CORE
Metadata, citation and similar papers at core.ac.uk
Provided by JKI Open Journal Systems (Julius Kühn-Institut)

Vitis **32**, 237–242 (1993)

Detection of grapevine fanleaf virus (GFLV) in infected grapevines by non-radioactive nucleic acid hybridisation

by

A. R. Gemmrich, G. Link and M. Seidel

Phytotechnologisches Labor, Fachhochschule Heilbronn, Weinsberg, Germany

Summary: The nucleic acid hybridisation technique was adopted for the detection of grapevine fanleafvirus (GFLV) in grapevine tissues using a non radioactive labeled cDNA. In crude plant sap a certain detection was not successful. Thus, a method was developed for the extraction of total RNA from a large number of samples in a microscale. By Northern blot hybridisation and by the more convenient slot blot technique GFLV infections could be detected. Comparing ELISA and slot blot hybridisation assay using identical plant material different results were obtained with some samples. This indicates different detection spectra for both techniques. The hybridisation assay has been found to be a suitable method by which a large number of samples from different grapevine tissues could be efficiently indexed for GFLV.

Key words: Vitis, nepovirus, virosis, non-radiactive cDNA probe, diagnosis.

Introduction

The use of healthy propagation material, free of viroids, viruses and bacteria is an important strategy in disease control in viticulture. Correct diagnosis is essential for the production of certified pathogen-free propagation stock. Virus infections can be detected by recognizable symptoms of disease but the detection of latent infections without any symptoms requires additional methods. Biological techniques for virus diagnosis like sap transmission on sensitive indicator plants have been substituted by rapid immunological methods (Clark and Adams 1977). The diagnosis of GFLV by enzyme linked immunosorbent assay (ELISA) has become a standard procedure. However, problems with undesired cross-reactivity in polyclonal antisera as well as uncertainty in autumn and winter months (Walter and Etienne 1987; Rowhani *et al.* 1992) made a further detection assay necessary.

Recently, an alternative method to ELISA, based on nucleic acid hybridisation has been developed (Gould and Symons 1983; Symons 1984). This technique has proved to be a very specific and sensitive method for the diagnosis of closterovirus infected grapevine (Minafra *et al.* 1992). In this paper we have adopted the slot blot procedure for the routine indexing for GFLV and have compared the sensitivity and practicability of the hybridisation assay with the standard ELISA method.

Materials and methods

Plant material: Grapevine cultivars Limberger, Riesling, Schwarzriesling, Spätburgunder and Trollinger and the rootstock 5 BB were provided by Landes-Lehr- und Versuchsanstalt für Wein- und Obstbau, Weinsberg, Germany. In the experiments plants from the field, potted plants and *in vitro* plants were used.

Extraction of nucleic acids: Total RNA was extracted from leaf tissue by a slightly modified method according to Rezaian and Krake (1987). 1-2 g of expanding leaves

Correspondence to: A. R. Gemmrich, Fachhochschule Heilbronn, Phytotechnologisches Labor, Öhringer Strasse 2, D-74189 Weinsberg, Germany

were powdered in liquid nitrogen and mixed with 10 vol. extraction medium A (6M Na-perchlorate, 5 % sodium dodecyl sulfate (SDS), 200 mM Tris-HCl, pH 7.5, 0.2 % 2-mercapto-ethanol, 10 % polyvinylpolypyrrolidon (PVPP). The homogenate was stirred for 30 min at room temperature and centrifuged at 8000 *g* for 10 min. The aqueous interphase was clarified by several centrifugations at 14,000 *g*. The nucleic acids were precipitated with 1 vol. of ethanol, washed with 70 % ethanol and resuspended in a small volume of DEPC-water (MANIATIS *et al.* 1982). This method was also used to extract RNA from shoots and roots. RNA prepared by this method was used in Northern blot and slot-blot hybridisation.

RNA mini-preparation: A rapid micro-scale method was developed for handling a large number of samples in microtubes. 70-100 mg of leaf tissue was homogenized in a mortar with 2 ml extraction medium B (3M Na-perchlorate, 1 % Triton X-100, 1 % N-lauroyl-sarkosine, 100 mM Tris-HCl, pH 7.5, 0.2 % 2-mercaptoethanol, 10 % PVPP).

