

## Molecular detection of Grapevine fleck virus-like viruses

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### Summary

**Molecular reagents have been developed for virus-specific and simultaneous (virus-non-specific) detection of Grapevine fleck virus (GFkV) and allied viruses, i.e. Grapevine asteroid mosaic-associated virus (GAMaV) and Grapevine red globe virus (GRGV). Degenerate primers designed on nucleotide sequences of the RNA-dependent RNA polymerase (RD) and methyltransferase (MTR) domains of the GFkV genome, were able to give amplification products of the expected size from total nucleic acid extracts of: (i) vines infected with GFkV, GAMaV, and GRGV; (ii) a Californian grapevine accession infected by a marafi-like virus; (iii) Greek grapevine accessions infected by an unidentified agent that induced symptoms reminiscent of those elicited by GAMaV in *Vitis rupestris*. Degenerate primers designed on the nucleotide sequence of the helicase (HEL) domain of the GFkV genome recognized all the above viruses except for GAMaV and the unidentified Greek viral agent. RD primer set worked well also with crude grapevine cortical scrapings, thus constituting a useful universal reagent for the non-specific molecular identification of GFkV-like viruses in *Vitis*. The marafi-like virus from California was amplified by all sets of primers, but was recognized only by the GRGV-specific probe, suggesting that it is a likely isolate of GRGV. Likewise, the unidentified virus from Greek vines shared sequence homology with GFkV and allied viruses (GAMaV and GRGV) but exhibited differences relevant enough that call for further investigations to establish its taxonomic position. While GRGV was identified, though with a very low incidence, in some 11 southern Italian grapevine cultivars, no evidence was obtained for infection by GAMaV in any of 50 cultivars analyzed.**

**Key words:** Grapevine fleck virus, Grapevine red globe virus, Grapevine asteroid mosaic-associated virus, diagnosis, riboprobes, hybridization, RT-PCR.

### Introduction

Grapevine (*Vitis vinifera* L.) is latently infected by a number of isometric, phloem-limited, non-mechanically trans-

missible viruses sharing similar morphological, biological, cytopathological and molecular features (SABANADZOVIC *et al.* 2000). Two of these viruses, Grapevine fleck virus (GFkV) and Grapevine asteroid mosaic-associated virus (GAMaV), but not Grapevine red globe virus (GRGV), induce characteristic symptoms in *Vitis rupestris*, an indicator extensively used for diagnosis (MARTELLI 1993). Serological detection is possible for GFkV (BOSCIA *et al.* 1991, 1995; RAMEL *et al.* 1993; WALTER and CORNUET 1993; SCHIEBER *et al.* 1997) but for none of the other viruses as no antisera are available.

Recently developed sets of degenerated primers have provided an insight of the molecular structure of GAMaV and GRGV genomes, showing their relationship with GFkV and members of the genera Marafivirus and Tymovirus and indicating the possible use of molecular tools for virus diagnosis (SABANADZOVIC *et al.* 2000).

As reported in this paper, RT-PCR and molecular hybridization tests have been developed for sensitive detection of GAMaV and GRGV in field-grown grapevines. Furthermore, the relationship of these viruses with a marafi-like virus from California (ZHANG and ROWHANI 2000) and another agent associated with asteroid mosaic-like symptoms in Greece (KYRIAKOPOULOU 1991; KYRIAKOPOULOU *et al.* 1993) was investigated, and a survey of the presence of these viruses was conducted in a grapevine germplasm collection of the University of Bari on 100 accessions from 50 different cultivars of Southern Italy.

### Material and Methods

**Virus source:** Virus sources used in this study were: (i) LN33 MT48 and *Vitis vinifera* BU51 infected with GFkV (BOULILA *et al.* 1990; BOSCIA *et al.* 1991); (ii) *Vitis rupestris* USA9 and USA11, infected with an authentic Californian isolate of GAMaV (BOSCIA *et al.* 1994); (iii) *V. vinifera* accessions RG40/5 and RG15/10, infected by GRGV (SABANADZOVIC *et al.* 2000); (iv) *V. vinifera* accessions GR7-21, GR7-23, and GR8-19, reported from Greece to induce asteroid mosaic-like symptoms in *V. rupestris* (KYRIAKOPOULOU 1991); (v) a *V. vinifera* accession from California (ZIV8) infected by a marafi-like virus which induces symptomless infection in *V. rupestris* (ZHANG and ROWHANI 2000). Mature canes from field-grown vines were used for total nucleic

acid (TNA) extraction and molecular testing. Apart from MT48 and BU51, none of these virus sources contained GFkV, as ascertained by repeated ELISA testing with GFkV-specific monoclonal antibodies (BOSCIA *et al.* 1995).

