Characterization and Detection of Grapevine Fanleaf Virus by Using cDNA Probes

M. Fuchs, M. Pinck, L. Etienne, L. Pinck, and B. Walter

First, third, and fifth authors: I.N.R.A., Station de Recherches Vigne et Vin, Laboratoire de Pathologie Végétale, 28 rue de Herrlisheim, 68021 Colmar, France. Second and fourth authors: Institut de Biologie Moleculaire des Plantes du C.N.R.S., Laboratoire de Virologie, 12 rue Gl Zimmer, 67084 Strasbourg, France.

Grateful appreciation is extended to P. Bass for excellent technical assistance. We would also like to thank Dr. K. Richards for improving the manuscript and all the suppliers who kindly provided virus strains.

Financial support was provided by INRA (AIP n° 4472) and the Region Alsace.

Accepted for publication 17 May 1990 (submitted for electronic processing).

ABSTRACT

Fuchs, M., Pinck, M., Etienne, L., Pinck, L., and Walter, B. 1991. Characterization and detection of grapevine fanleaf virus by using cDNA probes. Phytopathology 81:559-565.

Complementary DNA copies to the RNAs of grapevine fanleaf virus (GFLV) strain F13 were cloned in *Escherichia coli* plasmid pUC9 and used after nick-translation labeling as probes to detect and to characterize several GFLV isolates by molecular hybridization. Picogram amounts of viral RNA were detected specifically in RNAs extracted from plants with ^{32}P probes. Cross-hybridizations indicating nucleotide sequence homologies were obtained between GFLV probes and RNAs from arabis mosaic virus (ArMV). When a probe specific to the GFLV-F13 satellite RNA was used, it was possible to reveal the presence of satellite RNA associated with different GFLV and ArMV isolates, thus indicating strong homologies between these large satellite RNAs. The probes were also successful for viral detection directly on grapevine extracts. The RNA patterns obtained after northern hybridization were identical in grapevine leaves and rootlets.

Additional keywords: dot blot, immunoenzymatic assay.

Among the nepoviruses infecting grapevine, grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV) are responsible (in France and in many other countries) for the "court-noué" disease; this name indicates that shortening of the internodes occurs in some varieties of infected vine plants. Both viruses are members of the same subgroup of nepoviruses and are distantly related serologically (7). The infection can cause a decline or even destruction of the vinestocks; productivity and longevity are therefore affected.

GFLV occurs in all viticultural areas in the world and can infect all cultivated grapevine varieties. Detection of the viruses is of prime necessity for efficient sanitary selection of the vine plants. Polyclonal (35) or monoclonal antibodies (13) are commonly used for rapid and reliable virus detection in enzyme-linked immunosorbent assay (ELISA). Sensitive routine diagnosis of GFLV is therefore possible throughout the year by analyzing different grapevine organs (14,33).

Nucleic acid hybridization, the formation of a DNA/RNA duplex between two complementary nucleotide sequences, has become an attractive technique for detecting target viral nucleic acid sequences in plant tissues (8,21,30,32). The specificity, the sensitivity, and the speed of molecular hybridization allow this method to be a valuable complement to the more conventional immunological diagnostic approaches such as ELISA. cDNA probes are therefore powerful tools for plant indexing and viral genome identification (18).

Nucleic acid probes from cloned viral cDNA prepared with the RNAs of GFLV strain F13 have already proven to be very specific in the characterization and detection of each of the three RNAs of this isolate in northern blot experiments (25). Each probe recognized the homologous RNA in RNA preparations from purified virus, as well as in RNAs from infected *Chenopodium quinoa* Willd., whereas no reaction was detected with RNAs from healthy plants. The specificity of the F13 probes was first analyzed on *C. quinoa* since this herbaceous plant is the common host for all isolates studied and since the infectivity tests of the pathogen and the virus multipliction are realized on this plant in the laboratory.

We report here the use of radioactive probes to detect and to characterize several natural isolates of GFLV or ArMV by molecular hybridization with RNA samples from infected *C. quinoa* or grapevine grown in the glasshouse or from infected grapevine from the vineyard. As the virus multiplication and/or diffusion is very slow and the virus distribution uneven in infected grapevines, the analysis of the time course of virus multiplication on *C. quinoa* was a convenient way to test the sensitivity of the probes and to monitor the infection rate. Finally, hybridization techniques were used directly on RNA from infected grapevine in order to determine under which conditions the cDNA probes specific for each GFLV-F13 species infecting *C. quinoa* can give similar results on grapevines. To detect nucleotide sequence homologies between viruses belonging to different subgroups of nepoviruses, tomato black ring virus (TBRV), raspberry ringspot virus (RRV), strawberry latent ringspot virus (SLRV), and cassava American latent virus (CALV) were also analyzed with these GFLV probes.

MATERIALS AND METHODS

Plants and viral strains. Different grapevine varieties (*Vitis vinifera* L. 'Chardonnay', *V. vinifera* 'Pinot noir', and *V. riparia* Michaux × Berlandieri, Kober 5BB), naturally or experimentally infected with GFLV or ArMV (by heterografting or nematode transmission), were kept in the glasshouse or in the field. Healthy grapevines were obtained after heat treatment (2).

Ten GFLV and eight ArMV isolates from various hosts (grapevine, hop, raspberry, and sugar beet) and geographical origins (Bulgaria, England, France, Hungary, Italy, and Tunisia), indicated in Table 1, were analyzed. TBRV strain S (kindly provided by Dr. C. Fritsch, IBMP, Strasbourg, France), RRV strain red current spoon leaf (a gift from Dr. D. Z. Maat, IPR, Wageningen, The Netherlands), a grapevine isolate of SLRV (a gift from Dr. M. Rudel, Landes Lehr-und forschungsanstalt, Neustadt, Germany), and CALV (34) were also used. Some of these nepovirus strains were propagated in *C. quinoa*, a systemic herbaceous host.