Cell debris and proteins were separated by two centrifugations at 14,000 *g* for 5 min and the supernatant was extracted with 1 vol. of isobutanol. RNA was precipitated with 1 vol. of ethanolic perchlorate reagent (EPR: Na-perchlorate saturated in 80 % ethanol) from the clarified aqueous phase. The pellet was washed with 70 % ethanol and dissolved in 50 µl DEPC-water. The quality of RNA was improved when samples were kept on ice during preparation. The concentration of RNA was determined photometrically. The average yield was 15-125 µg RNA from 100 mg of leaf tissue depending on the material; from roots and sprouts RNA yields were not satisfying.

Northern blot: 5 µg total RNA were analyzed in formaldehyde containing denaturating agarose gels, then blotted onto a cationic charged nylon membrane (PALL Biodyne B, 1.2 µm or Boehringer Nylon 66, 0.45 µm) by capillary transfer with 20 x SSC (0.3 M sodium citrate, 3 M sodium chloride) and fixed for 30 min at 120 °C. Non-radioactive hybridisation was performed with a GFLV specific DNA fragment according to a slightly modified protocol from Boehringer, Germany (DIG luminescent detection kit): the membrane was incubated with 10 ng DNA probe per ml hybridisation solution containing 50 % formamide overnight at 42 °C followed by washing at low stringency (2·5 min at room temperature with 2·SSC/0.1 % SDS and 2·15 min at 68 °C with 0.5·SSC/0.1 % SDS). The membrane was then saturated with blocking reagent, incubated with a digoxigenin-specific antibody (30 min at room temperature) and washed.

Chemoluminescent reaction was started by adding AMPPD or CSPD in substrate buffer to the membrane and documented by exposition to a X-ray film. Bands became visible after 15-150 min. Alternative detection of viral RNA directly on the membrane was done with a colour reaction using the chromogenic substrates BCIP (bromochloroindoylphosphate) and NBT (nitrophenyltetrazolium). It took 12-14 h to get clearly visible bands.

Slot blot: 5 µg RNA extracted from grapevine was mixed with 3 vol. of a solution containing 12.9 M formamide/2.4 M formaldehyde/1.3·MOPS denaturated at 65 °C and adjusted to 10·SSC on ice. Samples were transferred by vacuum in a slot blot apparatus (BIO DOT SF, Biorad) and fixed at 120 °C. Hybridisation was performed as above.

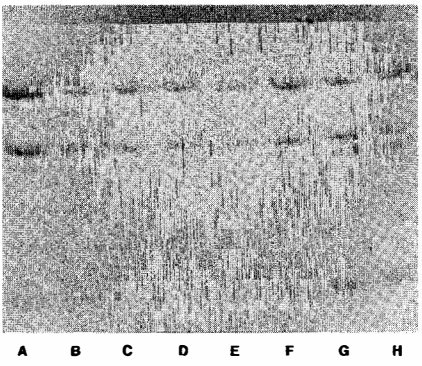
DNA probe: Probe was a cDNA fragment of 934 bp specific to GFLV. The probe is complementary to a part of GFLV RNA 2 which was sequenced by SERGHINI *et al.* (1991) and represents a part of the GFLV coat protein. This fragment cloned in a bluescribe vector was kindly provided by M. FUCHS (INRA, Colmar). Non-radioactive labeling of the DNA fragment was done by random priming with digoxygenin d-UTP according to the protocol of Boehringer (DIG DNA labeling kit).

ELISA: 0.2 g of grapevine leaves were homogenized in 2 ml extraction buffer containing 0.5 M Tris, 137 mM NaCl, 2 % PVPP, 1 % PEG, 0.02 % NaN_3, 0.05 % Tween 20, pH 8.2. Anti GFLV antibodies and AP conjugate was provided by Bioreba, Basel, Switzerland. The assay procedure was that of CLARK (1981). The test was evaluated with an ELISA reader (Biorad).

Results

Northern blot analysis of grapevine RNA using a viral specific DNA probe has proved successful for the detection of GFLV in fresh, frozen and *in vitro* material. In contrast to biotin labeled probes best results were obtained with digoxygenin labeled probes because of the much lower background.