**Total nucleic acid extraction:** TNA was extracted essentially according to WHITE and KAPER (1989), by grinding in liquid nitrogen in the presence of 3 ml of STE buffer containing 1 % SDS and 2 % mercaptoethanol, 300 mg of cortical scrapings obtained by shaving the cortex of mature grapevine canes with a scalpel. The extract was then mixed with phenol/chloroform (1:1) and centrifuged for 5 min at 10,000 *g*. After the addition of absolute ethanol to the supernatant to reach a concentration of 35 % and of cellulose CF-11, the mixture was kept at room temperature under gentle shaking for 45 min before washing twice with STE buffer containing 35 % ethanol and centrifuging at 10,000 *g* for 5 min. After a final washing, the cellulose pellet was allowed to dry before elution of nucleic acids with 100 µl of RNase-free water.

**RT-PCR:** 5 µl of TNA or cortical scraping extracts were denatured for 5 min at 95 °C, primed with 1 µg of random DNA hexanucleotides (Roche Biochemicals) and reverse transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) in a 30 µl reaction for 1 h at 39 °C. Three sets of degenerate primers were used for PCR (Table), *i.e.* the previously reported sets designed on the methyltransferase (MTR) and the RNA-dependent RNA polymerase (RD) domains of GFkV genome (SABANADZOVIC *et al.* 2000), and a new primer set, denoted HEL, designed on GFkV helicase sequences. Reverse transcription from crude sap extracts was as described by SABANADZOVIC *et al.* (1996).

**Preparation of riboprobes:** MTR- and RD-generated PCR products from GAMaV and GRGV, were cloned into pGEM-T Easy vector and the corresponding cRNA probes were transcribed using T7/Sp6 RNA Dig Labelling kit (Roche Biochemicals) after linearization of plasmids with *SacI*. The probes were denoted pMTRGV (572 nts) and pRpRGV (386 nts) for GRGV, and pMTRAM (575 nts) and pRpAM (386 nts) for GAMaV. All probes were successfully tested in early cross-hybridization trials. However, pRpRGV

and pRpAM gave more consistent results and were selected for use in later experiments.

**Molecular hybridization:** TNAs corresponding to 150 mg of tissue were denatured with 50 mM NaOH and 2.5 mM EDTA before spotting onto Hybond N+ membrane (Amersham). Molecular hybridization with Dig-RNA probes was done according to manufacturer's instruction (Roche Biochemicals) at 60 °C for pRpGV and pRpAM or 62 °C for pMTRGV and pMTRAM.

## Results and Discussion

**RT-PCR detection:** Degenerate primer sets MRT and RD amplified specific products from reverse-transcribed TNAs from GFkV-, GRGV-, and GAMaV-infected vines (Fig. 1 A, B). By contrast, primer set HEL was partially discriminative, as it yielded products of the expected size (296 bp) from GFkV- and GRGV- infected samples (Fig. 1 C lanes d and e) but gave no visible amplification from GAMaV sources.

Primer sets MTR and HEL failed to yield amplification products when used for RT-PCR on crude cortical scraping extracts (not shown). By contrast, set RD consistently gave amplicons of the expected size (Fig. 1 D, lanes c, d, f, and h).

Based on the above, and limited to the viruses considered in the present investigation, the conclusion appears tenable that the degenerate primer set designed on the RNA-dependent RNA polymerase domain of the GFkV genome, represents a useful universal reagent for the non-specific molecular identification of GFkV-like viruses in grapevines.

