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Detection of two strains of grapevine leafroll-associated virus 2

by

D. E. Goszczynski, G. G. F. Kasdorf, G. Pietersen and H. van Tonder

Plant Protection Research Institute, Agricultural Research Council, Pretoria, Republic of South Africa

Summary: Two strains of grapevine leafroll-associated virus 2 (GLRaV-2) were obtained by mechanical transmission from grapevines to *Nicotiana benthamiana*. The strains, designated 94/970 and 93/955, consistently differed with regard to the development of symptoms. The first induced chlorotic and occasional white-necrotic local lesions while the second induced chlorotic followed by metallic-opalescent, solid necrotic local lesions. The strains were indistinguishable with regard to the molecular weight of their capsid proteins or serologically. A difference in the pattern of minor dsRNA bands was consistently observed.

Key words: GLRaV-2, strains, symptoms, cytopathology, serology, dsRNA.

Introduction

Grapevine leafroll (GLR) is a viral disease known worldwide for many years but still of uncertain etiology (Bovey and Martelli 1992). Grapevine leafroll-associated virus 2 (GLRaV-2) is one of the 6 serologically distinct closteroviruses (GLRaV-1 - 6) frequently detected in grapevines affected by GLR (Gugerli *et al.* 1984; Rosciglione and Gugerli 1986; Hu *et al.* 1990; Zimmermann *et al.* 1990; Bovey and Martelli 1992; Gugerli and Ramel 1993; Boscia *et al.* 1995). It is the only grapevine closterovirus for which mechanical transmissibility to herbaceous species was confirmed by different laboratories (Monette and Godkin 1993; Boscia *et al.* 1995; Goszczynski, unpublished).

In this paper we report the existence of two biological strains of GLRaV-2.

Materials and methods

Virus isolates and their mechanical transmission: The two isolates of GLRaV-2 used in this study were recovered from GLR-affected *Vitis vinifera* L. cv. Muscat of Alexandria (Plant Quarantine Station, Stellenbosch) and hybrid LN33 (Nietvorbij Experimental Farm, Stellenbosch) by mechanical inoculation of *N. benthamiana*. Virus infection of LN 33 was achieved by chip-budding with buds from grapevine cv. Tinta Barocca (vine no. 10-27; Engelbrecht and Kasdorf 1990). The viruses for mechanical transmission were prepared as follows: 4 g of freshly collected petioles from potted grapevine plants were pulverised in liquid N_2 using a pestle and mortar and mixed with 40 ml of 0.1 M Tris-HCl buffer pH 7.6 containing 0.01 M $MgSO_4$, 0.2 % 2-mercaptoethanol, 2 % Triton X-100, 0.5 % bentonite and 4 % polyvinyl-polypyrrolidone (PVPP). 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 $MgSO_4$) at 26,000 rpm (TY30 rotor, Beckman) for 2 h 15 min at 8 °C. The pellet was resuspended in 0.7 ml of buffer containing 0.01 M K_2HPO_4, 0.01 M cysteine-HCl and 3 % nicotine (inoculation buffer) (Boscia *et al.* 1993), mixed with celite, and used for inoculation of *N. benthamiana*. Transmissions of viruses between *N. benthamiana* plants were done using sap of systemically infected plants extracted in inoculation buffer. Inoculations of *N. benthamiana* by isolates of GLRaV-2 were repeated more than 16 times over an 8-month period (January to September).

dsRNA analysis: GLRaV-2-infected *N. benthamiana* plants collected in February were used for extraction and analysis of dsRNA by the procedure of Valverde (1990). dsRNA of African horse sickness virus serotype 3 (AHSV-3) with Mr 3314, 3038, 2663, 2033, 1804, 1639, 1137, 1137, 1137 and 693 bp were used as Mr markers. Three dsRNA extractions and analyses were done.