Virus purification and separation of nucleoprotein components. Virus was purified by clarification with butanol, polyethyleneglycol precipitation, and differential centrifugations as described

by Pinck et al (25). The nucleoprotein components were separated by one centrifugation cycle in a 10–50% linear sucrose density gradient. Fractions containing the bottom component were collected with an ISCO 640 fractionator (Lincoln, NE), pooled, and sedimented at 302,000 *g* for 90 min. Virus concentration was evaluated by UV absorbance measurements by using an extinction coefficient $A_{260nm}0.1\% = 10$.

RNA preparations. Virion RNAs were extracted from purified viral particles and analyzed on formaldehyde-agarose gels in the presence of the RNAs from alfalfa mosaic virus strain S and GFLV strain F13 as molecular weight references (25). RNAs from *C. quinoa* were obtained from leaves ground to a fine powder in dry ice and homogenized with 2 volumes of the extraction buffer used by Jackson and Larkins (16) for polysome isolation. The brei was extracted with phenol/chloroform/isoamyl alcohol and precipitated from 70% ethanol in the presence of 0.3 M sodium acetate, pH 5.2. The precipitated nucleic acids were washed twice with 3 M sodium acetate, pH 5.2, and twice with 75% ethanol. The pellet of RNA was then dried briefly under vacuum and resuspended in sterile water.

Grapevine RNAs were prepared by using two extraction procedures; the first was identical to that reported above for *C. quinoa* RNAs and the second was as follows: Grapevine leaf or rootlet samples were powdered in dry ice and mixed with the buffer (1 g:4 ml, w/v) described by Newbury and Possingham (22). The crude extract obtained after low-speed centrifugation was homogenized with 8.5% insoluble polyvinylpyrrolidone and 1 volume of phenol/chloroform was added. The aqueous phase was then ethanol precipitated and washed as described above.

To evaluate the molecular hybridization sensitivity threshold, the kinetics of viral RNA multiplication was followed on *C. quinoa* during 3 wk. Celite-dusted leaves were rubbed with crude sap from GFLV strain F13-infected *C. quinoa* leaves. Mock-inoculated plants constituted the reference. At various times after inoculation, uninoculated leaves from infected and from mock-inoculated plants were randomly harvested from the apical zones of the plants. Total RNAs were extracted for each leaf sample as described above. Serial dilutions of cellular RNAs corresponding to 5 μg, 500 ng, 50 ng, and 5 ng for each sampling were then dot blotted on GeneScreen Plus membrane and hybridized with the labeled probes as in (25). In the same way, dilutions of total virion RNAs obtained from purified virus were spotted on the hybridization membrane and then hybridized with the same labeled probes as before, using the same hybridization conditions and time of autoradiographic exposure. After autoradiography, the intensity of the dots was quantified by transmission scanning on a Shimadzu CS9000 densitometer (Shimadzu Corporation, Kyoto, Japan). The values obtained with the viral RNA dilutions constituted a standard curve that allowed a precise determination of the amount of each viral RNA in RNAs extracted from infected plants, assuming RNA1 represents 8% of the virion RNAs in molar amount as previously determined (25).

TABLE 1. Origin of different grapevine fanleaf virus (GFLV) and arabis mosaic (ArMV) isolates

Virus	Isolate	Natural host	Country of origin	Supplier
GFLV	A 78	Grapevine	France	
	B 714	Grapevine	France	
	B 715	Grapevine	France	
	B 845	Grapevine	France	
	CB 844	Grapevine	France	
	F 13	Grapevine	France	
	F 13M[a]			
	GH	Grapevine	France	
	K 87	Grapevine	Hungary	Dr. Lehoczki
	Tu 92-14	Grapevine	Tunisia	
ArMV	862	Grapevine	Bulgaria	Dr. Gueorgieva
	S	Grapevine	Bulgaria	France
	6/29	Grapevine	Italy	Dr. Belli
	T 75	Grapevine	France	
	Tannat	Grapevine	France	
	AB 10	Sugar beet	England	Dr. Cooper
	C	Raspberry	England	Dr. Cadman
	H	Hop	England	Dr. Adams

[a] F 13M is a natural mutant that appeared spontaneously during cloning of F 13 on *Chenopodium quinoa* in the glasshouse.

GFLV cDNA synthesis and cloning. Partial cDNA copies were synthesized and cloned with pUC9-oligo (dT)-tailed plasmid as primer and GFLV-F13 RNA as template (12). The recombinant β-galactosidase$^-$ and ampicillin-resistant clones obtained after transfection of *Escherichia coli* strain JM 103 were selected. These clones were further characterized by restriction enzyme digestion for the presence of an insert. Their specificity against each virion RNA was analyzed by northern blotting after nick translation (25).

cDNA probes. The cDNA probes used corresponded to a part of the 3′ terminal region of the viral RNAs. To improve the hybridization signal and to decrease nonspecific background hybridization with the polyadenylated cellular RNAs, the poly(T) tail was deleted from each clone by appropriate restriction enzyme digestion. The different restriction fragments used as probes, named P_1, P_2, and P_3 hybridizing with RNA1, RNA2, and RNA3, respectively, have been previously described and localized on each RNA species (25). The restriction fragments used as probes and purified by electrophoresis on 8% nondenaturing polyacrylamide gels were nick-translated with DNA polymerase I (Kornberg fragment) and [α^{32}]P dCTP (3,000 Ci/mmole) (29). The unincorporated labeled nucleotides were removed by chromatography on a Sephadex G75 column.

To examine the ability of the cDNA probes corresponding to the RNAs of GFLV strain F13 to identify other GFLV and also ArMV isolates, each isolate was tested with probes P_1 and P_2 for sequence homologies between genomic RNAs. The presence of satellite RNAs associated with other GFLV and ArMV strains was checked with probe P_3, assuming these satellite RNAs share nucleotide sequences in common. Each vine plant or *C. quinoa,* individually infected with one strain, was therefore analyzed with the three GFLV-F13 probes.

