Grapevine virus A and grapevine virus D are serologically distantly related

by

E. Choueiri, N. Abou-Ghanem and D. Boscia

Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italia

S u m m a r y : Grapevine trichovirus A (GVA), B (GVB), and D (GVD) are not serologically related as ascertained by ELISA and IEM tests using polyclonal antisera. A study to investigate in detail their serological relationships was carried out with a larger number of reagents, including monoclonal antibodies (MAbs), and serological techniques (ELISA, IEM, tissue blot, Western blot). The results show that (i) polyclonal antisera to GVA, GVB and GVD cross-reacted in Western blot with all antigens; (ii) one out of 4 MAbs to GVA (MAb PA3.B9) reacted in ELISA, Western blot and tissue blot with the homologous virus and GVD but not with GVB. It is concluded that GVA, GVB and GVD are serologically distantly related and that the single antigenic determinant common to GVA and GVD is likely to be a cryptotope.

K e y   w o r d s : grapevine, trichovirus, GVA, GVB, GVD, serology, ELISA, Western blot, tissue blot.

Introduction

Grapevine virus D (GVD), a recently described new member of the trichovirus genus, closely resembles grapevine trichovirus A (GVA, Conti et al. 1980) in biological behaviour, particle size and morphology, cytopathology, dsRNA pattern, size of RNA, and organization of the 3' terminal genomic region (Abou-Ghanem et al. 1997). The coat protein of GVD has a M_r of 20.5 kDa, estimated by polyacrylamide gel electrophoresis, or 17.6 kDa, deduced from the amino acid sequence of the coat protein gene (Abou-Ghanem et al. 1997), values consistently lower than those reported for GVA, i.e., 22.5 and 21.5 kDa, respectively (Boccardo and D'Aquilio 1981; Castrovilli and Gallitelli 1985; Minafra et al. 1994). The coat proteins of GVA and GVD have a very high sequence homology (75 % of identical residues). However, the two viruses are not serologically related as ascertained by ELISA and immune electron microscopy (IEM) tests, using homologous and heterologous polyclonal antisera (Abou-Ghanem et al. 1997).

In the course of further studies, one of the monoclonal antibodies (MAb) of a panel of 4 raised to GVA (Boscia et al. 1992) gave a clear-cut response when tested in ELISA with sap extracted from GVD-infected *Nicotiana occidentalis*. This unexpected finding prompted additional investigations to establish whether, and to what extent, GVA and GVD are serologically related to each other, and to grapevine trichovirus B (GVB), MAbs to which are also available (Bonavia et al. 1996).

Material and methods

The present studies were based on comparative immunoenzymatic (DAS and TAS-ELISA) (Clark and Adams 1977) and IEM (Milne and Luisoni 1977) assays,

tissue blots (Hsu and Lawson 1991) and Western blots (Hu et al. 1990), for which the following reagents were used: (i) polyclonal antisera to GVA (Martelli et al. 1994), GVB (Boscia et al. 1993), and GVD (Abou-Ghanem et al. 1997); (ii) a panel of 4 MAbs to GVA denoted PA3.F5, PA3.C6, PA3.D11, and PA3.B9 (Boscia et al. 1992); (iii) a panel of 8 MAbs to GVB, named GB1E2G, GB5G11B, GB3H10C, GB6G11D, GB2H9D, GB6E5E, GB7C6E, and GB9C2E (Bonavia et al. 1996).

Antigens were viruses multiplied in *N. occidentalis* (GVB, GVD) or *N. benthamiana* (GVA) grown in a temperature-controlled glasshouse at 24-26 °C. In the trials, 11 GVA, 2 GVB and 1 GVD isolate were used.

E L I S A : In ELISA tests, carried out as described by Boscia et al. (1992), each virus isolate was tested separately with each of the polyclonal antisera and MAbs. The latter were used as supernatant culture fluids diluted 1/50 in phosphate buffered saline (PBS), pH 7.4. Plate coating was with polyclonal antisera, and MAbs were the revealing antibodies. Alkaline phosphatase-conjugated goat antimouse IgG was used at a dilution of 1:2000.

T i s s u e   b l o t s : Tissue blot assays (Hsu and Lawson 1991) were made using as reagents MAb PA3.B9 alone or in a cocktail with the other 3 anti-GVA MAbs (PA3.F5, PA3.C6, and PA3.D11). Symptomatic leaves of *Nicotiana* infected by GVA or GVD were rolled and transversely cut with a razor blade, then gently pressed on nitrocellulose membranes. Longitudinally or transversely sectioned petioles were prepared in the same way. The membranes were immersed in a blocking solution (PBS containing 1 % BSA) for 60 min at room temperature, then incubated at room temperature for 2 h with MAbs (primary antibodies) diluted in PBS with 0.5 % bovine serum albumin (BSA). After three washings of 10 min each in PBS-Tween 0.05 %, the membranes were exposed for 2 h at room temperature to alkaline phosphatase-labelled species specific secondary antibodies (anti-mouse IgGs), and

---

Correspondence to: Dr. D. Boscia, Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, I-70126 Bari, Italy. Fax: (080) 5442813.

stained by soaking in a solution of 14 mg nitroblue tetrazolium (NBT) and 7 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 40 ml of substrate buffer (0.1M Tris, 0.1M NaCl and 5mM $MgCl_2$, pH 9.5).

