cabernet Sauvignon (P136) were from KWV, Stellenbosch, South Africa; hybrid LN33 was from SFW, Stellenbosch, South Africa.

Mechanical transmission: Viruses for mechanical transmission were prepared as follows: 4 g of petioles freshly collected from grapevine plants were pulverised in liquid N₂ using a pestle and mortar and mixed with 40 mL of 0.1 M Tris-HCl buffer pH 7.6, containing 0.01 M MgSO₄, 0.2% 2-mercaptoethanol, 2% Triton X-100, 0.5% bentonite and 4% polyvinylpyrrolidone (PVP-P). After a low-speed centrifugation at 6000 g for 5 min the supernatant was centrifuged through a 20% sucrose cushion (in 0.1 M Tris-HCl buffer pH 7.6 with 0.01 M MgSO₄) at 26000 r.p.m. (rotor TY30, Beckman) for 2 h 15 min at 8°C. The pellet was resuspended in 0.7 mL of buffer containing 0.01M K₂HPO₄, 0.01 M cysteine-HCl and 3% nicotine (inoculation buffer) (Boscia *et al.*, 1993), mixed

* Paper presented at the first International SASEV Congress, 1995.

with celite and used for inoculation of *N. benthamiana*. Infected *N. benthamiana* used for purification of GLRaV-2 was prepared by inoculation of plants with extracts of systemically infected *N. benthamiana* using inoculation buffer.

Purification of viruses: GLRaV-2 was purified as follows: 80 g of virus-infected *N. benthamiana* plants with clear systemic symptoms were macerated using a pestle and mortar in 400 mL of 0.1 M Tris-HCl buffer pH 7.6 containing 0.01M MgSO_4 , 0.2% 2-mercaptoethanol and 5% Triton X-100 (extraction buffer). This was expressed through four layers of cheesecloth and the extract thoroughly shaken with 1/5 volume of chloroform and centrifuged at 10000 g for 15 min. The aqueous phase was collected and PEG 6000 and NaCl were added at concentrations of 8% and 0.2M, respectively. The mixture was stirred on ice for 2 h. Centrifugation was done at 12000 g for 25 min and the pellet diluted in resuspension buffer (extraction buffer without 2-mercaptoethanol) and resuspended with slow mixing overnight at 4°C. The preparation was centrifuged at 6000 g for 5 min, followed by ultracentrifugation at 26000 r.p.m. (rotor TY30, Beckman) for 2 h and 25 min at 8°C over a 20% sucrose cushion (in dialysis buffer = resuspension buffer without Triton X-100). The resulting pellet was diluted in 40 mL resuspension buffer and shaken with a marble at 4°C overnight. After centrifugation at 6000 g for 5 min (repeated twice) the supernatant was loaded on a Cs_2SO_4 gradient (prepared from 1 mL of each 15 and 25% and 2 mL of 35% Cs_4SO_4 in dialysis buffer) and centrifuged at 28000 r.p.m. for 3 h and 45 min at 8°C (rotor SW41, Beckman). Two clearly visible bands of the virus were collected and dialysed with the dialysis buffer. The preparations were then centrifuged at 12000 g for 10 min. Supernatants were stored at -75°C.

GVA and GVB were purified from systemically infected *N. benthamiana* plants as described above for GLRaV-2, except that 2% Triton X-100 was used in the extraction buffer. Partial purification of GLRaV from grapevine cane phloem was as described by Hu *et al.* (1990).

Treatment of GLRaV-2 with glutaraldehyde: Preparations of purified GLRaV-2 were treated with 0.2% glutaraldehyde as described by Francki & Habili (1972), except that 0.1 M Tris-HCl buffer pH 7.6 with 0.01 M MgSO_4 was used.

Antisera production: Rabbits were immunised by four intramuscular injections, 2 weeks apart, with 0.5 mL preparations of purified GLRaV-2 mixed 1:1 with complete (first injection) and incomplete (all subsequent injections) Freund's adjuvant. Blood was collected 2 weeks after the last injection.

Mice (outbred, Swiss white) were immunised as described for rabbits. Preparations of 0.1 mL purified virus mixed with adjuvant were subcutaneously injected at three-

week intervals. Blood was collected from the inferior vena cava (Campbell, 1984). Antisera was mixed with glycerol (1:1) and stored at 4°C.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE (Laemmli 1970) was done in 4.5% stacking and 12.5% resolving polyacrylamide gels at a constant voltage of 180 V for 50 - 60 min using a Dual-mini vertical unit (American Bionetics, Inc.). A prestained SDS-PAGE standards (Bio Rad, low range) was used for molecular weight determination.

Western Blot and immunoelectron microscopy (IEM): The methods used were as described by Goszczynski *et al.* (1995). A mini electroblotting unit (Biometra) was used. Antisera specific to the following viruses were used for the Western Blot and IEM experiments: GLRaV-1 and -3 (Goszczynski *et al.*, 1995) produced locally; GLRaV-4 (Hu *et al.*, 1990) supplied by Dr D. Gonsalves, Department of Plant Pathology, New York State Agricultural, Experimental Station, Geneva, NY, USA; GLRaV-5 (Zimmermann *et al.*, 1990) supplied by Dr B. Walter, Laboratoire de pathologie vegetale, INRA, Colmar-Cedex, France, and viruses of grapevine cv. Black Spanish (Pietersen & Kasdorf, 1993), GVA (Engelbrecht & Kasdorf, 1990), GVB and viruses of cv. Chasselas 8/22 (D.E. Goszczynski 1995, unpublished) produced locally. A monoclonal antibody to GLRaV-2 (Gugerli & Ramel, 1993) supplied by Dr P. Gugerli, Federal Agricultural Research Station of Changins, Nyon, Switzerland, was also used in IEM studies.