RNA denaturation and hybridization conditions. Northern hybridizations were performed as previously described (25). Otherwise, RNA samples were dissolved in 10 mM Tris-borate, 0.2 mM EDTA, pH 8.3, and 6% formaldehyde and denatured 10 min at 65 C before being blotted on 20× SSC presoaked GeneScreen Plus membranes (NEN, Boston, MA) using the BRL Hybri-Dot manifold (Bethesda Research Laboratories, Gaithersburg, MD) (1× SSC is 150 mM NaCl, 15 mM Na citrate, pH 7.0). After transfer or spotting of the RNA samples, the filters were air dried and hybridization was carried out according to the instructions of the manufacturer. After being washed, membranes were dried and exposed at −80 C to X-ray film (Fuji-RX) and intensifying screen.

Cell-free translation. Cell-free translation of virion RNAs in wheat germ extracts and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the [^{35}S]methionine-labeled translation products were carried out as previously described (25). Trypsin inhibitor (M_r 20,100), lactate dehydrogenase (M_r 36,500), glutamate dehydrogenase (M_r 55,400), and phosphorylase b (M_r 97,400) were used as calibration proteins.

ELISA. The presence of the virus was measured in a direct double antibody sandwich test (DAS-ELISA) with polyclonal antibodies (35). Antiserum was obtained from immunized rabbits after subcutaneous injections of purified bottom particles. IgGs were extracted with Rivanol (11) and conjugated with alkaline phosphatase by using glutaraldehyde (1). Coating and conjugate antibodies were used at 50 ng/ml and a 1/20,000 dilution, respectively. The absorbance values of the substrate hydrolysis were recorded at 405 nm with a Titertek Multiscan MCC/340 MK II spectrophotometer (Flow Laboratories, McLean, VA).

RESULTS

Specificity and sensitivity of detection by dot-blot hybridization. In attempts to determine a reliable sample preparation procedure,

plant extracts prepared by different means, crude saps, phenolic extracts, and formaldehyde-denatured samples (36) were spotted on hybridization membranes. In our experimental conditions, formaldehyde treatment of the RNAs from infected tissues before being spotted on GeneScreen Plus membranes gave sharp hybridization responses and phenolic extraction enhanced the hybridization intensity. Specific and reliable virus detection directly in crude sap from *C. quinoa* and grapevine, without any purification, was possible but the autoradiography exposure time had to be increased approximatively 10-fold.

To investigate the detection possibilities, the time course of viral RNA multiplication in *C. quinoa* was monitored by measuring the amount of RNA1 hybridized with probe P_1. After a 20-hr autoradiographic exposure, RNA1 was detected as early as at the fifth day after inoculation and the maximum of RNA1 was reached at the 12th day (Fig. 1). Increasing the autoradiographic exposure up to 3 days allowed RNA1 to be detected at the third day after infection. The detection limit for RNA1 was estimated between 0.5 and 1 pg from comparison of the hybridization signals with those obtained from a calibration curve. Absence of hybridization signal with extracts from healthy plant confirmed the high specificity of the probe used (Fig. 1). In ELISA tests, no coat protein could be detected in crude sap of leaf samples before the fifth day postinoculation.

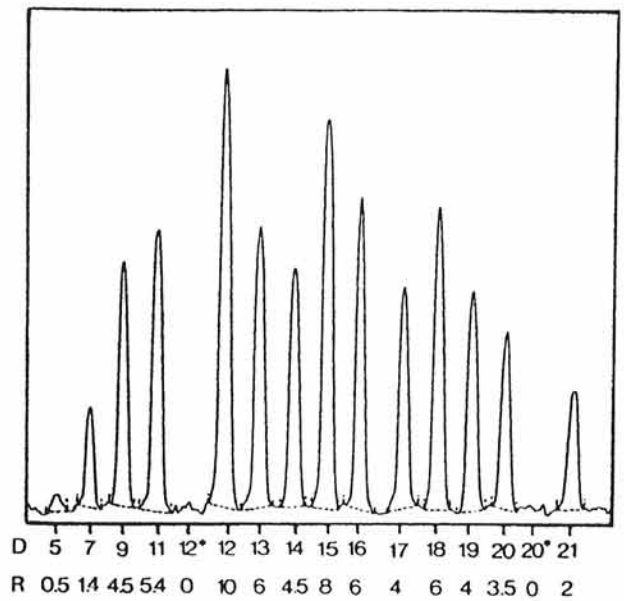

Fig. 1. Densitometric scanning of dot-blot hybridization pattern obtained for nucleic acids extracted from *Chenopodium quinoa* leaves harvested at various times after inoculation with grapevine fanleaf virus (GFLV)-F13 isolate. For each sample, 50 ng of plant RNA were blotted on the GeneScreen Plus membrane by using a BRL Hybri-Dot manifold. The blot was hybridized with ^{32}P-labeled cDNA corresponding to GFLV-F13 RNA1. The time after inoculation, in days, corresponding to each sample is indicated in lane D. Control samples taken 12 and 20 days after mock-inoculation are indicated 12* and 20*. Lane R indicates the amount in picograms of RNA1 in each sample as deduced from the area of each peak.

