Properties of a new isolate of grapevine leafroll-associated virus 2

N. Abou Ghanem-Sabanadzovic[1], S. Sabanadzovic[1], M. A. Castellano[2], D. Boscia[2] and G. P. Martelli[2]

[1] Istituto Agronomico Mediterraneo, Valenzano (Bari), Italia
[2] Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italia

Summary

A new isolate of grapevine leafroll-associated virus 2 (GLRaV-2-H4) was recovered by mechanically inoculating herbaceous hosts with concentrated tissue extracts from a North American accession of *Vitis rupestris*. Contrary to the Semillon isolate of GLRaV-2, isolate H4 elicited necrotic local lesions in *Nicotiana clevelandii* and infected systemically *N. occidentalis* inducing very severe symptoms. The migration rate of dissociated capsid protein of GLRaV-2-H4 in SDS-PAGE differed slightly from that of GLRaV-2-Sem. The coat protein sequence of GLRaV-2-H4 differed by about 12 % at the nucleotide level from capsid proteins of the other two GLRaV-2 isolates that have been sequenced to date. No serological differences could be detected. Isolate H4 is a biological variant of GLRaV-2, which can be distinguished from other mechanically transmitted isolates of the same virus because of differences in type and reactions of the herbaceous host range and in molecular traits of the coat protein cistron.

Key words: grapevine, closterovirus, leafroll, GLRaV-2, coat protein, nucleotide sequence.

Introduction

Grapevine leafroll-associated virus 2 (GLRaV-2) is one of the 7 closteroviruses found in vines affected by leafroll disease (Boscia *et al.* 1995; Choueiri *et al.* 1996) and the only one transmissible by inoculation of sap to herbaceous hosts (Boscia *et al.* 1995; Goszczynski *et al.* 1996 a and b). The genomes of GLRaV-2 isolates from cvs Pinot noir (Zhu *et al.* 1998), Semillon (Abou-Ghanem *et al.* 1998), and Muscat of Alexandria (Meng *et al.* 2000) have been more or less extensively sequenced, whereas isolates from cvs Semillon (Abou-Ghanem *et al.* 1998), Muscat of Alexandria, and LN33 (Goszczynski *et al.* 1996 a) have been partially characterised biologically and physico-chemically. A new GLRaV-2 isolate was recently recovered by mechanical inoculation from an accession of *Vitis rupestris* St. George originally introduced from California, and some of its properties were determined.

Material and Methods

Virus source, mechanical transmission and purification: The virus isolate under study was identified serologically in a Californian accession of *Vitis rupestris* St. George (H4) grown in a grapevine collection of the University of Bari, Italy. Concentrated virus preparations were obtained from cortical scrapings of infected *V. rupestris* as described by Namba *et al.* (1991) and inoculated onto celite-dusted leaves of *Chenopodium quinoa*, *Ch. amaranticolor*, *Nicotiana benthamiana*, *N. occidentalis*, *N. clevelandii* and *N. tabacum* cvs Samsun and White Burley. Transmission to a wider herbaceous host range was attempted with sap from infected *N. benthamiana* extracted in 0.1 M phosphate buffer pH 7.2. For further characterization, the virus was purified from systemically infected herbaceous hosts according to Namba *et al.* (1991).

Coat protein analysis: Coat protein (CP) was dissociated by boiling purified GLRaV-2-H4 virus preparations for 5 min in Laemmli's buffer, electrophoresed in 5-12.5 % polyacrylamide slab gels (PAGE) in a discontinuous buffer system, and stained with Coomassie brilliant blue (Laemmli 1970). Reference M_r markers were dissociated coat protein subunits of GLRaV-2 isolate from cv. Semillon (GLRaV-2-Sem) and a MW-SDS-70L kit (Sigma Chemical Co., St. Louis, USA).

dsRNA extraction and analysis: Double-stranded RNAs (dsRNAs) were recovered from infected *N. benthamiana* by phenol extraction and chromatography through CF-11 cellulose column (Dodds 1993). Further purification was by selective treatments with RNase-free DNase (60 $\mu g \cdot ml^{-1}$) and DNase-free pancreatic RNase (0.5 $\mu g \cdot ml^{-1}$) (Saldarelli *et al.* 1994). Electrophoretic pattern and mobility of isolate H4 dsRNAs were determined in 1.5 % TBE-agarose gels under native conditions and compared with those of GLRaV-2-Sem (Abou-Ghanem *et al.* 1998).