I m m u n o e l e c t r o n   m i c r o s c o p y : Decoration tests were done as described by MILNE and LUISONI (1977) by exposing virions extracted from infected *Nicotiana* sap to polyclonal antisera and MAbs, all undiluted.

W e s t e r n   b l o t s : Dissociated GVA, GVB and GVD capsid proteins (CP) were electrophoresed in SDS-PAGE discontinuous system (LAEMMLI 1970) and transferred to PVDF membranes with a semi-dry Trans-blot cell apparatus (Bio-rad Laboratories, Hercules CA) as described by Hu *et al.* (1990). After overnight blocking in 5 % non-fat milk powder, 1 % BSA and 0.05 % Tween-20 in TBS buffer (0.02M Tris, 0.5M NaCl) at 4 °C, the membranes were incubated for 2 h at 37 °C with polyclonal antisera to GVA, GVB and GVD, and monoclonal antibodies to GVA (PA3.B9; PA3.C6, PA3.F5 and PA3.D11) and GVB (GB6G11D) diluted 1:300 in the same buffer. The membranes were then washed in TBS buffer containing 0.5 % Tween-20 and incubated for 1 h at 37 °C in alkaline phosphatase-conjugated anti-rabbit and anti-mouse IgGs diluted 1:2000 in the blocking solution. After further washing, the membranes were stained by soaking in a solution of 14 mg nitroblue tetrazolium (NBT) and 7 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 40 ml of substrate buffer (0.1M Tris, 0.1M NaCl and 5mM $MgCl_2$ pH 9.5) (HSU and LAWSON 1991).

Results

E L I S A : In the trials, all polyclonal antisera recognized only the homologous antigens. Similarly, MAbs to GVA and GVB reacted specifically with the homologous viruses, regardless of the isolate, except for GVA MAb PA3.B9 which recognized also the single GVD isolate tested. The reaction of MAb PA3.B9 with GVD was positive regardless of whether polyclonal antisera to GVA or GVD were used for plate coating. However, the intensity (i.e., A_{405} readings) of the heterologous reaction (GVA MAb PA3.B9/GVD) was consistently lower than that of the homologous reaction (GVA MAb PA.3B9/GVA) (Table).

T i s s u e   b l o t s : Very strong positive reactions were obtained with blots from GVA-infected tissues exposed either to the GVA MAb cocktail (Fig. 1 A2, B3) or to MAb PA3.B9 alone. Much milder but still positive reactions were also obtained with blots from GVD-infected plants treated with MAb PA3.B9 (Fig. 1 B2), thus confirming the ELISA results.

I m m u n o e l e c t r o n   m i c r o s c o p y : In accordance with previous results (BOSCIA *et al.* 1993, 1994; BONAVIA *et al.* 1996; ABOU-GHANEM *et al.* 1997), polyclonal antisera decorated only homologous viruses (not shown). Similarly, MAb PA3.F5, but none of the others, decorated GVA particles (BOSCIA *et al.* 1992), whereas of the 8 MAbs to GVB, only 6 decorated homologous particles (BONAVIA *et al.* 1996). No heterologous decorations by any of the

Results of ELISA tests with 4 monoclonal antibodies to GVA and homologous (isolate GVA-PA3) and heterologous (GVD) antigens in infected sap of *Nicotiana* [a]

	Monoclonal antibodies to GVA			
	PA3.B9	PA3.C6	PA3.D11	PA3.F5
Coating with the polyclonal antiserum to GVA Antigens				
GVD	**0.703**	0.058	0.062	0.049
GVA-PA3	**1.871**	**1.859**	**1.896**	**1.071**
Control[b]	0.061	0.068	0.054	0.032
Coating with the polyclonal antiserum to GVD Antigens				
GVD	**0.546**	0.043	0.037	0.032
GVA-PA3	**1.611**	**1.104**	**1.925**	**0.451**
Control[b]	0.062	0.059	0.068	0.043

[a]   Figures are mean A_{405} values of seven replicates. Positive reactions are in bold.