Comparison with ELISA. To compare the detection sensitivities by molecular hybridization and by ELISA, GFLV-F13 was detected in a DAS-ELISA test with polyclonal antibodies and in dot-blot hybridization with probe P_1. Infected *C. quinoa* samples harvested 12–15 days postinfection were subjected to both procedures. When calibration standards established with known amounts of purified bottom particles added to crude sap from healthy *C. quinoa* were used, virions were detected in ELISA in amounts down to 1.5 ng. (Amounts tested were 50, 25, 12.5, 6, 3, 1.5, and 0.75 ng. Only 0.75 ng tested negative.) For molecular hybridization calibration with probe P_1, virion RNAs gave positive hybridization signals down to 15 pg (Amounts tested were 15,000, 1,500, 150, 15, and 1.5 pg.). This value is equivalent to 1.2 pg of RNA1, assuming RNA1 represents 8% of the virion RNAs in strain F13 (25) and constitutes the detection limit in the conditions used. With samples from infected plants, the detection values obtained were: 1, in ELISA down to a 1/108,000 dilution, with 1.5 ng of bottom component, which is equivalent to about 5 ng of virus; 2, for dot-blot hybridization down to 5 ng of RNAs from infected *C. quinoa*, i.e., about 15 pg of virion RNAs, which correspond approximately to 100 pg of virus, assuming the bottom particles contain 42% of RNAs and represent 34% of the virus (26). These results led to the same qualitative conclusions with both techniques for material harvested after 12–15 days post-inoculation: A positive sample in ELISA was also positive in hybridization and, conversely, a negative sample in ELISA was negative in hybridization. However, hybridization proved to be at least 10 times more sensitive than ELISA. The samples from healthy plants were negative with both methods.

Characterization of several isolates. The three F13 specific probes were assayed for the detection of several other GFLV and ArMV isolates. The results obtained after dot-blot hybridization with probe P_1 are shown in Figure 2. Similar results were obtained with probe P_2; each strain was recognized by probes P_1 and P_2 except the isolates GFLV-B714 and ArMV-Tannat. The results obtained with the three probes on all GFLV and ArMV infected tissues are summarized in Table 2.

Among the 18 isolates, only one GFLV isolate (GH) and two ArMV isolates (862 and C) hybridized with probe P_3, suggesting the presence of a satellite RNA associated with the latter strains for which no satellite RNA has been reported previously. The presence of a satellite RNA in these strains was visualized upon analysis of virion RNAs on denaturing agarose gels after ortho-toluidine blue staining. This is illustrated by the estimation of size for the virion RNAs of two isolates, GFLV-TU92-14 and ArMV-862, propagated on *C. quinoa*. For both isolates, the genomic RNAs were identical in size to the GFLV-F13 RNAs

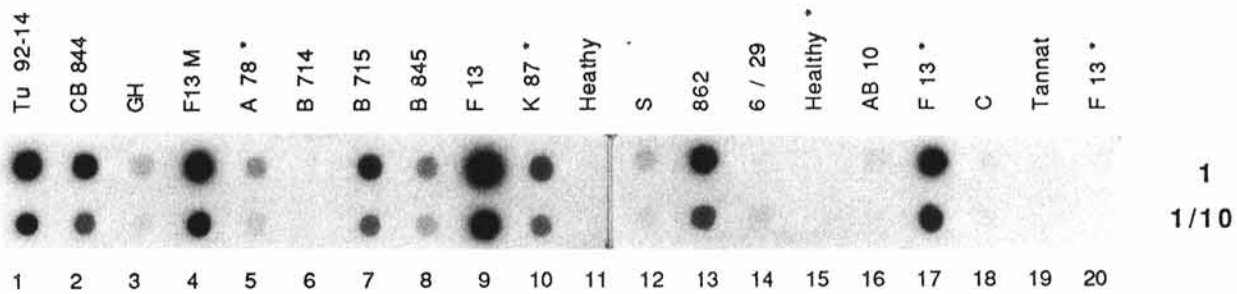

Fig. 2. Dot-blot hybridization for grapevine fanleaf virus (GFLV) and arabis mosaic virus (ArMV) RNAs in plant RNAs extracted from healthy and individually infected *Chenopodium quinoa* or grapevine (*) leaves. Blots were probed with P_1 in 50% formamide at 42 C, washed in 2× SSC + 0.1 % SDS at 60 C and autoradiographed overnight. Samples 1–10 correspond to different GFLV isolates (TU92-14, CB844, GH, F13M, A78, B714, B715, B845, F13, and K87) and samples 12–14, 16, 18, and 19 to ArMV isolates (S, 862, 6/29, AB10, C, Tannat). Samples 11 and 15 are from healthy plants. Samples from infected grapevine extracted with or without use of sodium perchlorate are shown in 17 and 20, respectively. Numbers beside the blots correspond to 5 μg (1) and 0.5 μg (1/10) of plant RNAs applied in each case.

(25) (Fig. 3, panel A) and the size of the satellite RNA of ArMV-862 and also of other isolates (not shown) was similar to that of GFLV-F13 RNA3. However, the translation products of the GFLV-GH and ArMV-862 satellite RNAs differed despite the similarity in size of the RNA: The GFLV-GH satellite RNA directed synthesis of a single protein (estimated 39K) as did GFLV-F13 satellite RNA (9), while the ArMV-862 satellite RNA directed synthesis of two proteins with apparent molecular weights 40K and 35K (Fig. 3B). Similar translation products have been reported for the satellite RNA of ArMV-lilac (37).

Nucleotide sequence homologies were also checked with virion RNAs from other nepoviruses with the GFLV probes. TBRV, RRV, SLRV, and CALV were tested, but none of these RNAs could be detected.

Detection of viral RNAs in grapevines. The GFLV-F13 probes were further used to detect the viral RNA in grapevine extracts. Preliminary experiments revealed that the RNA extraction procedure used for *C. quinoa* (see Materials and Methods) was unsuccessful when grapevine tissues were used, despite the fact that the plants analyzed indexed positively by typical symptom expression and ELISA. However, the detection of viral RNAs was possible in RNAs extracted from such tissue if sodium perchlorate and polyvinylpyrrolidone were used to eliminate polyphenols interfering with nucleic acid isolation (22). The efficiency of the RNA extraction procedure described in Material and Methods is illustrated in Figure 2, where the RNAs from GFLV-F13 infected grapevine leaves treated with both techniques were compared. Only the procedure involving sodium perchlorate extraction gave positive hybridization signals (compare sample 17 and sample 20 in Fig. 2).