Virus purification: The virus was purified as follows: 80 g of systemically infected *N. benthamiana* plants were macerated using a pestle and mortar in 400 ml of 0.1 M Tris-HCl buffer pH 7.6 containing 0.01 M $MgSO_4$, 0.2 % 2-mercaptoethanol and 5 % Triton X-100 (extraction buffer). This was expressed through cheesecloth and the extract thoroughly shaken with 1/5 volume of chloroform, before centrifugation at 10,000 *g* for 15 min. The aqueous phase was collected, PEG 6000 and NaCl were added to a concentration of 8 % and 0.2 M respectively, and the solution was stirred on ice for 2 h. After centrifugation at 12,000 *g* for 25 min, the pellet was diluted in resuspension buffer (extraction buffer without 2-mercaptoethanol) and resuspended slowly by stirring overnight at

Correspondence to: Dr. D. E. Goszczynski, Plant Protection Research Institute, Agricultural Research Council, Private Bag X134, Pretoria 0001, Republic of South Africa. Fax: (012) 329 3278.

4 °C. The suspension was centrifuged at 6000 *g* for 5 min, then ultracentrifuged at 26,000 rpm (TY30 rotor, Beckman) for 2 h and 25 min at 8 °C through 20 % sucrose cushion prepared with 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_2SO_4 in dialysis buffer) and centrifuged at 28,000 rpm for 3 h and 45 min at 8 °C (SW41 rotor, Beckman). Virus bands were collected and dialysed with dialysis buffer overnight at 4 °C. The preparations were then centrifuged at 12,000 *g* for 10 min. Supernatants were stored at -75 °C.

Antisera production: Antisera were produced by 4 intramuscular injections of rabbits, 2 weeks apart, with 0.5 ml preparations of purified virus emulsified with complete (first injection) and incomplete (subsequent injections) Freund's adjuvant. Blood was collected 2 weeks after the last injection.

SDS-PAGE, Western blot and IEM were done as described by GOSZCZYNSKI *et al.* (1995) with some minor modifications. Viruses were denaturated with an equal volume of SDS-PAGE disruption buffer containing 0.1 M Tris-HCl, pH 7.6, 0.01 M $MgSO_4$, 5 % SDS, 10 % glycerol, 10 % 2-mercaptoethanol. Prestained SDS-PAGE standards (Bio Rad, low range) were phosphorylase B (112,000), bovine serum albumin (84,000), ovalbumin (53,200), carbonic anhydrase (34,900), soybean trypsin inhibitor (28,700), and lysozyme (20,500). For IEM, the viruses were resuspended in 0.1 M sodium phosphate buffer pH 7.0. A Dual-mini vertical unit (American Bionetics, Inc.) and Mini trans-blot cell (Bio Rad) were used for electrophoresis (4.5 % stacking and 12.5 % resolving polyacrylamide gels, at 180 V for 50 min) and electro-transfer (at 100 V for 60 min).

Monoclonal antibody to GLRaV-2 (GUGERLI and RAMEL 1993) used in IEM was kindly donated by P. GUGERLI (Federal Agricultural Research Station of Changins, Nyon, Switzerland).

Cytopathology: For ultrastructural studies, pieces of tissue were collected in February from systemically virus-infected leaves of *N. benthamiana* showing vein clearing. They were double-fixed in 2.5 % glutaraldehyde and 1 % osmium tetroxide, en block stained with uranyl acetate, dehydrated in graded acetone series and embedded in Epon-Araldite resin (HAYAT 1972). Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a Joel JEM-100C electron microscope.

Results and discussion

Plants of *N. benthamiana* infected with viruses mechanically transmitted from cv. Muscat of Alexandria and hybrid LN33, contained only GLRaV-2 particles. This conclusion was based on detailed IEM examination of leaf dips and purified virus preparations using monoclonal antibody to this virus. The viruses were designated as isolates 94/970 and 93/955, respectively.

The development of symptoms in inoculated *N. benthamiana* plants consistently differed between isolates. Those inoculated with isolate 94/970 developed chlorotic and occasional white-necrotic local lesions followed by systemic vein clearing. In some plants vein clearing turned to vein necrosis. *N. benthamiana* inoculated with isolate 93/955 developed chlorotic local lesions which turned to metallic-opalescent, solid necrotic local lesions (Fig. 1). These necrotic local lesions occurred only in *N. benthamiana* inoculated by isolate 93/955. Vein clearing induced by this isolate was followed by strong vein necrosis.

