Development of a Polymerase Chain Reaction Technique for the Detection of Grapevine Fanleaf Virus in Grapevine Tissue

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We thank the American Vineyard Foundation, the California Table Grape Commission, and the California Raisin Advisory Board for their support.

We thank Bruce Kirkpatrick for suggestions and Patricia Hargis for technical assistance.

Accepted for publication 25 February 1993.

ABSTRACT

Rowhani, A., Chay, C., Golino, D. A., and Falk, B. W. 1993. Development of a polymerase chain reaction technique for the detection of grapevine fanleaf virus in grapevine tissue. Phytopathology 83:749-753.

A polymerase chain reaction (PCR) method has been developed to detect grapevine fanleaf virus (GFLV) in GFLV-infected grape tissue. Four sample extraction methods for infected plant tissues were compared. Although PCR could readily detect RNA in samples of GFLV RNA and virion in GFLV-infected leaf samples of *Gomphrena globosa* with all four extraction methods, only one method was useful for GFLV detection in grapevine tissue. Dilution of infected grape leaf samples by a 200-fold excess of healthy leaf tissue did not prevent GFLV detection by this method. Extracts from healthy grapevines prepared by methods 1, 2, and 3 prevented detection of extracted GFLV genomic RNAs by PCR, demonstrating that grape tissue extracts can inhibit either reverse-transcriptase reactions or PCR. Using method 4, GFLV could be detected in all tested cultivars of European grape, *Vitis vinifera*, and an American species, *V. rupestris*. Detection was possible in infected leaves, shoots, roots, and bark scrapings. PCR detection of as little as 128 fg of GFLV RNA was possible.

Grapevine fanleaf virus (GFLV) causes a severe disease in grapevines (*Vitis vinifera* L.). It is believed that this nepovirus has coexisted with grapes since the earliest cultivation of grapevines and has spread with the vegetatively propagated crop (20). Worldwide in distribution, it is nearly impossible to eradicate from vineyards that have been planted with infected vines and that are infested with the vector *Xiphinema index* (Thorne & Allen) (10). Spread can occur with either the use of infected propagating materials or vector feeding.

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The disease caused by GFLV, variously referred to as fanleaf, vein banding, yellow mosaic, or fanleaf degeneration (9,14), causes both malformations and discoloration of leaves. Berry set is reduced on infected vines. Yield losses of up to 80% (14) have been cited, in addition to loss of quality and reduction in vineyard longevity.

A primary control strategy for GFLV has been the use of virus-tested planting materials on uninfested land. Fortunately, the natural host range of the virus is limited to *Vitis* (9). Thus, land that has not been previously planted to grapes is unlikely to harbor viruliferous nematode populations. To produce virus-tested stock,

GFLV has been detected by techniques including herbaceous plant (9) and woody plant indexing (8), serology (e.g., enzyme-linked immunosorbent assay [ELISA] [1,17,22]), dsRNA analysis (A. Rowhani and D. A. Golino, *unpublished data*), and hybridization with cDNA probes (5). Reliability and practical applicability of GFLV testing could improve with the development of a more sensitive technique that is fast and, unlike other GFLV tests (11,18,21), free from seasonal fluctuations in sensitivity. In addition, progress in developing fanleaf-resistant grapevines, by either traditional breeding techniques or by genetic-engineering strategies, would be greatly accelerated by the development of techniques with increased sensitivity for GFLV detection.

Development of a polymerase chain reaction (PCR) assay for GFLV would provide a significant improvement in current detection technology, improving grapevine clean-stock programs and facilitating research to develop resistant cultivars. PCR assay has been modified for RNA viruses (12), and the necessary GFLV nucleotide sequences for PCR primers have been published (19). However, some problems with applying PCR technology to *Vitis* could be anticipated because of the presence of high levels of phenolic compounds, polysaccharides, and other substances that previously have been proven to make both nucleic acid extraction and virus detection difficult when grape tissue is processed (4,15,16).

This paper reports on the use of PCR to detect GFLV in samples, including viral RNA and infected herbaceous hosts and grapevines. Because standard extraction protocols were not effective for grape tissue, modifications of the sample preparation techniques were compared. A successful modification is reported. Using the modified protocol, the efficiency and sensitivity of PCR for detecting GFLV was evaluated for various grapevine tissues and in selected cultivars.