RNAs extracted from grapevine leaves or rootlets were tested with the three cDNA probes in northern blot hybridizations (Fig. 4). There was no difference between the pattern of stained RNAs obtained from healthy or infected grapevines, thus indicating that the amount of each viral RNA species was too low to be detected by staining. The hybridization results show that the genomic RNAs were clearly detected with probes P_1 and P_2 (Fig. 4A and B). No response was obtained in the virus-free samples. The RNAs extracted from a plant infected by a strain free of satellite RNA (GFLV-F13 M) revealed no hybridization response with probe P_3. The previous data obtained by northern hybrization on *C. quinoa* were therefore confirmed, that is, each genomic probe specifically detected its homologous viral RNA in total RNA from infected grapevine leaves or rootlets. RNA3 was also identified in the GFLV-F13 infected grapevine with probe P_3 (Fig. 4C). Morever, the size of the detected satellite RNA was identical in the grapevine leaves and rootlets.

DISCUSSION

The results reported in this paper show that hybridization of cDNA probes to immobilized plant RNA samples is a highly sensitive method for specific virus detection in plants. While no detectable signal is observed with healthy plants, GFLV RNAs are detected at the picogram level of RNA from infected plant. This detection threshold was estimated with the probe P_1 specific to RNA1. This probe was chosen because RNA1 is infectious alone (26) and contains the polymerase domain as determined from sequence analysis (L. Pinck, *unpublished results*). The amount of viral RNA1 in infected tissue therefore constitutes

TABLE 2. Hybridization of labeled grapevine fanleaf virus (GFLV) probes (P_1, P_2, P_3) with RNAs from plants infected with various isolates of GFLV and arabis mosaic virus (ArMV)

Inoculated isolate	GFLV Isolate								ArMV isolate									
	F13	F13M	A78	B714	B715	B845	CB844	GH	K87	TU92-14	862	S	6/29	Tannat	T75	AB10	C	H
Probe P_1	3[a]	3	1	0	2	1	2	1	1	2	2	1	1	0	1	1	1	1
Probe P_2	3	3	1	0	2	1	2	1	1	2	2	1	1	0	1	1	1	1
Probe P_3	3	0	0	0	0	0	0	2	0	0	1	0	0	0	0	0	1	1

[a] Strong (3), intermediate (2), low (1), no (0) hybridization.

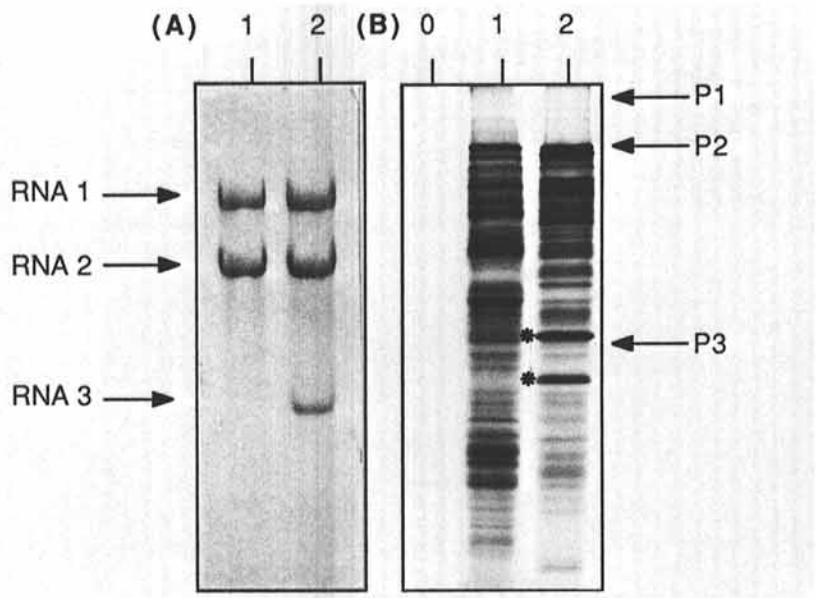

Fig. 3. Electrophoretic pattern of grapevine fanleaf virus (GFLV)-TU 92-14 (A, lane 1) and ArMV-862 (A, lane 2) RNAs analyzed in a formaldehyde-agarose gel and stained with ortho-toluidine blue. The arrows indicate the mobility in the same gel of the virion RNAs from GFLV-F13 used as size markers (25). Translation products analysed by SDS-PAGE in 12.5% gels of GFLV-TU 92-14 virion RNAs (B, lane 1) and ArMV-862 virion RNAs (panel B, lane 2). Lane 0 shows the endogenous proteins of the wheat germ system. P_1, P_2, and P_3 indicate the positions of the major translation products of GFLV-F13 RNA1 (225K), RNA2 (127K), and RNA3 (39K), respectively. The position of the 40K and the 35K proteins translated from the ArMv-862 satellite RNA are indicated by an asterisk.

