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Grapevine virus C and grapevine leaf roll associated virus 2 are serologically related and appear to be the same virus

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Summary

Protein extracted from grapevines infected with GLRaV-2 virus was subjected to electrophoresis, followed by Western blots. A protein band of about 23 kDa was detected in all infected plants. When GVC antibodies were used on blots obtained from the same infected plants, a similar protein band was detected in all infected plants. To address the possibility of the presence of another virus with the same molecular weight, the gene coding for the coat protein of GLRaV-2 was cloned and expressed in *E. coli*. The expressed protein reacted positively to both GLRaV- 2 and GVC antibodies. Using Immunosorbent Electron Microscopy (ISEM), polyclonal antibodies prepared against either GVC or GLRaV-2 trapped and decorated GLRaV-2 particles. The cDNA from GVC-infected grapevines and *Nicotiana benthamiana* were cloned and sequenced. All of the clones that were sequenced had the same sequence as GLRaV-2. Based on the data obtained, we concluded that GVC is the same virus as GLRaV-2.

K e y w o r d s : GVC, GLaV2, grapevine viruses.

Introduction

Grapevine virus C (GVC) is identified as one of the *trichoviruses* that affect grapevine. It was first reported in Canada (Monette and James 1990; Monette and James 1991) and in Italy (Castellano *et al.*1995). The virus was first isolated from grapevine cvs Mission, Chardonnay and Brant as one component of a multiple infection that caused corky bark disease. The virus can be transmitted from grapevine shoot tip culture to *Nicotiana benthamiana* which produces necrotic local lesions followed by leaf mottling, clearing of the veins, top necrosis and death of the plants (Monette and James 1990).

The virus is a flexuous rod of about 725 nm in length and 10 nm in width with striations of 3.4 nm (Monette and James 1990). Purified GVC preparations were used to produce polyclonal antibodies in rabbits. In Western blots, the coat protein was found to be about 25 kDa as estimated by PAGE (Monette and Green 1992). GVC is serologically distinct from *Grapevine virus A* (GVA) or *Grapevine virus B* (GVB) (Monette and Green 1992). Using ISEM, antisera prepared against several filamentous viruses such as *Apple stem grooving virus, Potato virus T*, and *Apple chlorotic leaf spot virus* failed to decorate GVC particles. This communication reports that the polyclonal antibodies produced against GVC reacted positively to *Grapevine leafroll associated virus 2* (GLRaV-2) and both viruses are related or identical.

Material and Methods

V i r u s s o u r c e : The corky bark-affected grapevine (*Vitis vinifera* L.) cultivars Mission 236-01B2, Mission 236-02B2, Semillon 1186-10A3, Semillon 1186-10C2, and Semillon 1186-10D3, were the same as those used in previous studies (Monette and James 1991; Monette and Green 1992; and Monette and Godkin 1995). *N. benthamiana* plants used in this study were from the same source as the *N. benthamiana* used by Monette (Monette and James 1991) to produce GVC antibodies.

M e c h a n i c a l i n o c u l a t i o n o f h e r b a c e o u s h o s t : *In vitro* shoot tip cultures were collected at the shoot proliferation stage. These were ground in a chilled mortar in 5 volumes (w/v) of 0.01 M potassium phosphate buffer containing 2.5 % nicotine final pH 9.5 according to the method of Monette *et al.* (1990). The extracts were rubbed onto *N. benthamiana* leaves, which had been placed in the dark overnight then dusted with Celite (Sigma, St Louis, USA), using sterile Q-tips. The plants were rinsed with distilled water and placed again in the dark overnight. They were then maintained in the greenhouse for 4-6 weeks and monitored for disease symptom expression.

A n t i s e r a : Polyclonal antibodies against GVC were the same used previously (Monette and Green 1992, Monette and Godkin 1995). The GLRV-2 polyclonal antibodies were obtained from Bioreba (Switzerland).

I m m u n o s o r b e n t e l e c t r o n m i c r o s c o p y (I S E M) : ISEM was performed as reported by Monette and Godkin (1995). Grids covered with Formvar-carbon films were coated with 1:100 dilution of either GVC or GLRaV-2 antiserum for 3 h and then washed with 1 ml of 0.1 M phoshate buffer pH 7.5 (PB). The grids were placed on a droplet of plant sap for one additional h and washed as before. The virus particles were decorated by the different antibodies and were washed with 1 ml of distilled water, stained with 25 % uranyl acetate and examined under the electron microscope.