In preliminary experiments a sensitive and reliable detection of viral RNA in crude plant saps or in partially purified plant extracts was not possible due to very weak and non reproducible signals and high backgrounds on the membrane (data not shown). For that reason it was necessary to extract RNA from the grapevine tissues. RNA preparations according to the modified method of REZAIAN and KRAKE (1987) were used to optimize hybridisation conditions of the Northern blot. This method however was not suitable for handling a large number of samples in microtubes. Therefore, a procedure for a RNA mini-preparation from small quantities of grapevine tissues (0.1 g) has been developed. Using this method it is possible to obtain enough grapevine RNA of good quality to detect the GFLV specific RNA. The RNA preparations showed no degradation after electrophoretic analysis (Fig. 1 a) and no difference to RNA prepared according to REZAIAN and KRAKE (1987) was detectable in Northern hybridisation (Fig. 1 b).



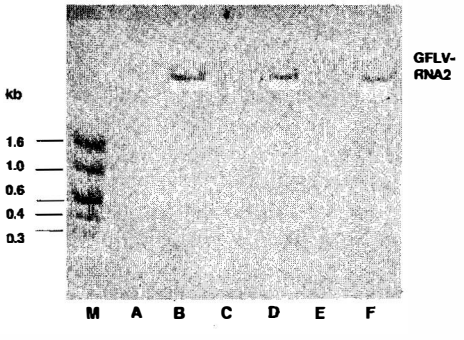


Fig. 1 a (left): Electrophoretic analysis of grapevine RNA. Samples A and B: RNA extracted according to REZAIAN and KRAKE (1987). Samples C, D, E, F, G and H: RNA extracted by the mini-preparation method described in „Materials and methods". Gel was stained with methylene blue.

Fig. 1 b (right): Detection of GFLV from different grapevine cultivars by Northern blot analysis. M: RNA molecular weight marker, digoxygenin labeled (Boehringer); A: Schwarzriesling, healthy control, RNA extracted according to REZAIAN and KRAKE (1987); B: Schwarzriesling, GFLV infected plant, same RNA extraction method as shown for sample A; C: Spätburgunder, infected with grapevine leafroll associated virus (GLRaV), GFLV negative by ELISA; D: Schwarzriesling, potted plant, GFLV infected; E: Müller-Thurgau, leaves stored at -20 °C, healthy control; F: Schwarzriesling, leaves stored at -20 °C, GFLV infected. C-F: RNA extracted by mini-preparation method, signals are of same intensity compared to sample B.

The sensitivity of the Northern blots was the same using colour substrate instead of chemoluminescent substrate. To simplify hybridisation analysis of a large number of samples a slot blot was performed. Compared to Northern blotting this procedure is faster, cheaper and less material-consuming, with reliable virus detection being possible (Fig. 2).

Leaves of 30 grapevine plants were tested for GFLV infections with ELISA and slot blot in a comparative study. Preliminary investigations revealed an inhomogenous distribution of GFLV in the whole infected plant as well as in single tissues. To compare the results of ELISA and slot blot hybridisation correctly the plant material was homogenized in liquid nitrogen, divided in two samples and mixed with the specific extraction buffers for RNA mini-preparation and ELISA, respectively. The use of identical original material was ensured by this approach. No direct correlation between expression of symptoms and the detection of GFLV

Table

Detection of GFLV from different grapevine cultivars: Comparison of ELISA and Northern Blot.

Sample No.	Vitis vinifera cultivar	Symptoms	ELISA OD_{405nm}		Northern Dot Blot
1	Riesling[2]	no	0.053	–	–
2	Trollinger[2]	no	0.070	–	–
3	Lemberger[2]	no	0.067	–	–
4	5BB[3]	no	0.048	–	–
5	Spätburgunder[3]	no	0.044	–	–
6	Riesling[4]	no	0.048	–	–
7	Lemberger[3]	no	0.194	+	–
8	Lemberger[3]	no	0.607	++	–
9	Lemberger[3]	no	0.053	–	–
10	Lemberger[3]	no	0.072	–	–
11	Riesling[2]	yes	0.089	–	–
12	Riesling[2]	yes	0.063	–	+
13	Schwarzriesling[3]	yes	0.836	++	+
14	Schwarzriesling[2]	yes	0.953	++	+++
15	Schwarzriesling[2]	yes	0.672	++	++
16	Schwarzriesling[2]	yes	1.683	+++	+++
17	Riesling[1]	yes	0.144	+	–
18	Riesling[1]	yes	0.055	–	–
19	Riesling[1]	yes	0.329	+	–
20	Riesling[1]	yes	1.627	+++	+
21	Riesling[1]	yes	1.913	+++	+
22	Trollinger[1]	yes	0.030	–	–
23	Lemberger[3]	no	0.238	+	–
24	Riesling[3]	no	0.023	–	–
25	Riesling[2]	yes	0.094	–	–
26	Riesling[2]	yes	0.076	–	+
27	Trollinger[3]	no	0.064	–	–
28	Riesling[3]	no	2.224	+++	+
29	Lemberger[3]	no	0.601	++	–
30	5BB[3]	no	0.020	–	–