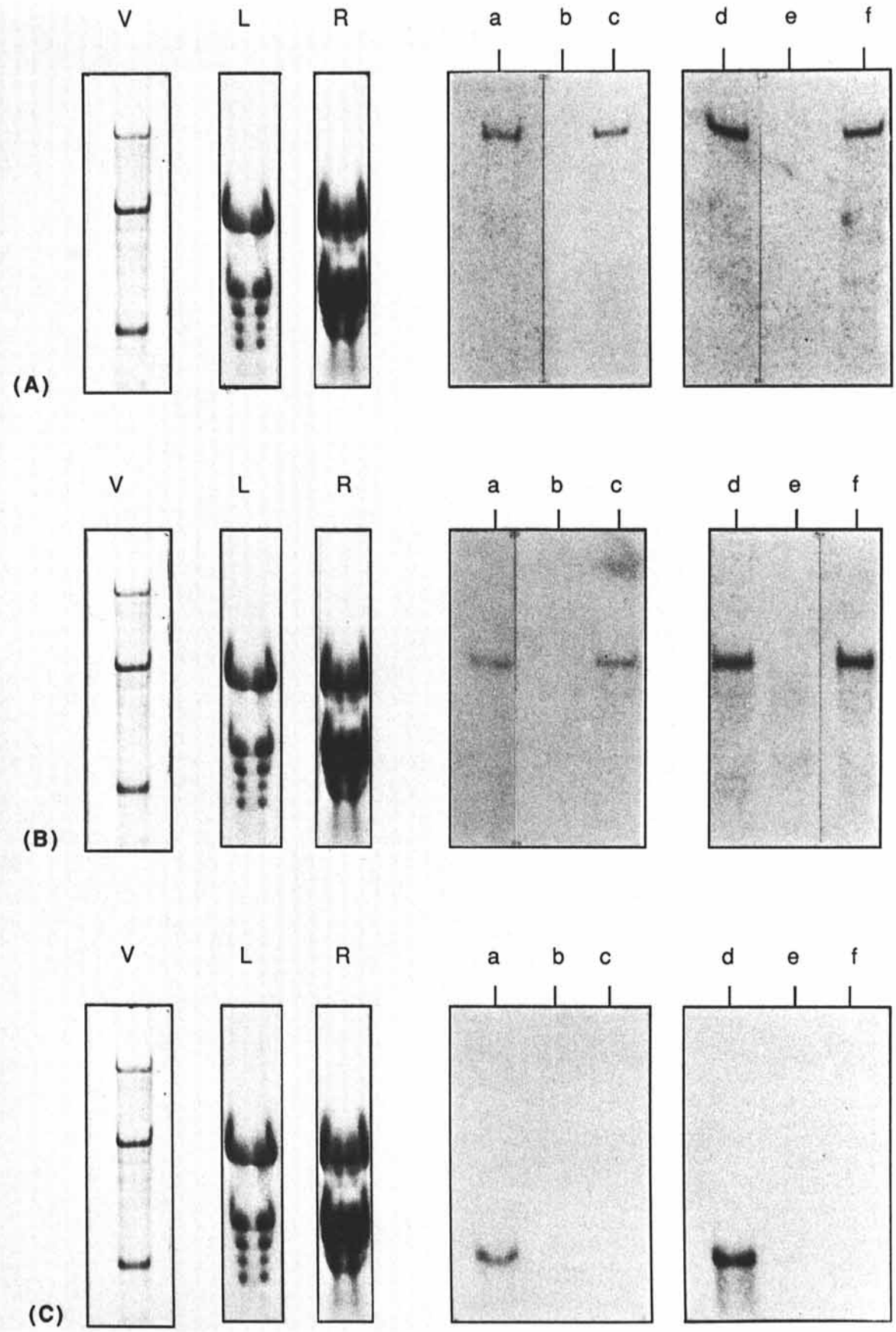

Fig. 4. Autoradiographic detection of viral RNAs after northern hybridization with ^{32}P-labeled probes P_1 (A), P_2 (B), and P_3 (C) by using nucleic acid extracted from grapevine fanleaf virus (GFLV)-F13 infected grapevine leaves (a), healthy grapevine leaves (b), GFLV-F13M infected grapevine leaves (c), GFLV-F13 infected grapevine rootlets (d), healthy grapevine rootlets (e), GFLV-F13M infected grapevine rootlets (f). Lanes V, L, and R show ortho-toluidine stained gels corresponding to GFLV-F13 virion RNA (V), RNAs from grapevine infected leaves (L), and RNA from infected grapevine rootlets (R), respectively.

a good indicator of the degree of virus multiplication and allowed detection as early as 3 days postinoculation at a moment where no coat protein is detectable by ELISA methods.

Among the genomic RNAs of the 10 GFLV isolates examined, all, except GFLV-B714, reacted with the probes P_1 and P_2. The RNA2 specific probe P_2 covers 42% in length of the 3′ end of the coat protein cistron (31); compared with the polyclonal antibodies this probe may behave in an approximately similar way. Hybridization with probes P_1 and P_2 indicates that nucleotide sequences are conserved between these GFLV isolates. These probes hybridized also with ArMV isolates that belong to the same subgroup of nepoviruses but that do not react with the GFLV polyclonal antibodies used. Attempts to detect homologies with other nepoviruses failed: Our GFLV probes revealed no cross-reactions with RNAs from TBRV, RRV, SLRV, and CALV.

The RNA3 specific probe P_3 proved very useful for screening various GFLV and ArMV isolates for the presence of satellite RNAs associated with various GFLV and ArMV isolates without preliminary virus extraction and purification. In this way, satellite RNAs were found in GFLV-GH and ArMV-862 isolated from grapevine and in ArMV-C isolated from raspberry, three isolates for which no satellite RNA has been described so far. Probe P_3 was unable to detect the satellite RNA of TBRV strain S. This absence of homology between GFLV-F13 and TBRV-S satellite RNAs was recently confirmed by comparing their nucleotide sequences (9). Moreover, no low molecular weight satellite RNA similar to that associated with ArMV-Ta responsible for hop nettlehead disease (6,17) or that associated with chicory yellow mottle virus (24) has so far been identified in any of the GFLV and ArMV origins analyzed by gel electrophoresis or hybridization experiments.

Our polyclonal antibodies obtained with purified GFLV-F13 particles used as immunizing antigens recognize all GFLV strains analyzed in ELISA tests. Serological distinction among GFLV isolates are only possible when monoclonal antibodies are used (13). The cDNA probes have a broader detection than antisera since GFLV and ArMV RNAs cross-react in hybridization with probes P_1 and P_2. When polyclonal antibodies are used, the detection limit is about 1 ng of bottom particles diluted in crude sap of healthy plants. With the radioactive probes, the sensitivity is of the order of 1 pg of viral RNA1 in RNAs extracted from infected plants. It is, however, not straightforward to compare the sensitivities of the two methods. First, since the preparation of bottom particles involves a single sucrose density gradient step, contamination with middle component particles is likely and can lead to an overevaluation of the virion concentration used to establish the ELISA calibration curve. Furthermore, since the ratio of RNA1 and RNA2 in bottom components is not well established (26), the proportion of RNA1 in bottom particles remains uncertain. Thus, it is not possible to calculate a precise relation between the detection limit of the two techniques by experiments performed with infected plant extracts. In that case, our results with molecular hybridization correlate well with those of ELISA. By comparing the detection thresholds of molecular hybridizaion and ELISA, it was estimated that cDNA probes are about 20-fold more sensitive than polyclonal antibodies in the standard conditions used here.