Direct double antibody sandwich (DAS) and indirect antibody-trapped antigen (ATA) ELISA: DAS-ELISA was performed as described by Clark & Adams (1977).

ATA-ELISA was done as described by Torrance (1992). Plates were coated with 1 µg/mL of immunoglobulins purified from rabbit antiserum to GLRaV-2. Mouse antisera, monoclonal antibody to GLRaV-2 and goat anti-mouse alkaline phosphatase conjugate (GAM-AP; Sigma) were used at dilutions of 1×10^{-4} , 1×10^{-3} and 2×10^{-3} , respectively. Absorbance values were measured at 405 nm (A_{405}) with a Titertek Multiscan photometer (Flow Laboratories).

Preparation of plant extracts for IEM and ELISA: Viruses for IEM examination were prepared from grapevine petioles as for mechanical transmission, except that the high-speed centrifugation pellets were diluted in 0.1 M Tris-HCl buffer pH 7.6 with 0.01 M MgSO_4 .

For ELISA, grapevine cane phloem was pulverised in liquid N_2 , then macerated with buffer (5 mL per 1 g of tissue) in a mortar, expressed through cheese-cloth and centrifuged at 3000 g for about 20 sec. The resulting supernatant (100 µL) was used in ELISA. Systemically infected leaves of *N. benthamiana* were ground in a mortar with extraction conjugate buffer (Clark & Adams, 1977) (5 mL/1g) and used directly in ELISA.

Cytopathology: For ultramicrotomy, tissues were cut from leaves of *N. benthamiana* plants systemically infected with GLRaV-2 showing vein-clearing symptoms. Pieces were double fixed in 2,5% glutaraldehyde and 1% osmium tetroxide, *en block* stained with uranyl acetate, dehydrated in a graded acetone series and embedded in Epon-Araldite resin (Hayat, 1972). Thin sections were stained with uranyl acetate and lead citrate and examined using a Joel JEM-100C electron microscope.

RESULTS

IEM examination of the virus preparation concentrated from petioles of grapevine cv. Muscat d'Alexandrie showed that the grapevine was infected with GLRaV-2 and GLRaV-3. *N. benthamiana* plants inoculated with viruses from this grapevine developed chlorotic local lesions and systemic vein clearing (Fig. 1A) in approximately 15 and 22 days respectively. Within 30 days a general yellowing of the leaves, necrosis of the stem from the base of the plant upwards, wilting and then death of infected plants were observed. In further mechanical transmission of the virus from *N. benthamiana* plants, local and systemic symptoms appeared in about 8 and 15 days respectively.

Leaf dips from systemically infected *N. benthamiana* showed a high concentration of closterovirus-like particles (Fig. 1B). Ultrathin sections of infected tissue showed many cells containing clusters of membraneous vesicles (Fig. 1C).

In IEM virus particles extracted from infected *N. benthamiana* plants were decorated only by the monoclonal antibody to GLRaV-2 among the GLRaV antisera tested. This confirmed that the mechanically transmissible virus was an isolate of GLRaV-2. The absence of undecorated particles suggested that the *N. benthamiana* plants were infected singly with GLRaV-2.

Virus purification: The virus appeared stable during treatment with 20% chloroform and 5% Triton X-100, which aided in its purification (Fig. 1D). Preparations of purified GLRaV-2 had an $A_{260} : A_{280}$ ratio of 1.48.

Purified virus preparations analysed by SDS-PAGE showed a single band of virus capsid protein (Fig. 2). This confirmed the IEM studies that the *N. benthamiana* plants had a single virus infection. The molecular weight of the capsid protein of the isolate of GLRaV-2 was estimated to be 25.4 kD.

Rabbit antiserum to GLRaV-2, detection of the virus by Western Blot, IEM and ELISA: Rabbit antiserum prepared to the isolate of GLRaV-2 clearly reacted with the 25.4 kD capsid protein of the virus in a Western Blot (Fig. 3, lane b). Molecular weights of capsid proteins of GLRaV-1, GLRaV-3, GVA and GVB determined in the same electrophoresis conditions were approximately 43.6, 50.6, 27.5 and 27.0 kD, respectively (Fig. 3 lanes a,c-e).

The antiserum strongly decorated GLRaV-2 particles in IEM (not shown).

SDS-PAGE and Western Blot analysis of extracts from systemically GLRaV-2-infected leaves of *N. benthamiana* showed that, besides the 25.4 kD virus capsid protein, the antiserum also clearly reacted with other antigens, especially those with a molecular weight of 43 - 57 kD (Fig. 4, lane a). The antiserum reacted strongly with an antigen of 56.2 kD in extracts from virus-free *N. benthamiana* (Fig. 4, lane b).