[1] leaves from plant in the vineyard, fresh
[2] leaves from plant in the vineyard, frozen -20°C
[3] leaves from potted plant, fresh
[4] leaves from in vitro plant

– negative result
+ weakly positive result
++ positive result
+++ strongly positive result

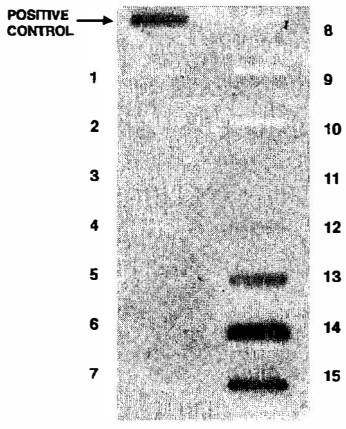


Fig. 2: Detection of GFLV from different grapevine cultivars by slot blot hybridisation. Sample numbers refer to Table. The identical original material was used for ELISA.

in grapevine plants could be found (Table). GFLV was detectable in some plants without symptoms while virus detection was not always possible in plants with GFLV symptoms.

The comparative investigations of GFLV infections by means of ELISA and slot blot hybridisation led to different results (sample nos. 7, 8, 12, 17, 19, 23, 24, 26, 28). With ELISA, GFLV could be detected in 13 of 30 samples, while testing the material with slot blot hybridisation only 9 of 30 samples showed positive results. GFLV detection by hybridisation does not seem to be less sensitive because a few samples reacting negatively in ELISA show positive results in the hybridisation assay (sample nos. 12 and 26). These results indicate differences in the detection spectra of ELISA and hybridisation assay.

Discussion

Results show that slot blot hybridisation assay combined with a quick RNA mini-purification procedure is an adequate method for the detection of GFLV infections in a large number of samples. A more simplified hybridisation assay without any RNA purification step showed no satisfying results because of the high amount of phenolic compounds in grapevine tissue. These compounds may interfere with hybridisation by binding to RNA and are also responsible for a heavy browning of the slots, impeding a colour reaction with NBT/BCIP on the membrane. Various components of unpurified plant sap tend to clog the membrane. Therefore it is often impossible to transfer the desired sample volume to the membrane, leading to a loss of viral RNA during transfer. Furthermore, since protein co-immobilisation competes with nucleic acids for binding sites on the membrane the amount of bound viral RNA is too low to get definite signals. For these reasons it was necessary to work with purified grapevine RNA.

Extraction buffers containing chaototropic salts like perchlorates which prevent binding of phenolic compounds to RNA (Newbury and Possingham 1979) showed best results. Using thiocyanates instead of Na-perchlorate, only RNA of poor quality was extracted. Because of the small quantity of grapevine tissue used for RNA extraction, the selection of convenient plant material, as well as the time of sampling, is of great importance.

Best results were obtained with young plant material. It is known that the GFLV content of field- and greenhouse-grown grapevine varies seasonally (Bovey *et al.* 1980). This finding is now supported by our slot blot hybridisation technique. With both methods, ELISA and slot blot hybridisation no virus was detected in the leaves of grapevine during late summer and autumn. A reliable detection of GFLV by ELISA or nucleic acid hybridisation is only possible in roots and sprouts at that time.