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Electrophoresis and Western blots: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on 12 % polyacrylamide (29.2:0.8 acrylamide-bisacrylamide) vertical gels with 3 % stacking gel in a Miniprotean II (Bio-Rad, Richmond, CA), using the method of Laemmli (1970). Sample preparation consisted of grinding approximately 0.1 g of dormant grapevine wood, or *in vitro* grapevine shoot tip tissue, or tissue from symptomatic *N. benthamiana* plants in 10 volumes (v/w) of Tris-buffered saline (TBS) consisting of 0.02 M Tris-HCl, 0.05 M NaCl, pH 7.5. The homogenate was centrifuged at 3000 x g for 2 min. The supernatant was collected and mixed with an equal volume of 0.06 M Tris-HCl, pH 6.8, containing 10 % glycerol, 2 % SDS, 5 % 2-mercaptoethanol, and 0.025 % bromophenol blue. The mixture was heated for 5 min at 95 °C and a 10 µl sample loaded onto the gel. Pre-stained molecular weight marker was used (Low molecular weight marker, Bio-Rad). Electrophoresis was performed for 70 min at 140 V. The resolved proteins were blotted onto PVDF membrane (Bio-Rad). The membranes were blocked for 1 h in TBS containing 5 % nonfat milk. The blots were developed using Opti 4CN (Bio-Rad).

Extraction of double stranded RNA: Double stranded RNA (dsRNA) was extracted from *N. benthamiana* according to Valverde (1990). In brief, 10 g of tissue was frozen in liquid nitrogen, pulverized in a coffee grinder and stirred in phenol-chloroform-isoamyl alcohol (50:48:2 %) for 1 h. The dsRNA was purified by absorbing the nucleic acid onto CF-11 cellulose. After precipitation, the dsRNA was analyzed on 6 % acrylamide gels in Tris-borate EDTA (TBE) buffer.

RT-PCR: Total RNA extractions were made from 0.1 g of ground dormant grapevine wood, or *in vitro* grapevine shoot tip tissue, or symptomatic *N. benthamiana*. The tissue was processed using a QIAGEN total RNA kit (Chatsworth, CA) according to the manufacturer's instructions. Reverse transcription (RT) was done on 10 µl of each RNA extract placed in a 0.5 ml tube containing 2 µl GLRaV-2 primers (5' GCC CTC CGC GCA ACT AAT GAC AG 3', Abou-Ghanem, unpubl.). The mixture was heated at 70 °C for 10 min, after which the tubes were cooled on ice for 2 min. This was followed by the addition of 10 µl of 5x first strand buffer, 2 µl of 10 mM dNTP mixture, 5 µl of 0.1 M dithiothriotol, and 18 µl of water and 1 µl (200U) of Superscript II reverse transcriptase (Invitrogen, CA). The tubes were incubated for 1 h at 42 °C. PCR reactions were performed using the following primers 5' ATA ATT CGG CGT ACA TCC CCA CTT 3' (primer 1) and 5' GCC CTC CGC GCA ACT AAT GAC AG 3' (primer 2) for the forward and the reverse primers respectively (Abou-Ghanem, unpubl.) in a volume of 50 µl PCR buffer supplied by the manufacturers, containing 1x cresol/sucrose solution (10Xstock: 1 mM cresol red, 0.2 $g.ml^{-1}$ sucrose), 5 µl cDNA (1-2 ng), 0.5 µM primer mix, 1.8 mM MgCl2, 0.2 mM dNTPs and 2.5 U of *Taq* polymerase using a 96 well gradient Robocycler (Stratagene). The cDNA was amplified for 35 cycles consisting of 94 °C for 50 s, 54 °C for 50 s and 72 °C for 70 s (Saiki *et al*. 1988).