We could not detect GLRaV-2 in sap extracted from petioles of virus-infected grapevine cv. Muscat d'Alexandrie using Western Blot (Fig. 4, lane c). The virus was detected only in preparations concentrated by ultracentrifugation (Fig. 4, lane d). An additional ten-fold concentration of these preparations by freeze-drying markedly improved the detection of the virus (Fig. 4, lane e). Although multiple bands were observed in SDS-PAGE with preparations concentrated by freeze-drying, only the virus capsid protein band was clearly detected in Western Blots in reaction with antiserum to GLRaV-2. An additional, faint 56.2 kD band was also observed. This band was probably fraction I protein of the grapevine, which is serologically related to the fraction I protein of *N. benthamiana* (Van Regenmortel, 1982), but occurred in a much lower concentration.

To test the reaction of the antiserum with different GLRaV-2 isolates, the viruses were partially purified from grapevines cvs. Shiraz, Tinta Barocca and Chasselas clone 8/22 infected with GLRaV-2 and analysed by using Western Blotting. Strong reaction of antiserum with bands of the same electrophoretic mobility as the homologous virus from cv. Muscat d'Alexandrie (Fig. 5, lane a-c and f) was observed. No reaction was observed with extracts from virus-free grapevines cvs. Shiraz and Tinta Barocca (Fig. 5, lanes d,e).

Immunoglobulins from the antiserum to GLRaV-2 used in standard DAS-ELISA (Clark & Adams, 1977) showed a high reactivity with sap from virus-free *N. benthamiana* (Table 1). The immunoglobulins could therefore not be used for detection of the virus in this host. The same DAS-ELISA, however, could clearly differentiate GLRaV-2-infected and virus-free grapevines (Table 1).

Influence of extraction buffers on detection of GLRaV-2 in grapevines by ELISA: Of the different buffers used for extraction of sap from cane phloem of grapevines, 0.1 M Tris-HCl buffer (pH 7.6) containing 0.01M $MgSO_4$, 0,2% 2- mercaptoethanol, 2% Triton X-100, 0,5% bentonite and 4% PVP-P gave the best results in an ELISA (Table 2). The ratio of absorbance values for infected to virus-free cv. Shiraz increased from 6.9 for the standard ELISA extraction buffer (Clark & Adams, 1977) to 15.4 for the extraction buffer described above. This buffer was used for extraction of viruses for all further ELISA tests.