Although GFLV detection by means of nucleic acid hybridisation has been simplified by RNA mini-preparation and slot blot procedure, virus detection with ELISA is still less laborious. However, in some cases the comparison of ELISA and hybridisation assay revealed contrary results. In a few samples virus was detected by slot blot hybridisation but not by ELISA. A non-specific hybridisation reaction is unlikely since non-infected negative controls never showed positive signals. The cDNA probe used in these experiments as well as other cDNA probes derived from GFLV RNAs show cross hybridisation with closely related viruses like AMV (Fuchs 1991). Thus it is possible to detect GFLV and AMV infections by using only one cDNA probe. Anti-GFLV-antisera used for ELISA were highly specific to GFLV showing no cross reactions with AMV. For some samples it was not possible to detect viral RNA by slot blot hybridisation, whereas ELISA led to positive results. In this case a release of viral RNA from the virus particle may be impeded by tanning of virion by phenolic compounds depending on the physiological state of the grapevine tissue.

These results indicate that both test systems exhibit a gradually different detection potential for GFLV and related viruses. To establish clean propagation stock, systemic viruses present the greatest problem, since the clones derived from them will also be infected. For routine testing of grapevine plants for GFLV infections the current indexing bioassay is too

time consuming. ELISA is reliable and less laborious but allows hybridisation assay to be a valuable adjunct, confirming ELISA results and indicating infections with closely related viruses like AMV. Especially for the production of pathogen-free mother plants using *in vitro*-techniques combined with thermotherapy, both detection assays should be used for safeguard.

Acknowledgements

Research supported by Land Baden-Württemberg, Schwerpunktprogramm für Fachhochschulen. Thanks are expressed to Dr. M. FUCHS, INRA, Colmar for supplying GFLV-specific cDNA.

References

BOVEY, R.; BRÜGGER, J. J.; GUGERLI, P.; 1980: Detection of fanleaf virus in grapevine tissue by enzyme-linked immunosorbent assay (ELISA) and immune electron microscopy (IEM). Proc. 7th Meeting ICVG, Niagara Falls, 259-275.

CLARK, M. F.; 1981: Immunusorbent assay in plant pathology. Ann. Rev. Phytopathol. **19**, 83-106.

——; ADAMS, A. N.; 1977: Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. **34**, 475-483.

FUCHS, M.; PINCK, M.; ETIENNE, L.; PINCK, L.; WALTER, B.; 1991: Characterisation and detection of grapevine fanleaf virus by using cDNA probes. Phytopathology **81**, 559-565.

GOULD, A. R.; SYMONS, R. H.; 1983: A molecular biological approach to relationships among viruses. Ann. Rev. Phytopathol. **21**, 179-199.

MANIATIS, T.; FRITSCH, E. F.; SAMBROOK, J.; 1982: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

MINAFRA, A.; RUSSO, M.; MARTELLI, G. P.; 1992: Further studies on the use of molecular probes to grapevine chlosterovirus A. Vitis **31**, 87-93.

NEWBURY, H. J.; POSSINGHAM, J. V.; 1979: Factors affecting the extraction of intact ribonucleic acid from plant tissues containing interfering phenolic compounds. Plant Physiol. **60**, 543-547.

REZAIAN, M. A.; KRAKE, L. R.; 1987: Nucleic acid extraction and virus detection in grapevine. J. Virol. Meth. **17**, 277-285.

ROWHANI, A.; WALKER, M. A.; ROKNI, S.; 1992: Sampling strategies for the detection of grapevine fanleaf virus and the grapevine strain of tomato ringspot virus. Vitis **31**, 35-44.

SERGHINI, M. A.; FUCHS, M. ; PINCK, M.; REINBOLT, J.; WALTER, B.; PINCK, L.; 1990: RNA 2 of grapevine fanleaf virus: Sequence analysis and coat protein cistron localisation. J. Gen. Virol. **71**, 1433-1441.

SYMONS, R. H.; 1984: Diagnostic approaches for the rapid and specific detection of plant viruses and viroids. In: KOSUGE, T.; NESTER, E. W. (Eds.): Plant Microbe Interactions, Vol. 1, chap. 5. Molecular and Genetic Perspective. Macmillan, New York.

WALTER, B.; ETIENNE, L.; 1987: Detection of grapevine fanleaf virus away from the period of vegetation. J. Phytopathol. **120**, 335-364.

Received April 16, 1993