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Molecular diagnosis of grapevine fleck virus

by

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Summary: A digoxigenin-labelled riboprobe was developed for the detection of grapevine fleck virus (GFkV) in infected tissues of grapevine leaves, roots and canes. The probe was GFkV-specific and was successfully used for virus identification both with dot spot assays, using alkali-treated crude sap, and tissue blot assays, using cross and longitudinal sections of leaf petioles. Primers designed for the amplification by reverse transcription-polymerase chain reaction of a viral genome fragment 243 nucleotides in size, gave also positive and repeatable results. These newly developed molecular-based detection tools extend the range of available procedures for the sensitive identification of GFkV in naturally infected hosts.

Key words: diagnosis, fleck, asteroid mosaic, molecular hybridization, riboprobes, digoxigenin-mediated chemiluminescence, RT-PCR, dot spot, tissue blot.

Introduction

The causal agent of fleck (GFkV), a worldwide occurring disease of the grapevine (*Vitis* spp.), latent in European cultivars and most American rootstocks, is a phloem-limited, non-mechanically transmissible, isometric virus (Boscia *et al.* 1991). GFkV resembles morphologically tymoviruses (Koenig *et al.* 1995) and a virus associated with grapevine asteroid mosaic (Boscia *et al.* 1994), but is taxonomically still unclassified.

A molecular characterization of GFkV was initiated by producing a library of DNA clones complementary to its genomic RNA. In this paper we report on the use of some of these clones for developing molecular tools for sensitive detection of GFkV in grapevine tissues.

Materials and methods

Virus source and purification: Three different GFkV isolates were used: MT48 from LN33, which was the object of previous studies (Boscia *et al.* 1991), UR4 and USA6, both from *Vitis vinifera* accessions. Two sources (H4 and L3) of asteroid mosaic propagated in *V. rupestris* were also used in hybridization tests.

GFkV was purified from young roots and petioles as described by Boulila *et al.* (1990). Viral RNA was extracted from purified virus by the SDS-phenol method (Diener and Schneider 1968) and electrophoresed in 1.2 % agarose-TBE (Tris 100 mM, boric acid 100 mM, EDTA 2.5 mM, pH 8.3) gels after denaturation for 5 min at 65 °C in 50 % deionized formamide.

Synthesis of cDNA and cloning: 5 µg of GFkV-MT48 RNA were reverse-transcribed with random hexanucleotides and complementary dsDNA synthesized using cDNA system plus (Amersham, UK). Transcribed cDNA was treated with T4 DNA polymerase and the DNA ligated to pGEM-4z-*Sma*I cut plasmids. Recombinant plasmids were obtained by transforming competent *Escherichia coli* DH5α and subsequent selection was done on ampicillin-containing plates (75 µg/ml) in the presence of 40 µl per plate of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; stock solution 20 mg/ml). X-gal negative colonies were grown in liquid medium, plasmids were recovered and the size of cloned inserts analyzed by agarose gel. cDNA clones were sequenced with a Termosequenase kit (Amersham, UK) using ^{35}S-ATP. All molecular techniques were performed according to Sambrook *et al.* (1989).

Preparation of riboprobes and molecular hybridization: Out of a library of 20 cDNA clones, spanning from about 250 to 1500 nucleotides (unpubl. information), clone GFkV20 was chosen as a template for the production of a cRNA digoxigenin-labelled probe (Dig-RNA). This clone, which contained a 270 bp insert between *Eco*RI and *Hind*III restriction sites, was linearized with *Eco*RI. Synthesis of Dig-RNA probes was done with a commercial kit (Boehringer Mannheim, Germany) using T7 or SP6 RNA polymerase on 1 µg of the selected plasmid. Quality and yield of transcribed RNA was analyzed in agarose gel electrophoresis by comparison with known quantities of marker RNAs.

Total nucleic acids from grapevine leaves, roots, and cortical scrapings of mature canes were extracted as previously described (Saldarelli *et al.* 1994 a). Double stranded RNAs were extracted from cortical scrapings of infected and healthy vines, fractionated by CF11 cellulose chromatography (Hu *et al.* 1990), and purified by enzymatic

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digestion with DNAse and RNAse (SALDARELLI *et al.* 1994 b). Molecular hybridization tests with Dig-RNA probes were performed as described by GALLITELLI and SALDARELLI (1995).