To successfully use molecular hybridization techniques directly on grapevine tissues, it was necessary to adapt the RNA extraction procedure. With conventional methods (22), it is impossible to extract RNAs as intact uncomplexed molecules from grapevine leaves or rootlets. To overcome this problem, a procedure was used employing high concentrations of a chaotropic agent and insoluble polyvinylpyrrolidone to inhibit polyphenolic compounds that otherwise quickly complex the released cellular RNAs during tissue homogenization. Recently, Rezaian and Krake (27) developed a grapevine RNA isolation procedure that involved a rapid two-step extraction followed by ethanol precipitation to avoid the use of phenol, which severely reduces the RNA yield during tissue homogenization. However, the RNA extraction conditions described here also proved suitable for virus indexing of grapevines and allowed specific identification of GFLV RNAs in grapevine leaves and rootlets. The RNA patterns obtained by northern hybridization are identical for RNAs from both organs and similar to those for RNAs from *C. quinoa* or from virions. These observations differ from the variable RNA patterns reported for the small BNYVV RNAs (4,19,28), where RNA3 and 4 patterns in sugar beet root extracts are identical for several virus origins but show great variation in number and size in leaf extracts from sugar beet or *C. quinoa*. In our case modification in length of the GFLV-F13 satellite RNA was never observed. Only one natural mutant (GFLV-F13M) occurred, which resulted from the spontaneous loss of the satellite RNA during cloning of GFLV-F13 on *C. quinoa* in the glasshouse.

The northern hybridization or dot-blot techniques used at present require radioactively labeled cDNA fragments, which impair large-scale applications. Among the possible methods to avoid radiolabeling, the use of biotin-labeled probes may be a good alternative (3,10). Biotin can be detected by fluorescence (23), by highly specific complex formation with avidin or streptavidin (5,30), or by enzyme-labeled antisera (20). Our preliminary experiments with the nonradioactive BRL DNA detection kit were successful with virion RNAs dotted on hybridization membranes, but the detection was about 100-fold less sensitive than with the radioactive probes.

Molecular hybridization is a powerful method for virus characterization and is complementary to the immunoenzymatic techniques. For large-scale diagnosis, a rapid and easy sample preparation procedure in needed. Therefore, ELISA is the most suitable technique because additional treatment of the crude sap is not necessary to allow highly sensitive virus detection, as it is for molecular hybridization. However, the use of cDNA probes to evaluate the amount of viral RNA in plants was a good complement to immunoenzymatic techniques in cross-protection studies between ArMV-S and GFLV-F13 isolates performed in *C. quinoa* (15). When cross-protection occurred between ArMV-S and GFLV-F13 for instance, we could demonstrate that a close correlation exists between the severity of the symptoms in doubly inoculated plants and the amounts of coat protein and nucleic acid of the challenge virus (15).

Many virus strains cannot be distinguished by immunological methods because of their wide antigenic cross-homology. As soon as the sequences of the genome of other GFLV isolates are characterized, it should be possible to construct either specific probes for the detection of individual GFLV isolates or "universal" probes for the widest detection range of viruses belonging to the nepovirus ArMV subgroup.

The satellite specifc probe P_3 allows a distinction between the different strains for the presence of satellite RNAs. It will be of special interest to investigate the biological properties of these satellite RNAs.

LITERATURE CITED

1. Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 6:43-52.
2. Bass, P., and Legin, R. 1981. Thermothérapie et multiplication in vitro d'apex de la vigne. Application à la séparation ou à l'élimination de diverses maladies de type viral et à l'évaluation des dégâts. C. R. Acad. Agric. France 11:922-933.
3. Bugawan, T. L., Saiki, R. K., Levenson, C. H., Watson, R. M., and Erlich, H. A. 1988. The use of non radioactive oligonucleotide probes to analyse enzymatically amplified DNA for prenatal diagnosis and forensic HLA typing. Biotechnology 6:943-947.
4. Burgermeister, W., Koenig, R., Weich, H., Sebald, W., and Lesemann, D. E. 1986. Diversity of the RNAs in thirteen isolates of beet necrotic yellow vein virus in *Chenopodium quinoa* detected by means of cloned cDNAs. J. Phytopathol. 115:229-242.
5. Cham, V. Y. W., Fleming, K. A., and McGee, J. O. D. 1985. Detection of subpicogram quantities of specific DNA sequences on blot hybridization with biotinylated probes. Nucleic Acids Res. 13:8083-8091.
6. Davies, D. L., and Clark, M. F. 1983. A satellite-like nucleic acid of arabis mosaic virus associated with hop nettlehead disease. Ann. Appl. Biol. 103:439-448.
7. Dias, H. F., and Harrison, B. D. 1963. The relationship between