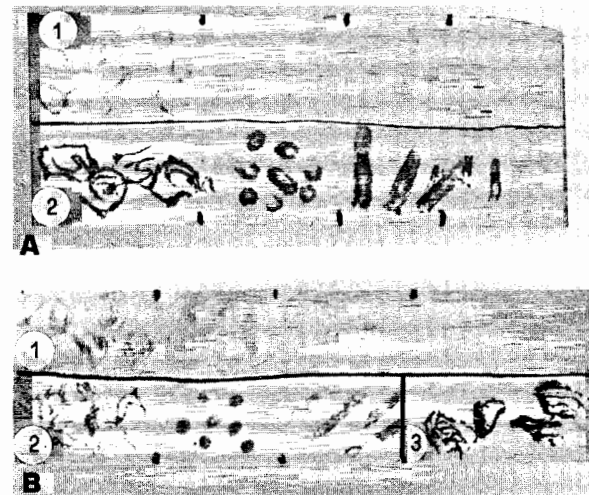[b]   Controls were extracts from healthy *Nicotiana*.

Fig. 1: Tissue blots of cross-sectioned rolled-up leaves and transversally- and longitudinally-sectioned petioles of symptomatic and healthy (control) *Nicotiana* leaves. Panel **A**: 1, control; 2, blots from GVA-infected plants exposed to a cocktail of 4 monoclonal antibodies to GVA. Panel **B**: 1, control; 2, blots from GVD-infected plants exposed to GVA MAb PA3.B9; 3, blots from GVA-infected plants exposed to GVA MAb PA3.B9. Note that the homologous reaction is much stronger than the heterologous.

MAbs were detected. GVD particles were not decorated by any of the heterologous polyclonal antisera nor by any of the MAbs to GVB or GVA, including MAb PA3.B9 (not shown).

W e s t e r n   b l o t s : Polyclonal antisera to GVA, GVB and GVD clearly recognized in Western blots dissociated CPs of homologous and heterologous viruses (Fig. 2 A, B, C). By contrast, virus specific reactions were given by most of the MAbs: anti GVA MAb PA3.C6 (Fig. 2, D) and MAbs PA3.D11 and PA3.F5 (not shown) recognized only GVA CP, whereas anti GVB MAb GB6G11D reacted only with GVB CP (Fig. 2, E). Anti
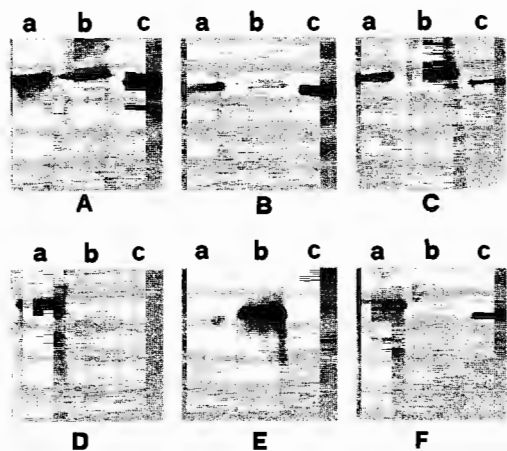
Fig. 2: Western blots of dissociated coat protein of GVA (lane a), GVB (lane b) and GVD (lane c) exposed to polyclonal antisera to GVA (A), GVB (B), and GVD (C) and to MAb PA3.C6 to GVA (D); MAb GB6G11D to GVB (E), and MAb PA3.B9 to GVA (F). All polyclonal antisera recognize dissociated coat protein of homologous and heterologous viruses, whereas of the monoclonal antibodies only MAb PA3.B9 to GVA reacts with the homologous virus and GVD.

GVA MAb PA3.B9 was an exception because it reacted with CPs of both GVA and GVD. The homologous reaction, however, was always stronger than the heterologous (Fig. 2, F).

Discussion

The present investigation has shown that GVA, GVB and GVD are serologically related. This conclusion, which apparently contradicts previous reports (BOSCIA et al. 1993; ABOU-GHANEM et al. 1997) is based on two lines of evidence: (i) the discovery that polyclonal antisera to GVA, GVB, and GVD cross-react with dissociated capsid proteins of homologous and heterologous viruses; (ii) the identification of a monoclonal antibody to GVA (MAb PA3.B9) that reacts also with GVD in ELISA and Western blot.

The lack of serological relationship between grapevine trichoviruses had been suggested because no cross-reactivity had ever been detected with virus-specific polyclonal antisera in both ELISA tests and IEM decoration assays (BOSCIA et al. 1993, 1994; BONAVIA et al. 1996; ABOU-GHANEM et al. 1997). The careful repetition of the same experiments in this study has largely confirmed earlier findings. Polyclonal antisera recognized only homologous viruses in ELISA and failed to decorate heterologous virus particles, and Mabs to surface antigenic determinants of GVA and GVB decorated only homologous virions. Anti GVA MAb PA3.B9 was no exception, because it did not decorate GVA, as previously reported (BOSCIA et al. 1992), nor GVD particles (present work). However, this monoclonal antibody reacted with dissociated GVD coat protein (Western blot) as well as with extracts from GVD-infected plants in ELISA and tissue blot, two conditions in which non-aggregated viral CP present in the cytoplasm of infected cells is available for serological recognition.

