Detection of two strains of grapevine leafroll-associated virus 2

by

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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. The two isolates of GLRaV-2 used in this paper were obtained 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.

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 (Nietvoorbij 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 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 % 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 MgSO4) 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 K2HPO4, 0.01 M cysteine-HCl and 3 % nicotine (inoculation buffer) (BOSCA 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 MgSO4, 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

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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 Cs2SO4 gradient (prepared from 1 ml of each 15 % and 25 % and 2 ml of 35 % Cs2SO4 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.

Antiseraproduction: 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.

SDSPAGE, Western blot and IEMwere 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 MgSO4, 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.

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 Doodds 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.

References


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