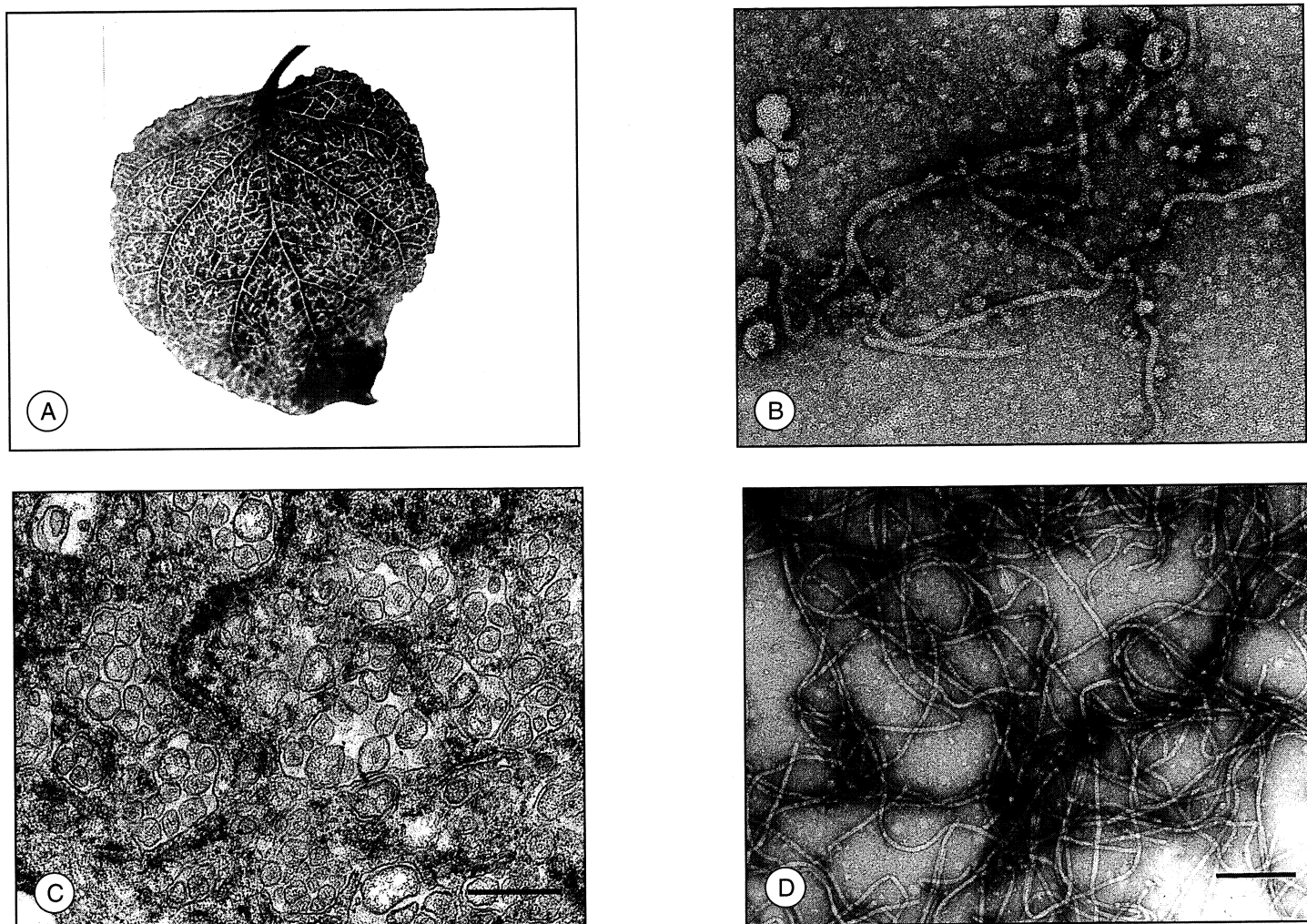


FIGURE 1

A = GLRaV-2-infected leaves of *Nicotiana benthamiana* with vein clearing. B = Leaf dip from infected plant. Bar = 100 nm C = Section of infected leaf cell showing clusters of membrane vesicles. Bar = 300 nm. D = Purified virus particles. Bar = 165 nm.

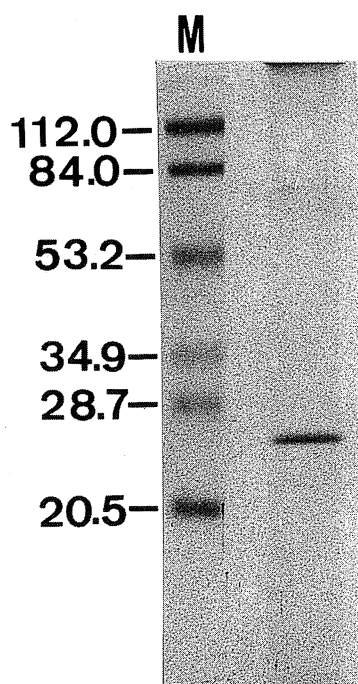


FIGURE 2

SDS-PAGE analysis of purified virus preparation. M = prestained SDS-PAGE standards.

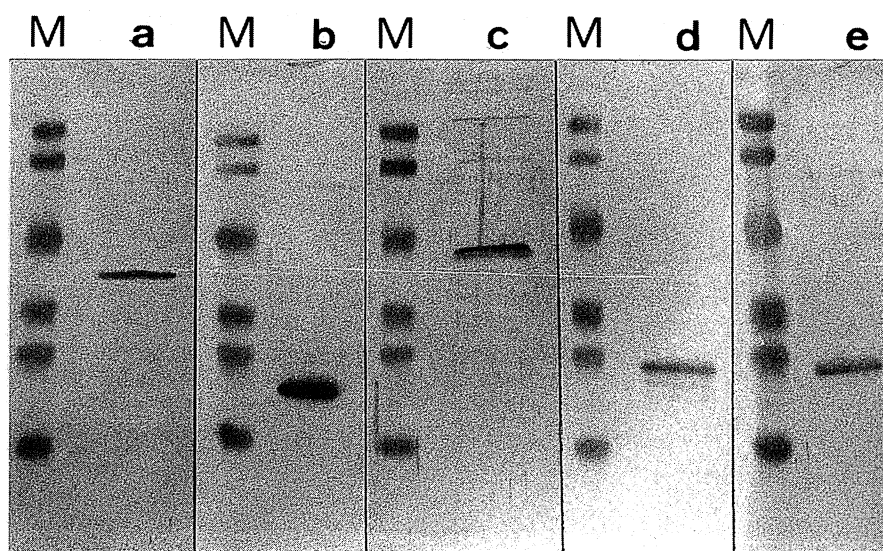


FIGURE 3

Comparison of molecular weight of capsid proteins of (a) GLRaV-1, (b) GLRaV-2, (c) GLRaV-3, (d) GVA and (e) GVB by Western Blot. M = prestained SDS-PAGE standards.