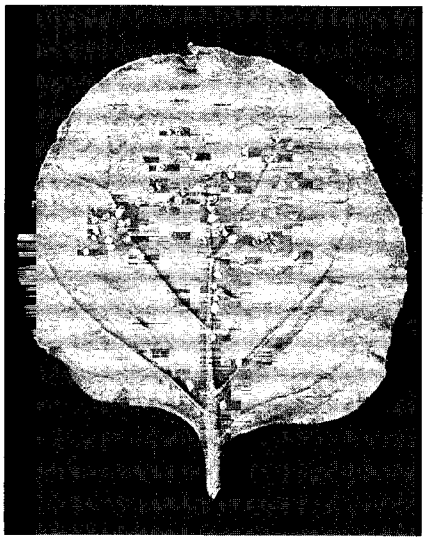

Fig. 1: Leaf of *Nicotiana benthamiana* with necrotic local lesions induced by GLRaV-2 isolate 93/955.

Cytological alterations observed in *N. benthamiana* infected with isolates of GLRaV-2 generally agreed with those described by CASTELLANO *et al.* (1995). While we did not find cytological alterations characteristic for only one of the GLRaV-2 isolates, the relative intensity showed marked differences between isolates. Isolate 93/955 induced a greater number of cytoplasmic vesicles, as well as stronger appositions of a callose-like substance than isolate 94/970. The extensive necrosis of vascular elements described by CASTELLANO *et al.* (1995) was observed only for isolate 93/955. EM observations indicated that the more severe cytological alterations induced by isolate 93/955 were not caused by higher numbers of viral particles present. In fact isolate 93/955 appeared to have a lower number of viral particles per cell than isolate 94/970. This was confirmed by analyses of leaf dips and purified virus preparations using IEM and SDS-PAGE. Fig. 2 A shows the typical difference in amount of purified capsid proteins between isolates of GLRaV-2 in SDS-PAGE.

Electrophoretic mobilities of capsid proteins were the same for both isolates (Fig. 2 A). In addition, no noticeable serological differences between isolates were detected in IEM and Western blots using homologous antisera.

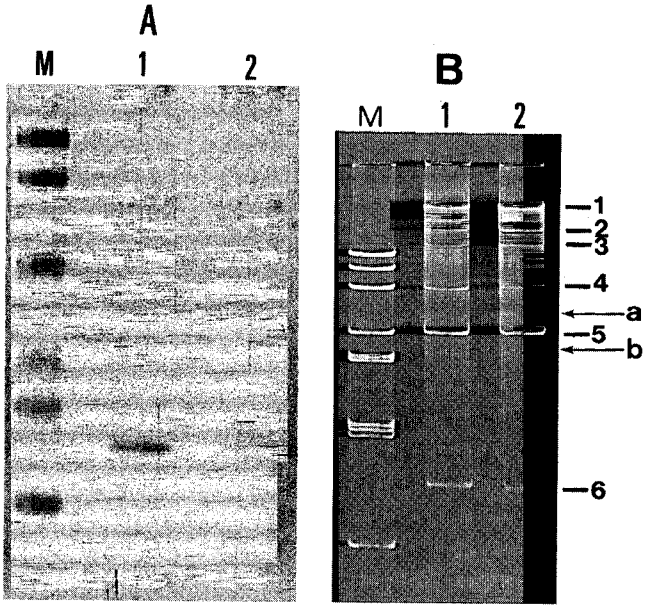

Fig. 2: SDS-PAGE (**A**) and dsRNA (**B**) analyses of GLRaV-2 isolates 94/970 (lane 1) and 93/955 (lane 2). M = Mr markers.

Polyacrylamide gel electrophoresis showed that both isolates contained the same pattern of 6 major dsRNA bands (Fig. 2 B). This was similar to the dsRNA pattern described for beet yellows virus (BYV) by DODDS and BAR-JOSEPH (1983). Consistant differences between isolates were, however, observed in minor dsRNA bands: dsRNA marked on Fig. 2 B as band "a" was clearly detected only in extracts of *N. benthamiana* infected with isolate 93/955, while dsRNA marked as band "b" was detected only for isolate 94/970.

The consistent differences in external symptoms, intensity of cytological alterations and minor dsRNA band patterns observed for GLRaV-2 isolates 94/970 and 93/955 indicate that they represent two strains of this virus. Isolate 93/955 is clearly more virulent for *N. benthamiana* than isolate 94/970. Whether a connection exists between dsRNAs differentiating these isolates and virulence has still to be determined.

This study represents the first identification of two biological strains of a grapevine closterovirus.

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