

Non-radioactive molecular probes for the detection of three filamentous viruses of the grapevine

by

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S u m m a r y : Digoxigenin-labelled riboprobes (DIG-RNA) were developed for the detection in infected tissue extracts of grapevine trichovirus A (GVA), grapevine trichovirus B (GVB) and grapevine leafroll-associated closterovirus III (GLRaV III). The probes were virus-specific and could be used for the identification of the respective viruses in sap expressed from infected *Nicotiana* species (GVA and GVB) and in total nucleic acid extracts from infected grapevines (GVA, GVB and GLRaV III). The efficiency of detection was the same as (GLRaV III), or slightly less than (GVA), with ELISA. No difference was found in detection efficiency between DIG-RNA and cDNA radioactive probes.

K e y w o r d s : closterovirus, trichovirus, leafroll, rugose wood, molecular hybridization, cold probes, digoxigenin-mediated chemiluminescence, diagnosis.

Introduction

Filamentous closterovirus-like viruses are among the most serious pathogens of cultivated grapevines (*Vitis* spp.). Grapevine leafroll-associated virus III (GLRaV III), a tentative species of the genus closterovirus (CANDRESSE and MARTELLI 1994) consistently occurs in leafroll infections and is thought to be one of the agents of this disease, while grapevine virus A (GVA) and grapevine virus B (GVB), both tentative species of the newly established genus trichovirus (MARTELLI *et al.* 1994), seem to be implicated, respectively, in the etiology of Kober stem grooving (CHEVALIER *et al.* 1993; GARAU *et al.* 1993) and corky bark (BOSCIA *et al.* 1993), two diseases of the 'rugose wood' complex (BOVEY and MARTELLI 1992).

These viruses can be detected by biological (indexing and mechanical transmission), serological (ELISA), and molecular hybridization assays (MARTELLI 1993). In particular, cloned probes to GVA (MINAFRA *et al.* 1991, 1992) GVB (SALDARELLI *et al.* 1993 a) and GLRaV III (SALDARELLI *et al.* 1993 b) were produced and used for diagnosis after ³²P labelling.

Utilization of radioactive probes requires properly trained personnel, provisions for safe handling and disposal of radioactive materials, and is not deprived of hazards. However, most of these constraints can be overcome by the development of cold probes whose successful hybridization with target nucleic acid is revealed by reactions other than those depending on radioactivity. Therefore, as reported in the present paper, a non-radioactive hybridization system was developed, in which digoxigenin-labelled RNA (DIG-RNA) probes were used for detection of GVA, GVB and GLRaV III in infected tissue extracts with an immunological chemiluminescent assay.

Materials and methods

Virus sources: GVA, isolate PA-3 (MINAFRA *et al.* 1991) and GVB, isolate 'Semillon' (BOSCIA *et al.* 1993), were multiplied and maintained, respectively, in *Nicotiana benthamiana* and *N. occidentalis* plants in a temperature-controlled glasshouse. Grapevine clones identified as 'pure virus sources' (i.e., infected by a single virus, i.e., either GVA, GVB or GLRaV III) or with multiple infections, were from a varietal collection of the University of Bari. The sanitary status of these clones had been determined by DAS-ELISA (CLARK and ADAMS 1977) and/or indexing. Controls were from a healthy LN33 clone.

Virus purification and nucleic acid extraction: Purification of GVA and GVB from herbaceous hosts was according to MONETTE and JAMES (1990) and BOSCIA *et al.* (1993), respectively. GVA and GVB genomic RNAs were extracted from purified virus with the standard SDS-phenol method (DIENER and SCHNEIDER 1968).

Total nucleic acids (TNAs) were obtained from grapevine (phloem or leaves with petioles) or herbaceous host tissues as described (WHITE and KAPER 1989), with slight modifications. Tissue samples of 0.1 g were ground in an Eppendorf tube with a teflon pestle in the presence of 0.5 ml of extraction buffer (0.1 M glycine, 0.001 M EDTA, 0.1 M NaCl, pH 9.5, 2 % SDS, 1 % sodium lauryl sarcosine). The slurry was extracted with phenol-chloroform, precipitated with ethanol, collected by centrifugation (11,000 g for 10 min) and resuspended in sterile water.

For further purification of TNAs from grapevine the standard extraction protocol was modified as follows: after phenol-chloroform extraction, the aqueous phase was made 35 % with ethanol and stirred for 1 h with 150 mg of

cellulose CF11 (Whatman, Maidstone, England) in a microfuge tube. Cellulose-bound nucleic acids were collected by centrifugation at 5,000 g for 3 min, were washed twice in STE buffer (0.1 M NaCl, 0.1 M Tris, 0.002 M EDTA, pH 8.0) and 35 % ethanol and were again centrifuged at 5,000 g for 3 min. Nucleic acids were eluted from cellulose by vortexing in the presence of 400 µl STE buffer. After a final centrifugation at 5,000 g for 3 min, the supernatant fluid was recovered, ethanol-precipitated and resuspended in 50 µl of sterile water.