Cloning and expression of GLRaV-2 coat protein: On the basis of the published sequence (Zhu *et al*. 1998), primers spanning the GLRaV-2 open reading frame 5 coding the entire coat protein were amplified by PCR using the following forward and reverse primers: 5' **GGA TCC** ATG GAG TTG ATG CCG ACA GCA ACC TTA G 3' containing the BAMH 1 restriction site (in bold) and 5' **CTC GAG** CTT CCC TTC TAC CTA GCT GAC GCA GAT TGC TCA C 3' containing XHO1 restriction site (in bold). The PCR product was cloned in TOPO (Invitrogen). The gene was recloned into the pET 28 plasmid behind the T7 promoter. The expressed protein was purified using a nickle agarose column (QIAGEN).

Results

Immunosorbent electron microscopy (ISEM): Virus particles obtained from the herbaceous host were successfully trapped and decorated using both GVC and GLRaV-2 antisera (Fig. 1 a, b). All the trapped and decorated virus particles were similar in size and affinity to the two antibodies. The same results were obtained from grapevine dried samples that were used previously by Monette and Godkin in1995 (data not shown).

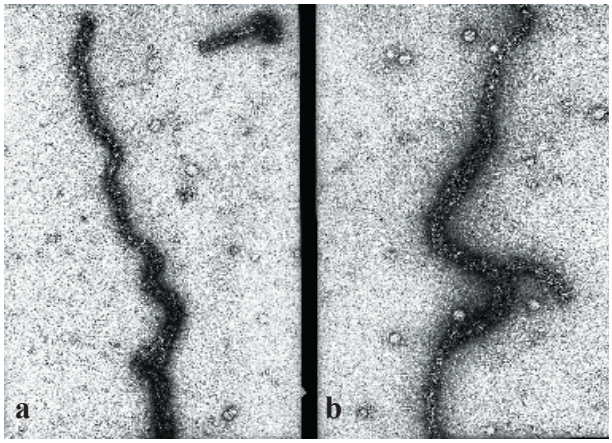

Fig. 1: Immunosorbent electron microscopy of *N. benthamiana* plant extract. Virus particles were trapped with GVC antibodies and decorated with GLRaV-2 antibodies (**a**) or trapped with GLRaV-2 and decorated with GVC antibodies (**b**).

Western blots: *N. benthamiana* seedlings inoculated with extracts from *in vitro* cultures of corky bark affected grapevines developed symptoms that were typical of both GVC and GLRaV-2. The seedlings developed necrotic local lesions followed by vein clearing and the collapse of the top leaves leading to death of all infected plants. A 22 kDa protein was detected by Western blot analysis of the infected *N. benthamiana* seedlings using the GLRaV-2 polyclonal antibodies (Fig. 2 a). A similar single virus protein band of about 22 kDa was also observed when the Western blot analysis was repeated using antibodies produced against GVC (Fig. 2 b). Western blot analysis of samples obtained from the different grapevine cultivars using either GVC antibodies or GLRaV-2 anti-