Virus detection in grapevine crude sap: 100 mg samples of young root, petiole, and leaf tissues were ground in 2 ml of 40 mM NaOH, 2.5 mM EDTA and the homogenate centrifuged at 6000 *g* for 3 min (MAULE *et al.* 1983). 50 µl of the supernatant were applied to a 2 x SSC (20 x SSC : 3 M NaCl, 300 mM tri-sodium citrate) prewetted Hybond N+ (Amersham, UK) membrane as such, or after serial dilution in the same extraction buffer up to 1:500.

Tissue blotting: Longitudinally and cross-sectioned grapevine leaf petioles were blotted by pressing on a membrane that was (method 1) placed dry on top of a Whatman paper strip saturated with 40 mM NaOH containing 2.5 mM EDTA. After incubation for 2 min for denaturing nucleic acids, the membrane was soaked for 5 min in 1 M Tris-HCl pH 7.5, then washed sequentially with 2 x SSC and absolute ethanol (ABOU JAWDAH *et al.* 1995); (method 2) pre-soaked in the 40 mM NaOH, 2.5 mM EDTA solution; (method 3) pre-equilibrated with 0.5 % SDS and 100 µg/ml proteinase K (NAVOT *et al.* 1989).

Reverse transcription-polymerase chain reaction: GFkV-specific oligonucleotides FkV1 (5'AGTACCTCCTCCACCGCACC3' sense primer) and FkV2 (5'TTTCTCGGGCAGAGAGCCGTCC3' antisense primer) were designed by computer analysis (Primer selection program, Henry M. Jackson Foundation, Bethesda, Maryland, USA) on the sequence of clone GFkV11rp (unpubl. information). These primers were supposed to amplify a 243-nucleotide fragment of the GFkV genome. About 100 mg of cortical scrapings from dormant cuttings were ground as described by WETZEL *et al.* (1992) and cDNA synthesized according to MINAFRA and HADIDI (1994). 5 µl (of a total of 50 µl) of cDNA were mixed with 44 µl of the amplification mixture (1 x Taq Promega buffer, 1.0 mM $MgCl_2$, 200 µM of each dNTP, 120 nM of each primer). After a hot start step at 80 °C for 1 min, 1 unit of Taq DNA polymerase (Promega Corporation, Madison USA) was added. Cycling was as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, extension for 1 min at 72 °C for 30 cycles and final extension for 7 min at 72 °C. PCR amplified products were analyzed by electrophoresis in 6 % polyacrylamide gel in 1 x TBE buffer and visualized by silver staining (BioRad Laboratories, Hercules, USA).

Results

Specificity of the probe: In dot spot assays, Dig-RNA probe GFkV20 specifically recognized homologous ssRNA, dsRNA and total nucleic acid (TNA) extracts from infected grapevines, but did react neither with healthy grapevine TNA, nor with dsRNA from two asteroid mosaic-affected vines (Fig. 1 A). In Northern blots, carried out after agarose gel electrophoresis under semi-denaturing conditions, the same probe hybridized to purified viral RNA preparations of the homologous (MT48) and two heterologous GFkV isolates (UR4 and USA6). A single band was detected, which was interpreted as the full size genomic RNA (Fig. 1 B).