Sap extracts from herbaceous hosts or grapevines (phloem or young leaves) were prepared by grinding 30 mg of tissues in 125 µl of 10 x SSC-0.01 % SDS buffer (SSC 2x = 0.3 M NaCl, 0.03 M trisodium citrate) in microfuge tubes with a teflon pestel (MONIS and DE ZOETEN 1990). The supernatant, recovered after a centrifugation at 10,000 g for 1 min, was used for hybridization assays as such or after dilution.

Double-stranded RNA purification:

dsRNA was purified from infected herbaceous host or grapevine tissues (phloem scraped from cuttings), according to HU *et al.* (1990). Elimination of DNA and RNA contaminants of plant origin was by enzymatic digestion with DNase and RNase (SALDARELLI *et al.* 1993 b).

Probe preparation: Complementary RNA probes (cRNA) labelled with digoxigenin-11-UTP were synthesized by SP6 or T7 transcription using a commercial kit (DIG-RNA labelling kit, Boehringer, Mannheim, Germany). Templates for transcription were Pst I- or Hind III-linearized plasmids containing DNA fragments complementary (cDNA) to the genome of GVA (pGA 42), GVB (pGB 5) and GLRaV III (pGEM 23). cDNAs were excised from the original plasmids pGA240a for GVA (MINAFRA *et al.* 1992), pGVB5 for GVB (SALDARELLI *et al.* 1993 a) and pGLRaV23ds for GLRaV III (SALDARELLI *et al.* 1993 b), and subcloned in the Sma I site of the transcription vector pGEM4z (for GVA and GVB) or pGEM3z (for GLRaV III).

Molecular hybridization and chemiluminescent detection: Nucleic acids (TNAs, purified viral RNAs and dsRNAs), previously denatured with 50 % formamide, 6.5 % formaldehyde and 1x MAE (MAE 1x: 3-N-morpholino-propane-sulphonic acid, 0.05 M sodium acetate pH 7.0, 0.01 M EDTA) by heating at 65 °C for 5 min (for TNAs and ssRNAs), or at 70 °C for 10 min (for dsRNAs), were spotted onto 10x SSC buffer prewetted Hybond N+ nylon membrane (Amersham, England) using a Manifold apparatus. TNAs extracted from about 100 mg of tissue were probed in a single spot.

Samples for target nucleic acid detection in sap were diluted serially with 10x SSC and 9.8 % formaldehyde, denatured at 65 °C for 5 min and spotted onto 10x SSC prewetted membrane. Nucleic acids were fixed to the membrane by 5 min exposure to UV light.

Hybridization was with a Boehringer Chemiluminescent Detection kit following manufacturer's instruction. Prehybridization and hybridization solutions consisted of 50 % formamide, 5x SSC, 0.02 % SDS, 0.1 % sodium lauryl sarcosine, 2 % blocking reagent. Hybridization was at 55 °C. After overnight incubation, the membranes were washed 3 times (30 min per washing) in 2x SSC-0.1 %

SDS, at 65 °C. Residual probe was eliminated with a further washing in 2x SSC containing 1 mg/ml of RNase A, for 30 min at room temperature, followed by a brief rinsing with 2x SSC. Digoxigenin-labelled hybrids were detected by anti-digoxigenin antibodies conjugated to alkaline phosphatase. Usually, short exposures (5-30 min) of the membrane to X-ray film were sufficient for impression.

ELISA: DAS-ELISA was as described (CLARK and ADAMS 1977), using locally produced antisera. With GVA, ELISA plates were first sensitized with 0.5 mg/ml protein A by incubation for 2 h at 37 °C (BOSCIA *et al.* 1992). Subsequent coating, washing and detection steps were the same for GVA and GLRaV III. Lack of a suitable antiserum did not allow to perform ELISA for the detection of GVB.

Results

Sensitivity of the probes: Serial dilutions of viral RNAs ranging from 4 to 0.031 ng were spotted onto a membrane and challenged with non-radioactive cRNAs probes. GVA genomic RNA (Fig. 1 A) was detected up to the lowest dilution tested (0.031 ng) whereas GVB genomic RNA (Fig. 1 B) was clearly detected up to a concentration of 0.125 ng and, occasionally, of 0.062 ng. No signals were observed with TNAs from healthy *N. benthamiana* and *N. occidentalis* controls. The GLRaV III riboprobe recognized viral dsRNA up to 0.125 ng and gave no reaction with healthy grapevine extracts (not shown).