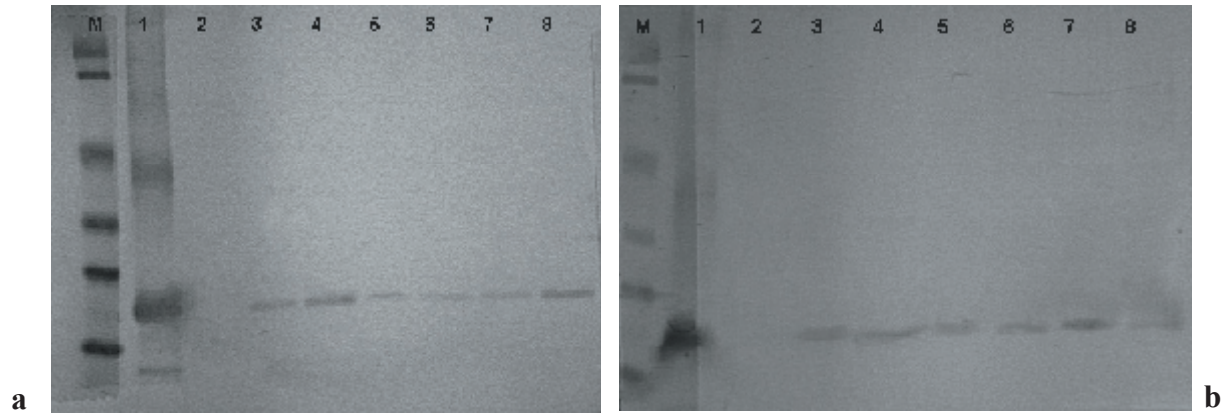

Fig. 2: Western blot analysis of the total protein from several grapevine species using rabbit anti-GVC antibodies (**a**) or anti- GLRaV-2 antibodies (**b**). Lane M pre-stained low mass molecular weight marker (Bio-Rad), lane 2 protein obtained from *N. benthamiana*, lane 2 protein obtained from LN 33 as a negative control, lanes 3-8 protein obtained from shoot tips of grapevine Mission 236-01B2, Mission 236-02B2, Semillon 1186-10A3, Semillon1186-10C2, and Semillon 1186-10D3, respectively.

bodies resulted in the detection of the same 22 kDa coat protein (Fig. 2 a, b).

The expressed and purified GLRaV-2 coat protein migrated as a single band on SDS-PAGE, with a molecular weight of about 22 kDa (Fig. 3). The expressed protein reacted positively to both GVC antibodies and GLRaV-2 antibodies when analysed on Western blots (Fig 3 a, b).

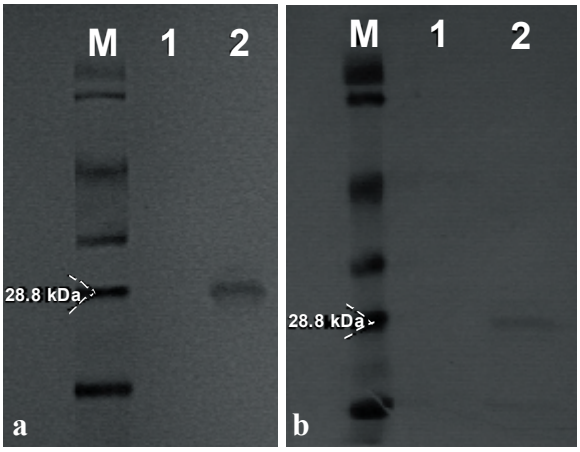

Fig. 3: Western blots on the expressed and purified GLRaV-2 coat protein. **a**: Western blot using polyclonal GLRaV-2 antibodies. Lane M, low mass pre-stained protein marker. Lane 1, total protein from *E. coli* without any plasmid as a negative control. Lane 2, protein extracted from *E. coli* expressing the coat protein of GLRaV-2. **b**: Western blot using GVC polyclonal anti bodies, lane M, low mass pre-stained protein marker. Lane 2, total protein from *E. coli* without any plasmid as a negative control. Lane 2, protein extracted from *E. coli* expressing the coat protein of GLRaV-2.

P C R : Using primers 1 and 2, which are specific for GLRaV-2, a PCR product of about 332 base pairs was obtained from all infected plants (Fig. 4). This PCR product is consistent with the predicted published sequence.

C l o n i n g o f d s R N A : A high molecular weight band of about 18,000 Da was consistently present in infected *N. benthamiana* plants, but not in healthy plants (data not shown). Several hundred cDNA clones were obtained from the dsRNA. Twenty clones, which are not host related, were selected at random for sequencing. All the clones selected from GVC infected plants have the same sequence as that of GLRaV-2 virus (data not shown).