This behaviour is consistent with the conclusion that the presently identified single antigenic determinant common to GVA and GVD is a cryptotope. It is also likely that the existence of common internal antigenic determinants accounts for the broader serological relationship between both these viruses and GVB as disclosed by Western blots with dissociated CP. The relationship among GVA, GVB, and GVD is too distant to question the taxonomic status of these viruses, which remain separate species. However, it constitutes further evidence for the closeness already shown by molecular studies (SALDARELLI et al. 1996; MINAFRA et al. 1997).

Acknowledgements

References

ABOU-GHANEM, N.; SALDARELLI, P.; MINAFRA, A.; BUZKAN, N.; CASTELLANO, M. A.; MARTELLI, G. P.; 1997: Properties of grapevine virus D, a novel putative trichovirus. J. Plant Pathol. 1 (in press).

BOCCARDO, G.; D'AQUILIO, M.; 1981: The protein and nucleic acid of a closterovirus isoated from a grapevine with stem pitting symptoms. J. Gen. Virol. 53, 179-182.

BONAVIA, M.; DIGIARO, M.; BOSCIA, D.; BOARI, A.; BOTTALICO, G.; SAVINO, V.; MARTELLI, G. P.; 1996: Studies on "corky rugose wood" of grapevine and on the diagnosis of grapevine virus B. Vitis 35, 53-58.

BOSCIA, D.; ABOU-GHANEM, N.; SALDARELLI, P.; MINAFRA, A.; CASTELLANO, M. A.; GARAU, R.; SAVINO, V.; MARTELLI, G. P.; 1994: A comparative study of grapevine virus B isolates. Riv. Patol. Veg. (S V) 4, 11-24.

- -; ASLOUJ, E.; ELICIO, V.; SAVINO, V.; CASTELLANO, M. A.; MARTELLI, G. P.; 1992: Production, characterization and use of monoclonal antibodies to grapevine virus A. Arch. Virol. 127, 185-194.

- -; SAVINO, V.; MINAFRA, A.; NAMBA, S.; ELICIO, V.; CASTELLANO, M. A.; GONSALVES, D.; MARTELLI, G. P.; 1993: Properties of a filamentous virus isolated from grapevines affected by corky bark. Arch. Virol. 130, 109-120.

CASTROVILLI, S.; GALLITELLI, D.; 1985: A comparison of two isolates of grapevine virus A. Phytopathol. Medit. 24, 219-220.

CLARK, M. F.; ADAMS, A. N.; 1977: Characteristics of the micro-plate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34, 475-483.

CONTI, M.; MILNE, R. G.; LUISONI E.; BOCCARDO, G.; 1980: A closterovirus from a stem pitting-diseased grapevine. Phytopathology 70, 394-399.

HSU, H. T.; LAWSON, R. H.; 1991: Direct tissue blotting for detection of tomato spotted wilt virus in impatiens. Plant Dis. 75, 292-295.

HU, J. S.; GONSALVES, D.; TELIZ, D.; 1990: Characterization of closterovirus-like particles associated with grapevine leafroll disease. J. Phytopathol. 128, 1-14.

LAEMMLI, U.K.; 1970: Cleavage of structural proteins during the assembly of the head of bacterial phage T4. Nature 227, 680-685.

MARTELLI, G.P.; BOSCIA, D.; CHOUEIRI, E.; DIGIARO, M.; CASTELLANO, M.A.; SAVINO, V.; 1994: Occurrence of filamentous viruses and rugose wood of grapevine in Yemen. Phytopathol. Medit. 33, 146-151.

MILNE, R. G.; LUISONI, E.; 1977: Rapid immune electron microscopy of virus preparations. In: MARAMORSCH, K.; KOPROWSKI, H. (Eds.): Methods in Virology 6, 265-281. Academic Press, New York, USA.

MINAFRA, A.; SALDARELLI, P.; GRIECO, F.; MARTELLI, G. P.; 1994: Nucleotide sequence of the 3' terminal region of the RNA of two filamentous grapevine viruses. Arch.Virol 137, 249-261.

- -; - -; MARTELLI, G. P.; 1997: Grapevine virus A: nucleotide sequence, genome organization, and relationships in the Trichovirus genus. Arch. Virol. 142, 417-423.

SALDARELLI, P.; MINAFRA, A.; MARTELLI, G. P.; 1996: The nucleotide sequence and genomic organization of grapevine virus B. J. Gen. Virol. 77, 2645-2652.