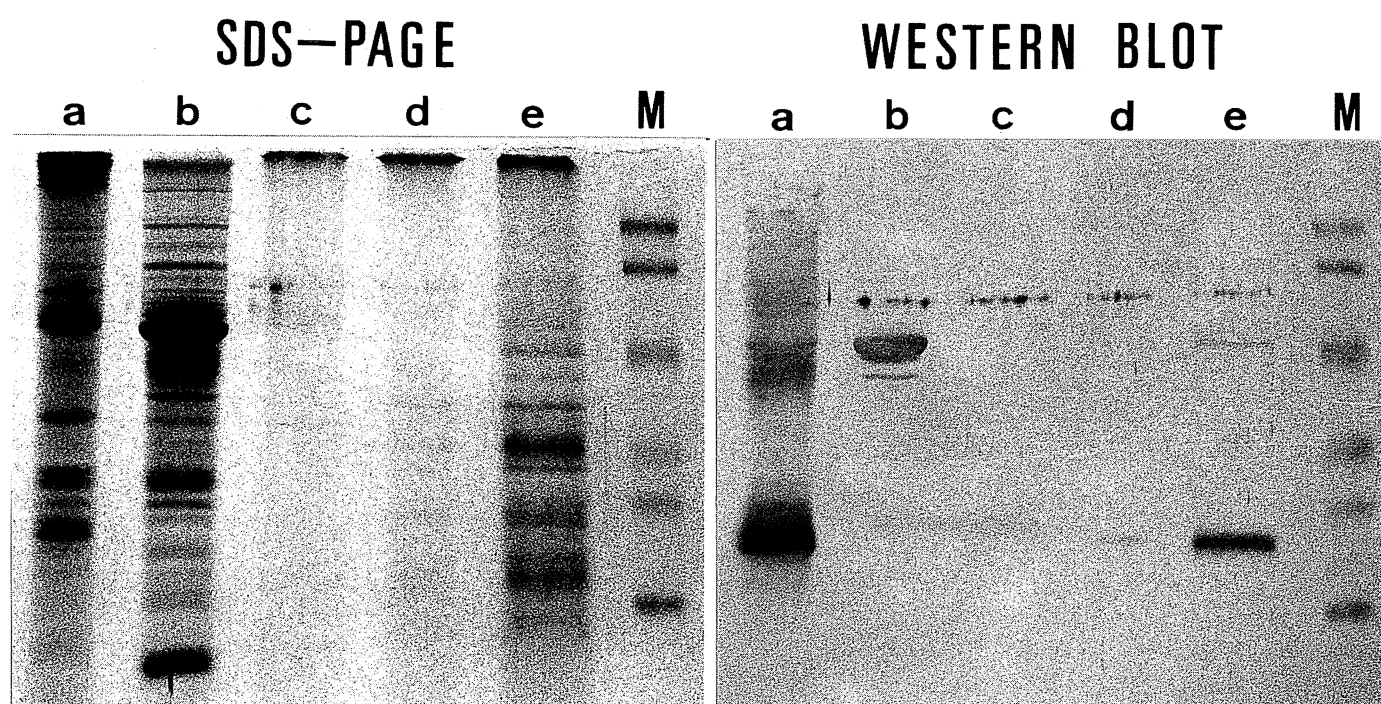


FIGURE 4

SDS-PAGE and Western Blot analysis of extracts from leaves of (a) GLRaV-2-infected and (b) virus-free *Nicotiana benthamiana* and (c, d, e) petioles of virus-infected grapevine cv. Muscat d'Alexandrie (c) unconcentrated and (d) concentrated by ultracentrifugation and (e) concentrated by ultracentrifugation and freeze-drying. M = prestained SDS-PAGE standards.

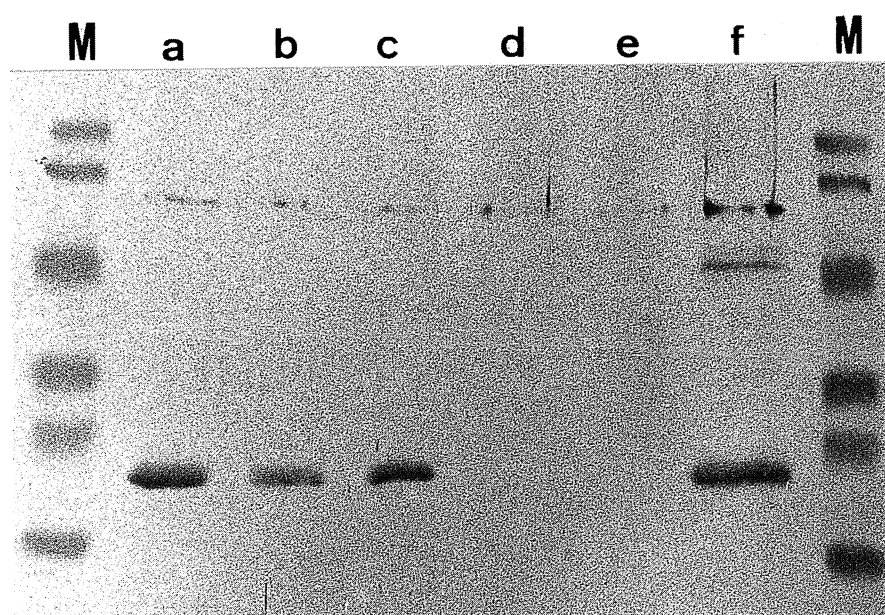


FIGURE 5

Western Blot analysis of reactivity of antiserum to GLRaV-2 with different isolates of the virus partially purified from grapevine cvs. (a) Shiraz, (b) Tinta Barocca, (c) Chasselas and with extracts from virus-free grapevine cvs. (d) Shiraz and (e) Tinta Barocca. (f) Reaction of antiserum with concentrated extract from cv. Muscat d'Alexandrie was used as control. M = prestained SDS-PAGE standards.