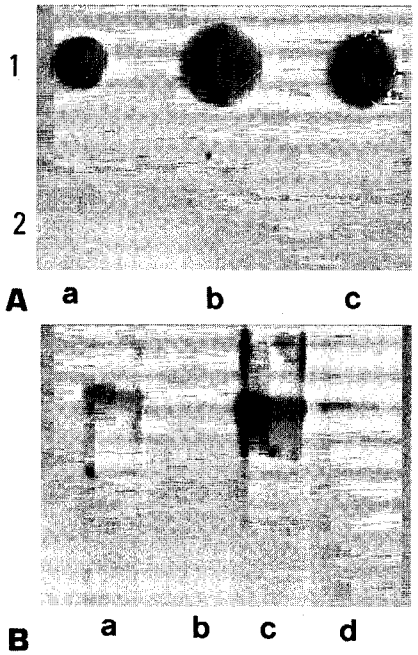

Fig. 1: Specificity of Dig-RNA probe GFkV20. **A.** Dot spot hybridization of total nucleic acids (TNA), genomic RNA, and dsRNA from fleck- and asteroid mosaic-infected grapevines. 1a: TNA from a GFkV MT48-infected vine; 1b: dsRNA from a GFkV MT48-infected vine; 1c: GFkV MT48 genomic RNA; 2a: TNA from a healthy vine; 2b: dsRNA from a vine infected by asteroid mosaic H4; 2c: dsRNA from a vine infected by asteroid mosaic L3. Positive hybridization is only with GFkV-infected sources. **B.** Northern blot of purified genomic RNA of three different GFkV isolates: UR4 (lane a), blank (lane b) MT48 (lane c), and USA6 (lane d).

The genomic RNA of isolates MT48 and UR4 had the same electrophoretic migration rate. By comparison with the mobility of RNA markers, a size of ca. 8800 nucleotides was estimated for both of them (Fig. 2). This value differs from that of ca. 7400 nucleotides reported for GFkV RNA in previous studies (BOULILA *et al.* 1990).

Virus detection by dot spot in grapevine sap: Sap from infected grapevine roots, petioles, and leaves extracted with alkali (GALLITELLI and SALDARELLI 1995; SALDARELLI *et al.* 1996) reacted with Dig-RNA probe GFkV20 up to the last dilution tested (1:500) (Fig. 3, rows 1, 3, 5), which corresponds to a tissue sample weight of 0.1 mg. Healthy controls gave no hybridization signals.

Virus detection by tissue blotting: Of the three blotting methods described method 2 yielded

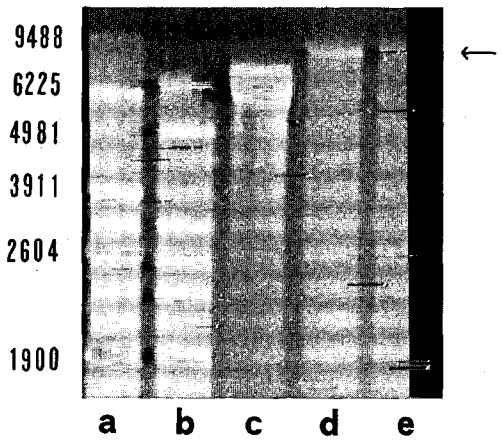

Fig. 2: Agarose semi-denaturing gel electrophoresis of purified genomic RNA from GFkV isolates UR4 (lane d), MT48 (lane e) (arrow), and turnip yellow mosaic tymovirus (lane c). Markers in lanes a and b are a mixture of ssRNA from a commercial kit (Promega Corporation, Madison, USA).

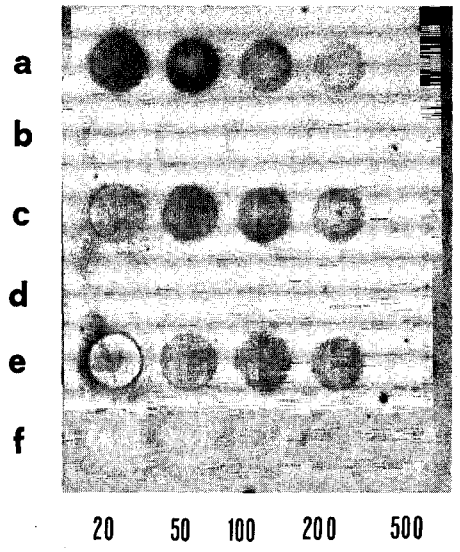

Fig. 3: Detection by dot spot hybridization of GFkV MT48 in sap from different grapevine organs diluted from 1:20 to 1:500. Infected roots (row a); healthy roots (row b); petioles from infected leaves (row c); petioles from healthy leaves (row d); infected leaf blades (row e); healthy leaf blades (row f). Hybridization signals are visible till the last dilution and only with infected material.

the best results. The other two methods gave either a weak hybridization signal (method 1), or no signal at all (method 3) (data not shown). Positive hybridization was obtained with infected petioles, both in transverse and longitudinal section (Fig. 4 A, B) while healthy samples did not react. In agreement with the phloem-limited nature of GFkV (CASTELLANO and MARTELLI 1984), the signal was localized in the outer region of both types of sections, i.e. at the level of conducting tissues.