**Molecular hybridization:** Dig-RNA probes to GAMaV (pRpAM) and GRGV (pRpRGV) recognized specifically homologous viruses (Fig. 2 A, row 1 c, 1 d; 2 B, row 1 a, 1 b) but not two GFkV isolates (Fig. 2 A, B rows 2 a and 2 b) nor the healthy controls (Fig. 2 A and B, rows 2 c and 2 d). The same results were obtained using a mixture of the two probes on mix-infected samples (Fig. 2 C).

Dot blot hybridization with Dig-RNA probes showed the presence of GRGV in 12 accessions of 11 different cultivars out of 50, *i.e.* cvs Barbarossa, Biancolella, Bianco

Table

Degenerate primers for the amplification of the methyltransferase, polymerase and helicase coding sequences of grapevine fleck virus-like viruses

Primer	Nucleotide sequence	MgCl <sub>2</sub> (mM)	Annealing temp. (°C)	Product (bp)
MTR1	5'TTCATGCAYGAYGCYMTSATGT3'	3.0	50	572-575
MTR2	5'TCCCAVGCNBHBGVRGTGACCCA3'			
RD1	5'CYCARCAYAARGTVAACGA3'	2.0	50	386
RD2	5'GCGCATGCABGTSAGRGGG3'			
HEL1	5'GYTTYSCHGGYTGYGGHAA3'	3.0	55	296
HEL2	5'TGCAGRGGRTCCCRAGVRBKAT3'			

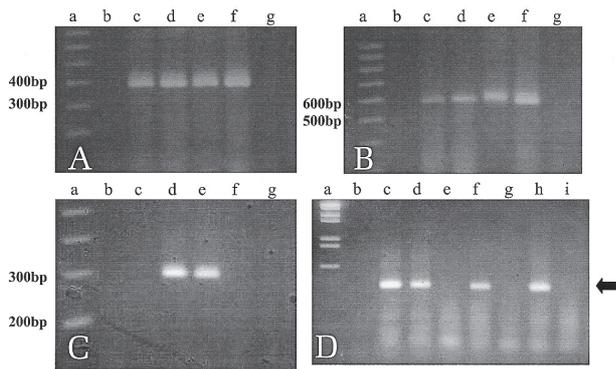


Fig. 1: Gel electrophoresis of PCR products from reverse-transcribed total nucleic acid extracts from grapevine sources infected by different viruses using degenerate primers. In all panels but D, SIGMA mol. wt ladder is in lane a, healthy control in lane b, water control in lane g. **A:** Evidence that RD primer set amplifies a product of the expected size (386 bp) from: GAMaV-infected sources USA 9 and USA 11 (lanes c and f); GFkV-infected source MT48 (lane d); GRGV-infected source RG40/5 (lane e). **B:** Evidence that MRT primer set amplifies a product of the expected size (575 bp) from: GAMaV-infected sources USA 9 and USA 11 (lanes c and f); GFkV-infected source MT48 (lane d); GRGV-infected source RG40/5 (lane e). **C:** Evidence that HEL primer set amplifies a product of the expected size (296 bp) from: GFkV-infected source MT48 (lane d) and GRGV-infected source RG40/5 (lane e), but not from GAMaV-infected sources USA 9 and USA 11 (lanes c and f). **D:** RT-PCR products from reverse-transcribed crude extracts from cortical scrapings from grapevine sources infected by different viruses using the RD primer set. Positive amplification from GAMaV-infected sources USA 9 and USA 11 (lanes c and d); GRGV-infected source RG40/5 (lane f), and GFkV-infected source MT48 (lane h). Arrows points to the 386 bp amplicon.

d'Alessano, Greco di Tufo, Montepulciano, Ottavianello, Pagadebiti, Sangiovese, Sanguinello Uva Carrieri, Uva della Scala. GAMaV was not detected in any of 100 grapevine accessions analyzed.