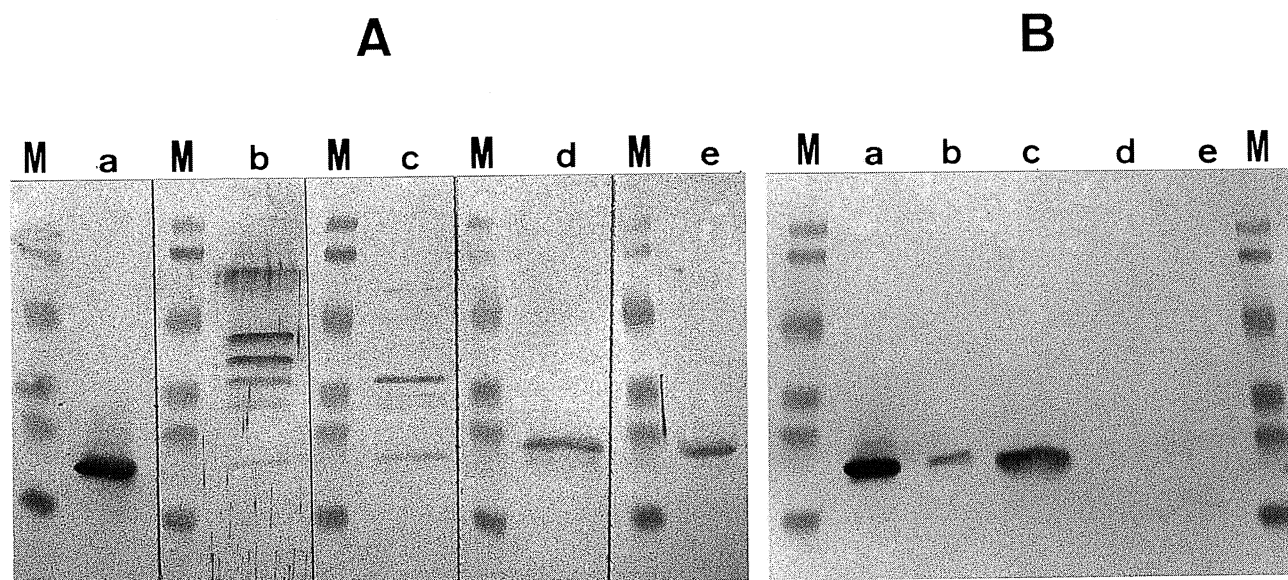


FIGURE 6

Western Blot analysis of reactivity of (a) GLRaV-2, (b) viruses of grapevine cv. Black Spanish, (c) viruses of grapevine cv. Chasselas, (d) GVA and (e) GVB with (A) homologous antisera and (B) antiserum to glutaraldehyde-treated GLRaV-2. M = prestained SDS-PAGE standards.

TABLE 1

Comparison of reactivity of antiserum to GLRaV-2 with extracts from infected and virus-free *Nicotiana benthamiana* and grapevine cv. Shiraz by DAS-ELISA.

Plant	Coating immunoglobulins ($\mu\text{g/mL}$)		
	2	1	0.5
<i>Nicotiana benthamiana</i> ^a	0.914 ^c	0.617	0.410
	0.326 ^d	0.195	0.119
<i>Vitis vinifera</i> cv. Shiraz ^b	0.617	0.320	0.190
	0.095	0.043	0.027

^a, ^bSap was extracted from (a) leaves and (b) cane phloem using standard ELISA extraction-conjugate buffer (Clark & Adams, 1977).

^c, ^dAbsorbance values (A_{405}) for (c) infected and (d) healthy plants obtained after 1 h incubation of substrate.

TABLE 2

Comparison of the effects of extraction buffers on the detection of GLRaV-2 in grapevine^a by DAS-ELISA.

Buffer ^b	Absorbance (A_{405})		
	Infected	Healthy	Inf./Health.
1	0.346 ^c	0.050	6.9
2	0.308	0.020	15.4
3	0.410	0.040	10.2
4	0.153	0.022	6.9

^aExtract from cane phloem of *Vitis vinifera* L. cv. Shiraz was used.

^bBuffer: (1) PBS (pH 7.4) containing 2% PVP (MW 44000), 0.2% ovalbumine, 0.5% Tween 20, 0.02% NaN_3 (Clark & Adams, 1977); (2) 0.1 M Tris-HCl (pH 7.6) containing 0.01 M MgSO_4 , 0.2% 2-mercaptoethanol, 2% Triton X-100, 0.5% bentonite, 4% PVP-P; (3) 0.2 M Tris-HCl (pH 8.2) containing 2% PVP (MW 25000), 0.05% Tween 20, 0.8% NaCl, 0.02% NaN_3 (Zimmermann *et al.*, 1990); (4) 0.5 M Tris-HCl (pH 8.2) containing 0.01 M MgSO_4 , 0.2% 2-mercaptoethanol, 5% Triton X-100, 0.5% bentonite, 4% PVP-P (Hu *et al.*, 1990). In the case of buffers 2 and 4, extracts were expressed through cheese-cloth and centrifuged 3000g for about 0.5 min.

^cAbsorbance values (A_{405}) obtained after 1 h incubation of substrate.

TABLE 3
Comparison of reactivity of various antisera and a monoclonal antibody to GLRaV-2 with extracts from infected and virus-free grapevine and *Nicotiana benthamiana* plants by ATA-ELISA.

Plant	Antisera								Monoclonal antibody
	Native virus				Glutaraldehyde-treated virus				
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7	Mouse 8	
<i>Nicotiana benthamiana</i> ^a	>2.00 ^c	0.832	1.572	1.730	1.987	1.013	1.737	1.737	0.585
	1.837 ^d	0.042	0.524	0.042	0.056	0.043	0.035	0.035	0.098
<i>Vitis vinifera</i> cv. Shiraz ^b	1.706 ^c	0.451	1.433	0.267	1.963	1.951	1.024	1.904	0.768
	0.028 ^d	0.022	0.016	0.014	0.013	0.027	0.023	0.014	0.026

a,b,Sap was extracted from leaves (a) and cane phloem (b) using, buffer 1 and 2 respectively, described in Table 2.

a,b,Absorbance values (A₄₀₅) obtained for infected (a) and healthy (b) plants after 30 min incubation of substrate.

TABLE 4

Detection of GLRaV-2 in cane phloem of different grapevine cultivars by ATA and DAS ELISA using various antisera.