These results are in agreement with literature records reporting comparable sensitivities for digoxigenin-labelled and radioactive probes (KANEMATSU *et al.* 1991; PODLECKIS and HAMMOND 1992). In the present experience, DIG-cRNA probes were as effective as conventional cDNA ³²P-labelled probes for the detection of GVA (MINAFRA *et al.* 1992), or more effective, in the case of GVB (SALDARELLI *et al.* 1993 a).

Virus detection in crude sap: Viral RNA detection was attempted in crude, non-concentrated sap extracts from herbaceous hosts (GVA and GVB) or grapevines (GLRaV III).

Clear-cut hybridization signals were obtained with

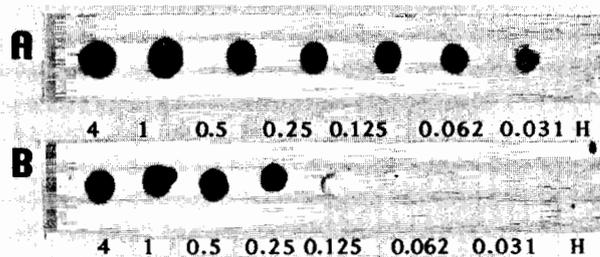


Fig. 1: Sensitivity of DIG-RNA probes as assessed by dot blot hybridization. Purified genomic RNA from GVA and GVB was serially diluted, spotted onto a nylon membrane and hybridized with GVA probe pGA 42 (A) and GVB probe pGB 5 (B). Controls (H) were TNA extracts from healthy *N. benthamiana* (A) and *N. occidentalis* (B).

samples from *Nicotiana* plants infected with GVA (Fig. 2 A, row b) and GVB (Fig. 2 B, row b), but not with the respective healthy controls (Fig. 2 A and B, row a). In these tests, the intensity of hybridization signals appeared to increase with increasing sap dilution, which was interpreted as the consequence of the progressive elimination of plant components that may have interfered with absorption or detection of target RNA sequences.

No hybridization was evident with sap extracts from

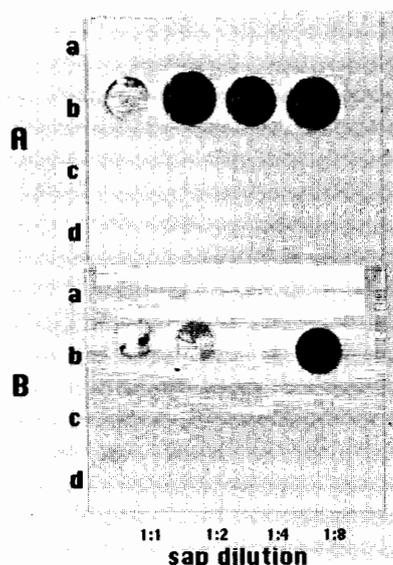


Fig. 2: Dot blot hybridization with DIG-RNA probes of sap expressed from herbaceous hosts and grapevine cortical tissues. A. GVA pGA 42 probe. Row a, healthy *N. benthamiana*; row b, GVA-infected *N. benthamiana*; row c, healthy vine; row d, GVA-infected vine. B. GVB pGB 5 probe. Row a, healthy *N. occidentalis*; row b, GVB-infected *N. occidentalis*; row c, healthy vine; row d, GVB-infected vine.

GVA- (Fig. 2 A, row d) and GVB- (Fig. 2 B, row d) infected grapevines, nor with healthy grapevine controls (Fig. 2 A and B, row c). Likewise, sap expressed from GLRaV III-infected vines did not yield hybridization signals, much the same as the healthy grapevine control (not shown).

Detection of target RNA sequences in grapevine: Cellulose CF11 treatment was used for purifying grapevine TNA extracts from residual unwanted plant components, so as to obtain higher amounts of nucleic acids deprived as much as possible of materials

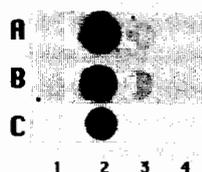


Fig. 3: Dot blot hybridization with DIG-RNA probes of total nucleic acid extracts purified with cellulose CF11 treatment. Rows A, B, and C were probed with GVA-pGA 42, GVB-pVB 5 and GLRaV III-pGEM 23, respectively. 1A, healthy *N. benthamiana*; 2A, GVA-infected *N. benthamiana*; 3A, GVA-infected vine; 4A, healthy vine. 1B, healthy *N. occidentalis*; 2B, GVB-infected *N. occidentalis*; 3B, GVB-infected grapevine; 4B, healthy vine. 1 C, healthy vine; 2C GLRaV III-infected vine; 3 and 4 C, void.

liable to interfere with hybridization. To verify the reliability of the method, the quality of grapevine TNAs was checked by agarose gel electrophoresis and found to be comparable to that of TNA extracts from herbaceous host tissues (not shown).