**Analysis of Californian and Greek grapevine accessions:** RT-PCR on TNA extracts from Californian (ZIV8) and Greek grapevines using degenerate MTR and RD primers confirmed the presence of GFkV-like viruses in both, yielding amplification products of the expected size, *i.e.* 386 bp with set RD (Fig. 3 A, lanes c and f) and 575 bp with set MTR (Fig. 3 B, lanes c and f), that matched the amplicons from grapevine sources infected by GFkV (Fig. 3 A, lane e; 3 B, lane e) and GRGV (Fig. 3 A, lane d; 3 B, lane d). However, HEL primers amplified extracts from Californian ZIV8 (Fig. 3 C, lane c), GFkV-infected MT48 (Fig. 3 C, lane e), GRGV-infected RG15/10 (Fig. 3 C, lane d), but not from any of the Greek accessions (Fig. 3 C, lane f), thus indicating the possible existence of a difference in the sequence homology of the viral helicase domain between the unidentified Greek virus and all the others.

Dot blot hybridization tests clearly showed that the Dig-RNA probe specific to GRGV reacted with TNA from ZIV8 (Fig. 4 A, row 2 a) and the positive control (Fig. 4 A, row 1 a) but with none of the TNAs from sources infected by GFkV (Fig. 4 A, row 1 a, 1 c), GAMaV (Fig. 4 A row 1 d) and by the unidentified Greek virus (Fig. 4 A, row 2 b, 2 c). The probe

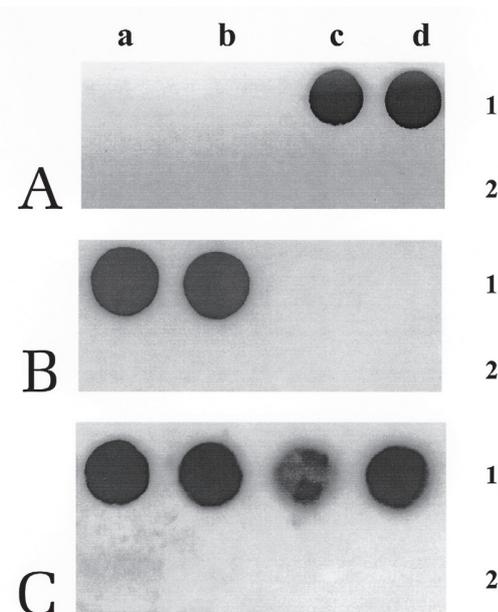


Fig. 2: Results of dot blot hybridization with Dig-RNA probes pRpAM and pRpRG of total nucleic acid extracts from sources infected by different viruses. **A:** Evidence that GAMaV probe pRpAM hybridizes only with extracts from sources USA 9 (row 1 c) and USA 11 (row 1 d) infected by the homologous virus. 2 a and 2 b are GFkV-infected sources MT48 and BU51; 1 a and 1 b are GRGV-infected sources RG15/10 and RG40/5; 2 c and 2 d are healthy controls. **B:** Evidence that GRGV probe pRpRG hybridizes only with extracts from sources RG15/10 (row 1 a) and RG40/5 (row 1 b) infected by the homologous virus. Extracts from: GFkV-infected sources MT48 and BU51 in 2 a, and 2 b; GAMaV-infected sources USA 9 and USA 11 in 1 c and 1 d; healthy controls in 2 c and 2 d. **C:** Evidence that a mixture of the two probes used on a mixed sample of TNAs from sources infected by GAMaV, GRGV and GFkV hybridize specifically with homologous viruses (GRGV, row 1 a and 1 b: GAMaV, row 1 c and 1 d) but not with GFkV (row 2 a and 2 b). Healthy controls are in 2 c and 2 d.

specific to GAMaV, gave hybridization signals only with the source infected by the homologous virus (Fig. 4 B, row 1 d).

In conclusion, the present study has shown that RT-PCR with degenerate primer sets, especially those designed on GFkV polymerase domain, can detect non-specifically a number of grapevine viruses bearing molecular similarities to GFkV, and that this procedure can also be utilized for picking up still unidentified viral agents. On the other hand, the use of virus-specific riboprobes allows identification at the species level apparently with a high degree of confidence.