Grapevine	ATA-ELISA					DAS-ELISA ^C
	1 ^a	3	5	8	MAb ^b	
GLRaV-2-infected:						
Tinta Barocca	0.770 ^d	0.673	0.867	1.030	0.187	0.386 ^e
Pinot noir	1.314	0.971	1.336	1.435	0.745	1.440
Barlinka	0.938	0.781	1.006	1.127	0.247	0.561
Chasselas	1.231	1.014	1.112	1.293	0.484	0.565
GLRaV-2-free:						
Tinta Barocca	0.017	0.011	0.011	0.012	0.016	0.052
Pinot noir	0.016	0.008	0.007	0.009	0.016	0.057
Cabernet franc	0.020	0.016	0.015	0.016	0.024	0.062
Cabernet Sauvignon	0.020	0.015	0.014	0.015	0.023	0.065
Merlot	0.017	0.011	0.010	0.010	0.013	0.039
Mission	0.016	0.012	0.012	0.012	0.014	0.036
Gamay	0.019	0.013	0.012	0.012	0.016	0.040
LN33	0.019	0.012	0.013	0.015	0.030	0.058

a, b Mouse antisera (a) and monoclonal antibody (b) as described in Table 3.

^c Based on rabbit antibodies.d, e Absorbance values (A_{405}) obtained after 30 min (d) and 180 min (e) incubation of substrate.

Mouse antisera to GLRaV-2 treated with glutaraldehyde and their activity in ELISA, IEM and Western Blots: Treatment of some viruses with glutaraldehyde increase their immunogenicity, and production of antisera with substantially higher antibody titre than for native viruses can be obtained (Francki & Habili, 1972; Hajimorad & Francki, 1991). To test whether this applies to GLRaV-2, preparations of purified virus were treated with 0.2% glutaraldehyde and then dialysed to remove the glutaraldehyde before immunising four mice. Four control mice were immunised with native virus. Antisera obtained to native and glutaraldehyde-treated GLRaV-2 preparations were tested in ATA-ELISA using extracts of infected and virus-free *N. benthamiana* and grapevine cv. Shiraz. Results presented in Table 3 show that the absorbance values obtained for infected plants did not differ markedly between the two sets of antisera. The absorbance values obtained with antisera to native GLRaV-2 for virus-free *N. benthamiana* were highly variable. The values ranged from 0.042 to 1.8 depending on the mouse, whereas the absorbance values obtained with the antisera to glutaraldehyde-treated preparations were fairly uniform. The values remained low and ranged between 0.035 and 0.056. Surprisingly, we observed relatively high levels of nonspecific reaction with the monoclonal antibody to extracts of virus-free plants compared to the polyclonal antisera (Table 3). In contrast to the results obtained for virus-free *N. benthamiana*, the absorbance values to virus-free grapevine cv. Shiraz were low for both the antisera to glutaraldehyde-treated preparations and to the native GLRaV-2 preparations and were similar to the results obtained with the monoclonal antibody.

The possibility existed that the treatment of GLRaV-2 with glutaraldehyde had changed the antigenic structure of the virus. New epitopes on the GLRaV-2 capsid proteins could potentially elicit antibodies cross-reactive to other viruses of grapevine. To investigate this possibility, mouse antiserum to glutaraldehyde-treated virus (mouse 5) was tested against known GLRaV by IEM. Partially purified viruses from grapevine cultivars Black Seedless, Barlinka, Ohanez and hybrid LN33 infected with GLRaV-1, GLRaV-4, GLRaV-5 and GLRaV-3 (Goszczynski *et al.*, 1995) respectively, were used. GVA and GVB purified from infected *N. benthamiana* were also used. No decoration of the above viruses by the antiserum to glutaraldehyde-treated GLRaV-2 was observed. Reaction of this antiserum with capsid proteins of different GLRaV types was also tested in Western Blot experiments. In these experiments viruses partially purified from the grapevine cvs. Black Spanish and Chasselas clone 8/22 were used. Cultivar Black Spanish was shown to be infected with GLRaV-3, GLRaV-1, a closterovirus serologically related to GLRaV-6 (Goszczynski *et al.*, 1995; Goszczynski & Kasdorf, 1995) and GLRaV-2. These viruses were detected in Western Blots as bands with molecular weights of 50.6, 43.6, 38.9 and 25.4 kD, respectively, in reaction with a homologous antiserum (Fig. 6, lane b). Cultivar

Chasselas clone 8/22 was known to be infected with GLRaV-6 and GLRaV-2 (Gugerli & Ramel, 1993). These viruses were detected in Western Blots as two bands with molecular weights of about 38.9 kD and 25.4 kD in reaction with a homologous antiserum (Fig. 6, lane c). Preparations of purified GVA, GVB and GLRaV-2 were also included (Fig. 6). Results showed that the antiserum to glutaraldehyde-treated GLRaV-2 reacted only with capsid proteins of GLRaV-2. No reaction of this antiserum was observed with bands of capsid proteins of GLRaV-1, GLRaV-3, GLRaV-6, GVA and GVB (Fig. 6).