Serology: An antiserum was raised in a rabbit immunized with intramuscular and intravenous injections of density gradient purified virus (about 1 mg of nucleoprotein per injection) at weekly intervals. The rabbit was bled one week after the last injection. The specificity and titre of the antiserum were determined by Western blot-

Correspondence to: Prof. Dr. G. P. Martelli, Università degli Studi di Bari, Dipartimento di Protezione delle Piante e Microbiologia Applicata, Via Amendola 165/A, 70126 Bari, Italy. Fax: +39-080-544-2911. E-mail: martelli@agr.uniba.it

ting (HU *et al.* 1990) and immunoelectron microscopy (MILNE and LUISONI 1977).

Molecular analysis: A set of primers GLR2CP1 (5' ATGGAGTTGATGTCCGAC3') and GLR2CP2 (5'TACATAACTTCCCTTCTACC3'), based on published nucleotide sequences of two GLRaV-2 isolates (ABOU-GHANEM *et al.* 1998; ZHU *et al.* 1998), was designed for amplifying the entire CP cistron. Substrate for amplification were dsRNAs extracted from infected *N. benthamiana,* denatured by 20 mM methyl mercuric hydroxide, random primed and reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT, Gibco BRL) prior to PCR. Cycling was as follows: cDNAs were denaturated at 94 °C for 2 min and the target region was amplified by 35 cycles of PCR (94 °C for 30 s, 50 °C for 35 s and 72 °C for 1 min) followed by final extension at 72 °C for 7 min. Amplified products were cloned into pGEM-T Easy vector, inserted in *Escherichia coli* DH5α cells, and sequenced by the dideoxynucleotide chain termination method (SANGER *et al.* 1977) using ^{35}S-ATP and the Thermo Sequenase cycle sequencing kit (Amersham). Nucleotide and protein sequences were analysed using the Strider 1.1 (MARCK 1988) and CLUSTAL W (THOMPSON *et al.* 1994) programs.

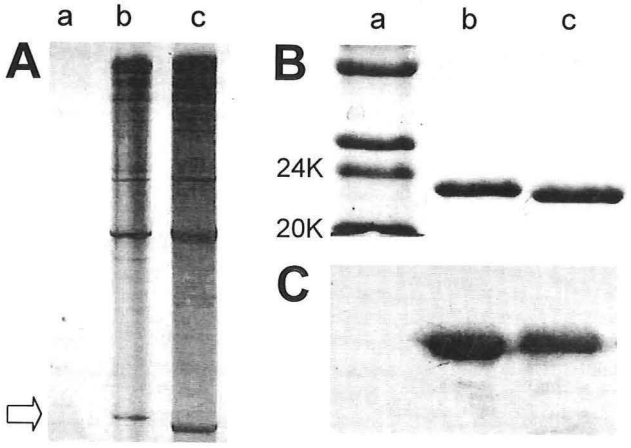

Fig. 1: **A:** Electrophoretic pattern of dsRNAs extracted from GLRaV-2-H4 and GLRaV-2-Sem infected *N. benthamiana* (lanes b and c). Healthy *N. benthamiana* control is in lane a. Note slight difference in the migration rate of the smallest dsRNA (arrow). **B:** Electropherogram of dissociated coat proteins of GLRaV-2-H4 (lane b) and GLRaV-2-Sem (lane c). M_r markers are in lane a. **C:** Western blots of dissociated coat proteins of GLRaV-2-Sem (lane b) and GLRaV-2-H4 (lane c) equally well recognized by the GLRaV-2-H4 antiserum. Lane a contains total proteins from healthy *N. benthamiana* (control).

Results and Discussion

Host range and symptomatology: Whereas GLRaV-2-Sem infected only *N. benthamiana,* GLRaV-2-H4 invaded systemically *N. benthamiana* inducing systemic vein clearing and curling of the leaves, and *N. occidentalis* in which it elicited chlorotic/necrotic local lesions followed by strong systemic clearing of the veins, apical necrosis and death of the plants. In *N. clevelandii* the virus induced local necrotic lesions followed by latent systemic infection as ascertained by back-inoculation onto *N. benthamiana*. Attempts to extend the herbaceous host range were unsuccessful.

Virus purification and physico-chemical properties: The virus was readily purified from both *N. occidentalis* and *N. benthamiana*. In sucrose density gradients it sedimented as a single band, which contained flexuous filamentous particles with prominent transverse striations, resembling closely to those of closteroviruses.