Assays aiming at detecting GVA, GVB and GLRaV III in TNAs from grapevine by DIG-RNA probes, showed that clarification with cellulose CF11 greatly enhanced hybridization signals as compared to non-treated TNAs. As shown in Fig. 3, GVA and GVB probes strongly recognized homologous RNA sequences in TNAs from infected *N. benthamiana* and *N. occidentalis* (lane 2, A and B) but not in the corresponding healthy controls (lane 1, A and B). Lighter but clear-cut signals were also obtained with TNA extracts from grapevines infected by GVA and GVB (lane 3, A and B). By contrast, extracts from GLRaV III-infected vines gave hybridization signals (lane 2, C), as strong as those obtained with TNAs from GVA- and GVB-infected herbaceous hosts. None of the probes reacted with healthy grapevine controls (lane 4, A, B, C).

To test the reliability of DIG-RNA probes, the detection of GVA, GVB and GLRaV III was attempted on a somewhat larger scale, using phloem or leaves with petioles collected from field- or glasshouse-grown vines. The results of these hybridization trials were compared with the outcome of ELISA tests for GLRaV III and GVA (Table), or with the results of indexing for GVB. As shown in the Table, there was complete agreement between ELISA and dot blot assays for the detection of GLRaV III in 35 samples, whereas as many as 28 out of 35 ELISA-positive GVA samples hybridized successfully in dot blot assay. The remaining 7 samples gave either doubtful, or no visible hybridization signals. As to GVB, 3 out of 5 grapevine clones that had induced corky bark symptoms in LN33 reacted with a digoxigenin-labelled GVB probe.

Positive hybridization reactions were also obtained with 6 additional grapevine clones for which no indexing data on LN33 were available.

Table

Comparative results of ELISA and dot blot hybridization with DIG-RNA probes of total nucleic acid extracts, for the detection of GVA and GLRaV III in grapevine samples

	GLRaV III	GVA
Samples positive with both ELISA and dot blot	35	28
Samples positive in ELISA only	0	7
Total samples tested	35	35

Discussion

The successful development of a reliable non-radioactive method of molecular hybridization applicable to the detection of three different grapevine clostero-like viruses represents the major output of the present study.

Advantages of non-radioactive over radioactive probes are many. Besides safety and absence of dangerous waste, digoxigenin-labelled probes are not subject to radiolysis and can be stored for several months at -20 °C. Furthermore, the chemiluminescent reaction is very rapid and impresses X-ray films as neatly as radioactive emissions and, after hybridization, a probe can be removed from the substrate, which can be challenged with another probe. Fair detection limits and reproducibility of results can now be achieved with commercial kits, so that their utilization for viral diagnosis is becoming increasingly popular (see among others, KIMPTON *et al.* 1989; CRESPI *et al.* 1991; MUSIANI *et al.* 1992; PODLECKIS and HAMMOND 1992) also for grapevine viruses (GEMMICH *et al.* 1993).

Significant results were obtained with DIG-RNA probes in the identification of GVA, GVB and GLRaV III, with limits of detection of purified viral single-stranded or double-stranded RNAs comparable to those of ³²P-labelled probes (MINAFRA *et al.* 1992; SILDARELLI *et al.* 1993 a, b). By using an improved method of TNAs purification, higher consistency and reproducibility of target RNA sequence detection in grapevine extracts was also achieved. Cellulose CF11 treatment, which allowed spotting of higher amounts of nucleic acids little contaminated by plant interfering substances, made detection of GVA and GVB possible in TNAs from infected vines. This result was of particular importance for GVB, because in absence of an antiserum usable in ELISA, molecular probes represent the only tool currently available for sensitive detection of this virus in naturally infected hosts.

Comparison of the results of dot-blot hybridization with those of ELISA tests showed a complete agreement for GLRaV III detection and about 80 % agreement for GVA. This is remarkable considering the difficulties experienced earlier with the identification of GVA sequences by radioactive probes in grapevine extracts (MINAFRA *et al.* 1991).

The ultimate goal, i.e. viral RNA sequence detection directly in crude grapevine sap extracts has not yet been achieved. This is probably due to the low titer of the viruses object of this study in the tissues of naturally infected vines and, perhaps, to inefficient nucleic acid denaturation and/or extraction. Nevertheless improvements, however slow, are being made and non-radioactive probes, due to their reliability, seem now to qualify for use in the detection of target nucleic acid sequences amplified by one of the currently available amplification systems (e.g., polymerase chain reaction).

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