Virus detection by RT-PCR: As shown in Fig. 5 a DNA fragment of the expected length was amplified from: (i) GFkV RNA (lane b); (ii) sap from three different grapevines infected by GFkV (lanes c, d and e);

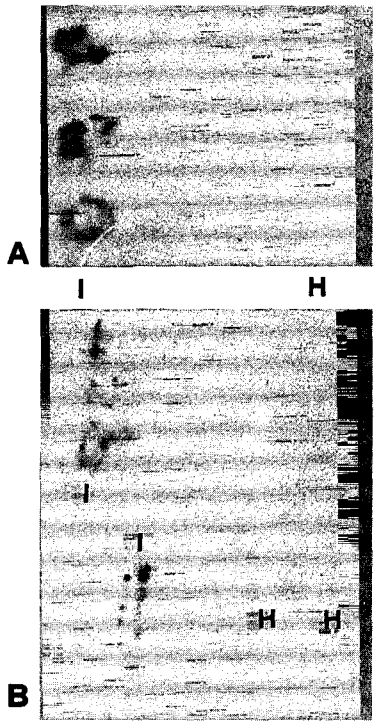

Fig. 4: Detection of GFkV MT48 by tissue blotting. Positive hybridization signals are visible in blotted transverse (A lane I) and longitudinal (B, I) sections of infected grapevine leaf petioles, but not in blots from healthy vines (pale shadows in H of both A and B panels).

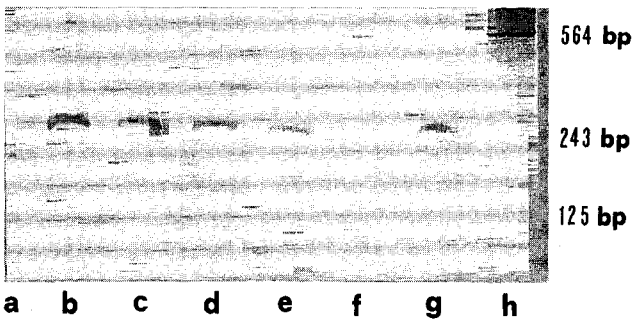

Fig. 5: Polyacrylamide gel electrophoresis of RT-PCR products amplified from grapevine sap infected by GFkV isolates MT48 (lane c), USA6 (lane d), and UR4 (lane e). Positive controls are in lanes b (GFkV RNA) and g (recombinant plasmid). Negative controls are in lanes a (water) and f (healthy plant extract). Markers are in lane h. The 243 bp band is the amplified target RNA sequence.

(iii) the plasmid (positive control, lane g). No amplified products were obtained from negative controls consisting of healthy LN33 extracts (lane f) and water (lane a).

Discussion

Current techniques for detection and diagnosis of GFkV are biological and serological assays. Indexing on *Vitis rupestris* is a reliable and widely used field test (MARTELLI 1993), however, it is time-consuming and expensive. The development of immunoenzymatic assays based on the use of polyclonal antisera (BOSCIA *et al.* 1991; WALTER and

CORNUET 1993; RAMEL *et al.* 1993) and monoclonal antibodies (BOSCIA *et al.* 1993, 1995; RAMEL *et al.* 1993) has widened the range of available diagnostic tools, providing dependable and easily applicable protocols. The results of the present investigation are a further contribution to sensitive laboratory detection of GFkV. The use of a virus-specific digoxigenin-labelled riboprobe allowed the establishment of three different procedures (i.e. dot spot, tissue blot and RT-PCR) all of which consistently identified GFkV in leaf and root tissues of infected grapevines. A remarkable improvement of these procedures with respect to those previously used for molecular detection of other phloem-limited grapevine viruses (MINAFRA *et al.* 1992 a and b; SALDARELLI *et al.* 1993, 1994 a and b, 1996) is that hybridization tests were successfully carried out with samples that had undergone minimal manipulation, i.e. crude sap (dot spot and RT-PCR) or hand-made sections of leaf petioles (tissue blots).

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