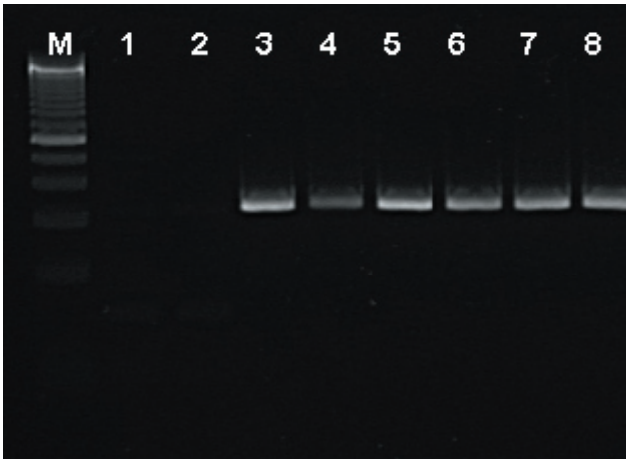

Fig. 4: PCR results from cDNA obtained from grapevine plants infected with GLRaV-2. Lane M, 100 base DNA marker (Bio-Rad), lane 1 DNA extracted from *N. benthamiana* as a negative control, lane 2, LN 33 as a negative control, lane 3 PCR results obtained by the amplification of cDNA extracted from *N. benthamiana* infected with GLRaV-2 as a positive control, lanes 4-8 amplification of cDNA extracted from shoot tips of grapevine Mission 01B2, Mission 02B2, Semillon10A3, Semillon 10C2, and Semillon 10D3, respectively.

Discussion

The classification of GVC was based on the best techniques available at the time such as ISEM, Western blot and the size of the virus particles (Monette and James 1990; Monette and Green 1992; Monette and Godkin 1995; Abou-Ghanem *et al*. 1995). Due to advances in molecular techniques, such as cloning and protein expression that allowed the expression and purification of the coat protein of the GLRaV-2, our results were different.

ISEM results show that GVC antibody can trap and decorate GLRaV-2 virus particles. There were no differences in trapping or decoration between using either GVC or GLRaV-2 antibodies. This shows clearly that GVC and GLRaV-2 are serologically related. This was confirmed by Western blots where we found that the coat protein of the GLRaV-2 can be detected by both antibodies (Fig. 2). To prove that there were no other viruses present containing a coat protein of the same molecular weight, we cloned and expressed the gene coding for the GLRaV-2 coat protein. The recombinant protein reacted positively to both antibodies (Fig. 3). Our data differs from the previously published data regarding the size of the virus particles. It was reported previously that the size of the GVC particles was about 800 nm (Monette *et al*. 1990), while our data shows that the particle size is about 1200 nm. This discrepancy could be due to the presence of more than one virus in the original plant material from which the virus was isolated. To resolve this, the original tissue culture, which was used previously to inoculate *N. benthamiana*, was tested by PCR using GVB and GLRaV-2 primers. That particular sample was found to be contaminated with both GVA (Monette and Green 1992) and GVB (Fig. 5). Both these viruses have the same particle size as the reported GVC, and together with GLRaV-2 are mechanically transmissible to *N. benthamiana*. In subsequent publications, ISEM was applied successfully to detect GVC but the size of the virus particles was not cited (Monette and Godkin 1995). It is possible that the decorated virus particles in that particular publication were that of GLRaV-2. In conclusion, the results of these experiments indicate that GVC and GLRaV-2 are either very closely related or the same virus.

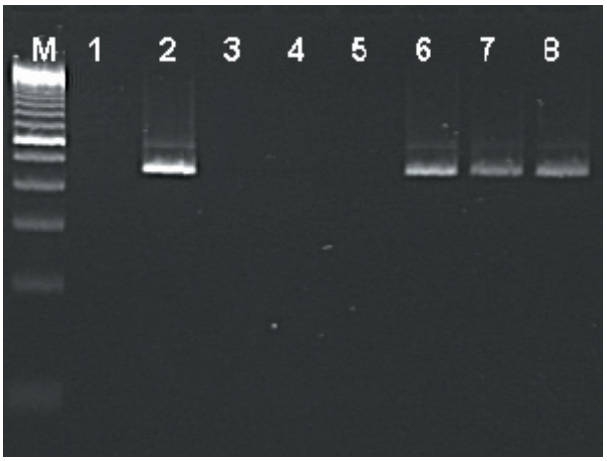

Fig. 5: PCR results from grapevine plants infected with GVB. Lane M, 100 base DNA marker (Bio-Rad), lane 1, PCR results obtained from amplification of cDNA extracted from LN 33 as a negative control, lane 2, PCR results obtained from amplification of cDNA extracted from N. benthamiana as a positive control, lane 3 DNA extracted from *N. benthamiana* infected with GVB as a positive control, lanes 4-8 DNA extracted from shoot tips of grapevine Mission 01B2, Mission 02B2, Semillon10A3, Semillon 10C2, and Semillon 10D3, respectively.

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