grapevine fanleaf, grapevine yellow mosaic and arabis mosaic viruses. Ann. Appl. Biol. 51:97-105.
8. Gallitelli, D., Savino, V., and Martelli, G. P. 1985. The use of a spot hybridization method for the detection of grapevine virus A in the sap of grapevine. Phytopathol. Mediterr. XXIV:221-224.
9. Fuchs, M., Pinck, M., Serghini, M.A., Ravelonandro, M., Walter, B., and Pinck, L. 1989. The nucleotide sequence of satellite RNA in grapevine fanleaf virus, strain F13. J. Gen. Virol. 70:955-962.
10. Habili, N., Mc Innes, J. L., and Symons, R. 1987. Non radioactive, photobiotin labelled DNA probes for the routine diagnosis of barley yellow dwarf virus. J. Virol. Methods 16:225-237.
11. Hardie, G., and Van Regenmortel, M. H. V. 1977. Isolation of specific antibody under conditions of low ionic strength. J. Immunol. Methods 15:305-315.
12. Heidecker, G., and Messing, J. 1983. Sequence analysis of zein cDNAs obtained by an efficient mRNA cloning method. Nucleic Acids Res. 11:4891-4906.
13. Huss, B., Muller, S., Sommermeyer, G., Walter, B., and Van Regenmortel, M.H.V. 1987. Grapevine fanleaf virus monoclonal antibodies: Their use to distinguish different isolates. J. Phytophatol. 119:358-370.
14. Huss, B., Walter, B., Etienne, L., and Van Regenmortel, M. H. V. 1986. Grapevine fanleaf virus detection in various grapevine organs using polyclonal and monoclonal antibodies. Vitis 25:178-188.
15. Huss, B., Walter, B., and Fuchs, M. 1989. Cross-protection between arabis mosaic virus and grapevine fanleaf virus in *Chenopodium quinoa*. Ann. Appl. Biol. 114:45-60.
16. Jackson, A. O., and Larkins, B. A. 1976. Influence of ionic strength, pH and chelation of divalent metals on isolation of polyribosomes from tobacco leaves. Plant Physiol. 57:5-10.
17. Kaper, J. M., Tousignant, M. E., and Steger, G. 1988. Nucleotide sequence predicts circularity and self-cleavage of 300-ribonucleotide satellite of arabis mosaic virus. Biochem. Biophys. Res. Comm. 154:318-325.
18. Koenig, R., An, D., and Burgermeister, W. 1988. The use of filter hybridization techniques for the identification, differentiation and classification of plant viruses. J. Virol. Methods 19:57-68.
19. Koenig, R., Burgermeister, W., Weich, H., Sebald, W., and Kothe, C. 1986. Uniform RNA pattern of beet necrotic yellow vein virus in sugarbeet roots, but not in leaves from several plant species. J. Gen. Virol. 67:2043-2046.
20. Langer-Safer, P. R., Levine, M., and Ward, D. C. 1982. Immunological method for mapping genes on Drosophila polytene chromosomes. Proc. Natl. Acad. Sci. USA 79:4381-4385.
21. Maule, A. J., Hull, R., and Donson, J. 1988. The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. J. Virol. Methods 6:215-224.
22. Newbury, H. J., and Possingham, J. V. 1977. Factors affecting the extraction of intact ribonucleic acid from plant tissues containing interfering phenolic compounds. Plant Physiol. 60:543-547.
23. Oser, A., Rothe, W. K., and Valet, G. 1988. Sensitive non radioactive dot-blot hybridization using DNA probes labelled with chelate group substituted psoralen and quantitative detection by europium ion fluoresence. Nucleic Acids Res. 16:1181-1196.
24. Piazzolla, P., Rubino, L., Tousignant, M. E., and Kaper, J. M. 1989. Two different types of satellite RNA associated with chicory yellow mottle virus. J. Gen. Virol. 70:949-954.
25. Pinck, L., Fuchs, M., Pinck, M., Ravelonandro, M., and Walter, B. 1988. A satellite RNA in grapevine fanleaf virus strain F 13. J. Gen. Virol. 69:233-239.
26. Quacquarelli, A., Gallitelli, D., Savino, V., and Martelli, G. P. 1976. Properties of grapevine fanleaf virus. J. Gen. Virol. 32:349-360.
27. Rezaian, M. A., and Krake, L. R. 1987. Nucleic acid extraction and virus detection in grapevine. J. Virol. Methods 17:277-285.
28. Richards, K., Jonard, G., Guilley, H., Ziegler, V., and Putz, C. 1985. In vitro translation of beet necrotic yellow vein virus RNA and studies of sequence homology among the RNA species using cloned cDNA probes. J. Gen. Virol. 66:345-350.
29. Rigby, P. W. J., Dieckman, M., Rhodes, C., and Berg, P. 1977. Labelling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. J. Mol. Biol. 113:237-251.
30. Roy, B. P., AbouHaidar, M. G., Sit, T. L., and Alexander, A. 1988. Construction and use of cloned cDNA biotin and ^{32}P-labeled probes for the detection of papaya mosaic potexvirus RNA in plants. Phytopathology 78:1425-1429.
31. Serghini, M.A., Pinck, M., Fuchs, M., Walter, B., and Pinck, L. 1989. RNA2 of grapevine fanleaf virus: Cloning, sequence analysis and coat protein location. The 4th International Plant Virus Epidemiology, Workshop, Montpellier, France. Abstr. book, page 144.
32. Varveri, C., Ravelonandro, M., and Dunez, J. 1987. Construction and use of cloned cDNA probe for the detection of plum pox virus in plants. Phytopathology 77:1221-1224.
33. Walter, B., and Etienne, L. 1987. Detection of the grapevine fanleaf viruses away from the period of vegetation. J. Phytopathol. 120:355-364.
34. Walter, B., Ladevéze, I., Etienne, L., and Fuchs, M. 1989. Some properties of a previously undescribed virus from cassava: cassava american latent virus. Ann. Appl. Biol. 115: 279-289.
35. Walter, B., Vuittenez, A., Kuszala, J., Stocky, G., Burckard, J., and Van Regenmortel, M. H. V. 1984. Détection sérologique des virus du court-noué de la vigne par le test ELISA. Agronomie 4:527-534.
36. White, B. A., and Bancroft, F. C. 1982. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. J. Biol. Chem. 257:8569-8572.
37. Yuanyi, L., and Cooper, J. I. 1989. A disease-modifying satellite RNA from arabis mosaic nepovirus. Abstracts of the NATO Advanced Research Workshop, Recognition and Response in Plant-Virus Interactions, 12-16 April, Chichester.