Detection of GLRaV-2 by ELISA using various antisera:

Selected mouse antisera to native- (mice 1 and 3) and glutaraldehyde-treated GLRaV-2 (mice 5 and 8) and the monoclonal antibody were used to test the reaction of the antisera with extracts from various infected and virus-free grapevine cultivars. The grapevines were also tested by DAS-ELISA based on the rabbit antiserum to native virus. Results presented in Table 4 showed that mouse antisera to both native and glutaraldehyde-treated GLRaV-2 detected the virus with a high degree of sensitivity and specificity. Each mouse antiserum gave absorbance values below 0.02 for virus-free grapevines and was comparable with that of the monoclonal antibody. The sensitivity of detection of GLRaV-2 by DAS-ELISA based on the rabbit antiserum was much lower than that of the ATA-ELISA. The absorbance values for virus-infected grapevines in ATA-ELISA after 30 min of substrate incubation were only obtained in DAS-ELISA after more than 3 hours incubation. In spite of this, absorbance values for different GLRaV-2-free grapevine cultivars obtained in DAS-ELISA were lower than 0.065 (no visible colour reaction), and therefore this ELISA is still useful for detection of the virus.

DISCUSSION

The first reports of mechanical transmission of GLRaV-2 were published by Monette & Godkin (1993) and Castellano *et al.* (1995). For the transmission of the virus these authors used an extract from *in vitro* shoot tip culture of grapevines infected with GLRaV-2. In our experiments, the virus was mechanically transmitted from extracts of petioles of infected grapevine concentrated by ultracentrifugation. Using this relatively short procedure we also transmitted the virus from grapevine cvs. Cabernet Sauvignon, Weldra, Kerner, Shiraz, Queen of the Vineyard, Colombard, Jubily, Opsimo Soufliou and from hybrid LN33 (unpublished).

Yellowing of the GLRaV-2 infected *N. benthamiana* plants and the membraneous vesicle inclusions observed in the cells of these plants resembled those of beet yellows virus (BYV). This indicated that GLRaV-2 belong to the closterovirus group with BYV as a "type member" (Bar-Joseph *et al.*, 1979; Francki *et al.*, 1985). The extensive vesiculation of cytoplasm of *N. benthamiana* cells infected with GLRaV-2 was also reported by Castellano *et*

al. (1995). Yellowing and death of infected plants approximately one month after inoculation clearly differentiated GLRaV-2-infected *N. benthamiana* plants from those infected with other mechanically transmissible grapevine viruses - trichoviruses GVA and GVB (Martelli *et al.*, 1994). The latter plants remained alive and green for at least four months (unpublished).

The molecular weight of the capsid protein of our isolate of GLRaV-2 (25.4 kD) differed from that reported by Gugerli & Ramel (1993) (26.5 kD). This may be due to differences in electrophoresis conditions and the use of different protein molecular weight markers. The molecular weights of the capsid proteins of GLRaV-1 (43.6 kD), GLRaV-3 (50.6 kD) and GVA (27.5 kD) determined by us also differed from those reported by the above authors (38.8, 44.3 and 25.1 kD, respectively).

The Western Blot results showed that purification of GLRaV-2 from infected *N. benthamiana* plants according to the procedure described in this report did not remove all plant proteins. Especially the protein with molecular weight of 56.2 kD, probably fraction I protein (Van Regenmortel, 1982), appeared to be highly immunogenic. Although this protein was not visible in the purified virus preparations analysed by SDS-PAGE, it gave a clear immunological response. Due to the antibody activity against this protein the antisera to GLRaV-2 were not suitable for the detection of the virus in extracts on *N. benthamiana*. However, detection of GLRaV-2 in extracts from grapevines petioles and cane phloem was not affected by the presence of antibodies to this protein. Our results indicate that sap extracted from these grapevine tissues according to the method described in this report contain relatively low amounts of this protein. Petioles and cane phloem are commonly used for detection of GLRaV in grapevines because of the high concentration of viruses in this infected material (Hu *et al.*, 1990).

Treatment of preparations of GLRaV-2 with glutaraldehyde before immunisation clearly improved the quality of the antisera. Antisera to glutaraldehyde-treated GLRaV-2 showed increased specific and decreased non-specific antibody activity in ELISA. Rybicki & Von Wechmar (1981) suggest, on the basis of results obtained for brome mosaic (BMV) and cowpea chlorotic mottle (CCMV) viruses, that treatment of viruses with glutaraldehyde can alter their serological properties to the extent that it could influence the study of plant viruses. This is especially important for the diagnosis of grapevine viruses since there is the possibility that new epitopes created by this method could elicit antibodies which cross-react with other serologically unrelated grapevine viruses which occur frequently in mixed infections (Hu *et al.*, 1990; Zimmermann *et al.*, 1990). Our IEM and Western Blot analyses did not show any reactivity of our antiserum to glutaraldehyde-treated GLRaV-2 with known GLRaV and GVA and GVB. Aldehyde-fixed tobacco mosaic virus (TMV) (Van Regenmortel & Le Large, 1973), cucumber mosaic virus (CMV) (Francki & Habili, 1972) and alfalfa

mosaic virus (AMV) (Hajimorad & Francki, 1991) did not show any significant changes of antigenic structure either. These results support the assumption that antisera to glutaraldehyde-treated viruses have full diagnostic value. Although the results of Rybicki & Von Wechmar (1981) indicate a change of the antigenic structure of the viruses, their results also suggest that antisera produced to such viruses have increased specificity. Mechanical transmission of GLRaV-2 directly from grapevine plants to *N. benthamiana* and the development of a highly sensitive ELISA for the detection of GLRaV-2 described in this manuscript undoubtedly open new possibilities for the study of this closterovirus.

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