MATERIALS AND METHODS

Cultivar, virus, and primer sources. GFLV isolates were obtained from the Davis Grapevine Virus Collection (6): fanleaf deformation (GFLV 100 and GFLV 103), vein banding (GFLV 105), and yellow mosaic (GFLV 107). Grapevine cultivars tested included *Vitis rupestris* Scheele 'St. George' and *V. vinifera* cvs. Chenin Blanc, French Colombard, and Thompson Seedless. Types of tissue tested included young leaves and shoot tips collected from the field, shoots and roots from dormant cuttings forced in water in the laboratory, and bark scrapings from dormant cuttings. The herbaceous host of GFLV, *Gomphrena globosa* L., was grown in the greenhouse and mechanically inoculated with GFLV; symptomatic leaves were used in the experiments.

RNAs from the purified preparation of GFLV virions were released by phenol extraction (13). Fivefold serial dilutions (40 ng/ml to 2.4 pg/ml) in water were made from the RNA, starting at OD_{260} of 0.001 (equal to 40 ng of RNA per milliliter), and 2 μl of each dilution was used in the PCR reaction.

The PCR primers were selected from the GFLV coat protein-gene region, located at the 3′ end of RNA2 (19). Primer C1 was complementary to nucleotides 1,064–1,083 (5′-CCAAAGTTGGTTTCCCAAGA-3′). Primer V1 corresponded to nucleotides 762–781 (5′-ACCGGATTGACGTGGGTGAT-3′). Preliminary PCR experiments with GFLV cDNA clones showed that these primers were sufficient to amplify DNA of the expected size (321 bp).

Sample preparation. Preliminary experiments using method 1 for sample preparation indicated that although GFLV could be detected in GFLV-infected *G. globosa* or GFLV RNA by PCR, problems were encountered when infected grape tissue was used. Therefore, different procedures were evaluated for extraction of total nucleic acids from grapevine tissues. Unless otherwise mentioned, all steps were performed at 4 C, and all centrifugations were carried out in a Sorvall SA600 rotor (E. I. Du Pont De Nemours & Co., Inc., Sorvall Instruments Division, Newtown, CT) at 4 C and 11,700 *g*.

Method 1. One gram of tissue was ground to a powder with liquid nitrogen in a mortar and pestle; 4 ml of extraction buffer (10 mM Tris-HCl, 10 mM EDTA, and 5 mg of sodium dodecyl sulfate [SDS] per milliliter, pH 8.0) was added and mixed well. Four milliliters of water-saturated phenol and 4 ml of chloroform were added to the extract and mixed by vortexing. The homogenate was centrifuged for 10 min, and the aqueous phase was reextracted with phenol and chloroform. The aqueous phase was transferred into a clean tube, and the nucleic acid was precipitated with ethanol. The pellet was resuspended in 0.5 ml of water, and 2 μl was used in the reverse-transcriptase reaction.

Method 2. Method 1 was followed until the second phenol-chloroform extraction, then the aqueous phase was transferred into a clean tube and an equal volume of 5 M lithium chloride was added, mixed well, and stored at 4 C overnight. The tube was centrifuged for 15 min at 16,800 *g*. The pellet was saved and resuspended in 0.4 ml of water, and the single-stranded RNA was recovered by precipitation with ethanol. This pellet was resuspended in 0.5 ml of water, and 2 μl was used in the reverse-transcriptase reaction.

Method 3. One gram of tissue was ground in liquid nitrogen and mixed with 9.0 ml of extraction buffer (100 mM Tris-HCl, pH 8.2; 20 mM EDTA; 2% polyvinyl polypyrrolidone [PVPP]; 1.4 M NaCl; and 2% activated charcoal). Ten milliliters of water-saturated phenol and 10 ml of chloroform were added to the extract, vortexed several times, and centrifuged for 10 min. The aqueous phase was transferred into a clean tube, an equal volume of chloroform was added, and the tube was vortexed and centrifuged for 10 min. The aqueous phase was transferred into a clean tube and the nucleic acids were precipitated with ethanol. The pellet was resuspended in 0.5 ml of water, and 2 μl was used in the reverse-transcriptase reaction.