The electrophoretic pattern of dsRNAs extracted from infected *N. benthamiana* was virtually identical to that of GLRaV-2-Sem, except for the different mobility of the lowest band, interpreted as the double-stranded form of a subgenomic RNA, which migrated at a slightly slower rate (Fig. 1 A, arrow).

Dissociated CP preparations of GLRaV-2-H4 migrated at a slightly slower rate than those of GLRaV-2-Sem (Fig. 1 B). The difference in M_r was estimated to be 0.3 kDa (average of three determinations).

Serology: GLRaV-2-H4 was poorly immunogenic. In immunoelectron microscopy the antiserum decorated homologous and GLRaV-2-Sem virus particles up to a dilution 1:50. In Western blots, the same antiserum recognized equally well dissociated CPs of both the homologous virus and GLRaV-2-Sem (Fig. 1 C).

RT-PCR and sequencing: In different PCR runs, primers designed for amplifying the entire CP cistron gave products that were sequenced and found to have the same size (597 bp) of the comparable genes of the other two GLRaV-2 isolates sequenced so far (ABOU-GHANEM *et al.* 1998; ZHU *et al.* 1998). Thus, the slight variation in the electrophoretic migration rate of isolate H4 compared with that of GLRaV-2-Sem, is likely due to CP conformational reasons rather than to differences in protein size. Nucleotide sequence analysis showed that whereas the CP of GLRaV-2-H4 differed by about 12 % from CPs of both GLRaV-2-Sem and the GLRaV-2 isolate from Pinot noir from USA (ZHU *et al.* 1998), the two latter isolates had a virtually identical CP (about 99 % identity) (Fig. 2). The identity level between GLRaV-2-H4 and and the two mentioned GLRaV-2 isolates was 88 % and 94 % at the nucleotide and amino acid level, respectively (Fig. 2). Most of the variability was located in the N terminus of the CP.

Based on the present results it can be concluded that isolate H4 is a GLRaV-2 variant morphologically and serologically very close if not indistinguishable from GLRaV-2-Sem but differing in biological and molecular characteristics. Also its ultrastructural features conform to those reported for GLRaV-2-Sem (CASTELLANO *et al.* 2000) and the South African isolate of GLRaV-2 from Muscat of Alexandria (GOSZCYNSKI *et al.* 1996 b). Thus, the traits that characterize H4 as a GLRaV-2 isolate distinct from those described so far are the wider host range (ability to infect *N. occidentalis* and *N. clevelandii)* , the higher virulence to *N. benthamiana* and *N. occidentalis,* and sequence variation in the CP cistron.

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GLRaV-2 USA   MELMSDSNLSNLVITDASSLNGVDKKLLSAEVEKMLVQKGAPNEGIEVVFGLLLYALAAR
GLRaV-2 Sem   MELMSDSNLSNLVITDASSLNGVDKKLLSAEVVKMLVQKGAPNEGIEVVFGLLLYALAAR
GLRaV-2 H4    MELMSDENLNNLVITDATSLNGVDKKLLSAEVIKMLVQKGAPSDGIEVVFGLLLYALAAR
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GLRaV-2 USA   TTSPKVQRADSDVIFSNSFGERNVVVTEGDLKKVLDGCAPLTRFTNKLRTFGRTFTEAYV
GLRaV-2 Sem   TTSPKVQRADSDVIFSNSFGERNVVVTEGDLKKVLDGCAPLTRFTNKLRTFGRTFTEAYV
GLRaV-2 H4    TTSPKVQRADSDLIFSNNFGERNIAVTEGDLKKVLDGCAPLTRFTNKLITFGRTFTEAYI
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GLRaV-2 USA   DFCIAYKHKLPQLNAAAELGIPAEDSYLAADFLGTCPKLSELQQSRKMFASMYALKTEGG
GLRaV-2 Sem   DFCIAYKHKLPQLNAAAELGIPAEDSYLAADFLGTCPKLSELQQSRKMFASMYALKTEGG
GLRaV-2 H4    DFCIAYKHKLPQLNAAAELGIPAEDSYLAADFLGTCPKLSELQQSRKMFASMYALKTEGG
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GLRaV-2 USA   VVNTPVSNLRQLGRREVM
GLRaV-2 Sem   VVNTPVSNLRQLGRREVM
GLRaV-2 H4    VVNTPVSNLRQLGRREVM
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Fig. 2: Alignment of the predicted amino acid sequences of CP cistrons of GLRaV-2-H4, GLRaV-2-Sem and the GLRaV-2 isolate from Pinot noir. Asterisks indicate identical residues.

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