Thus, the marafi-like virus from California (ROWHANI *et al.* 2000; ZHANG and ROWHANI 2000) was amplified by all sets of primers, but was recognized only by the GRGV-specific probe, suggesting that it is a likely isolate of GRGV. Likewise, the unidentified virus from Greek vines appeared to share sequence homology with GFkV and allied viruses (GAMaV and GRGV), so as to be regarded as a GFkV-like virus. However, this virus had biological (*i.e.* *V. rupestris* reaction differing from that induced by GFkV, GAMV, and GRGV) and molecular (*i.e.* no amplification by HEL set of primers, no recognition by virus-specific riboprobes) differences relevant enough to call for further investigations to establish its taxonomic position.

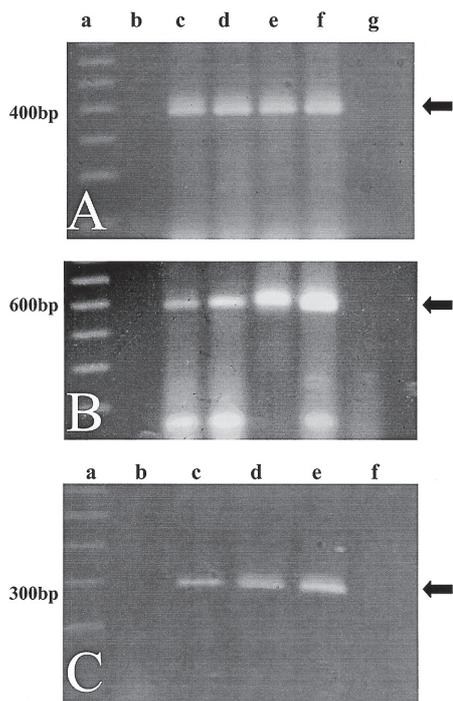


Fig. 3: PCR products from reverse-transcribed total nucleic acid extracts from grapevine sources infected by different viruses using degenerate primers. SIGMA mol. wt ladder is in lane a, healthy control in lane b, water control in lane g. **A:** Evidence that the RD primer set amplifies a product of the expected size (386 bp) from: Californian source ZIV8 infected by a marafi-like virus (lane c); GRGV-infected source RG40/5 (lane d); GFkV-infected source MT48 (lane e); Greek source 8-19 infected by an unidentified virus (lane f). **B:** Evidence that the MRT primer set amplifies a product of the expected size (575 bp) in tests with the same arrangement as in panel A. **C:** Evidence that the HEL primer set amplifies a product of the expected size (296 bp) in tests with the same arrangement as in panel A.

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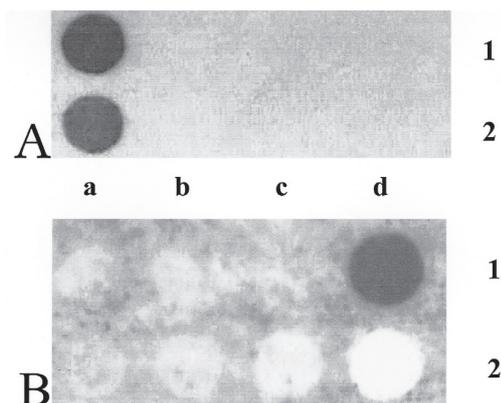


Fig. 4: Results of dot blot hybridization with Dig-RNA probes pRpAM and pRpRG of total nucleic acid extracts from sources infected by different viruses. **A:** Evidence that GRGV probe pRpRG hybridizes only with extracts from source RG15/10 (row 1 a) infected by the homologous virus and the Californian source ZIV8 infected by a marafi-like virus (row 2 a). No hybridization signal is visible with extracts from: GFkV-infected sources MT48 and BU51 (row 1 b, and 1 c); GAMaV-infected source USA 9 (row 1 d); Greek sources GR 8-19 and GR7-21 infected by an unidentified virus (row 2 b and 2 c). Healthy control in 2 d. **B:** Evidence that GAMaV probe pRpAM hybridizes only extracts from sources USA 9 (row 1 d) infected by the homologous virus. No hybridization signal is visible with extracts from: GRGV-infected source RG15/10 (row 1 a); GFkV-infected sources MT48 and BU51 (row 1 b, and 1 c); Californian source ZIV8 infected by a marafi-like virus (row 2 a); Greek sources GR 8-19 and GR7-21 infected by an unidentified virus (row 2 b and 2 c). Healthy controls in 2 d.

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