Method 4. A modified method of Dellaporta et al (2) was used (B. Kirkpatrick, Univ. Calif., Davis, *personal communication*). One gram of fresh tissue was cut in small pieces in a cold mortar containing 5 ml of cold extraction buffer (21.7 g of $K_2HPO_4 \cdot 3H_2O$, 4.1 g of KH_2PO_4, 100 g of sucrose, 1.5 g of bovine serum albumin [BSA] fraction V, 20 g of polyvinyl pyrrolidone (PVP 10), and 5.3 g of ascorbic acid per liter (ascorbic acid was added, and the pH was adjusted to 7.6 before use). The tissue was incubated in the buffer 10–20 min to plasmolyze the cells before grinding. The tissue was ground thoroughly, 5 ml more of fresh extraction buffer was added, and the tissue was ground again. The extract was transferred into a cold centrifuge tube and was centrifuged for 3–4 min at 1,050 *g*. The supernatant was transferred into a clean centrifuge tube, and the pellet was discarded. The supernatant was centrifuged for 20 min at 16,800 *g*. The supernatant was discarded, and the tube was drained briefly and placed in an ice bucket. The pellet was resuspended in 2 ml of a solution containing 10 mM EDTA; 50 mM Tris, pH 8.0; and 0.1% of 2-mercaptoethanol (added immediately before use). After resuspension, 250 μl of 10% SDS was added to the sample and incubated for 10 min at 60 C. Then 800 μl of 5 M potassium acetate was added and mixed thoroughly, and the tube was incubated on ice for at least 30 min (or overnight at 4 C). The tube was centrifuged for 15 min at 16,800 *g*. The clear supernatant was transferred to a clean, sterile corex tube. One-tenth of the volume of 3 M sodium acetate, pH 5.4, and 1 volume of ice-cold isopropanol were added to the supernatant, mixed thoroughly, and incubated at −20 C for 1 h or more. The tube was centrifuged for 20 min at 16,800 *g*, the supernatant was discarded, the pellet was washed with 1–2 ml of cold 80% ethanol, and the tube was inverted on a paper towel at room temperature to dry. The pellet was resuspended in 0.5 ml of TE, pH 7.4 (10 mM Tris base and 1 mM EDTA), on ice, and 2 μl was used for reverse-transcriptase reaction.

PCR procedure. The reverse-transcriptase reactions and PCRs were done with the GeneAmp RNA kit (Perkin-Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions. GFLV RNA (without any denaturing treatments) served as template for first-strand cDNA synthesis in the presence of reverse transcriptase and 0.2 μg per reaction of C1 primer. Cocktail was made for reverse transcription from the reagents provided in the kit, as suggested by the manufacturer. Sample or virus genomic RNA

was added to the cocktail, and the DNA thermal cycler (model 480, Perkin-Elmer Cetus) was programmed for reverse transcription at 42 C for 15 min, 99 C for 5 min, and 5 C for 5 min. For PCR, the cocktail was made as suggested by the manufacturer, and V1 primer at 0.2 μg per reaction was added. Then 79 μl of the cocktail was added and mixed with the contents of the tube from the reverse-transcriptase reaction. The PCR was performed in the DNA thermal cycler programmed for an initial cycle of 95 C for 2 min; 35 cycles of 1 min at 95 C, 1 min at 53 C, and 1 min at 72 C; and a final cycle of 7 min at 72 C prior to holding the samples at 4 C until removal from the thermal cycler.

Analysis of PCR products. Ten microliters of each PCR product was analyzed by electrophoresis through a 2% agarose gel in TBE (10.8 g of Tris, 5.5 g of boric acid, and 0.4 ml of 0.5 M EDTA per liter) at 5 V/cm for 1.5 h. The gels were stained in ethidium bromide (0.5 μg/ml of water) 20–30 min, rinsed briefly in deionized water, and photographed on a UV-transilluminator with a Polaroid camera.

Detection of different GFLV isolates and the effects of different cultivars. The effects of grapevine cultivar on PCR detection was tested with two species, *V. vinifera* (cvs. Chenin Blanc, French Colombard, and Thompson Seedless) and *V. rupestris* (cv. St. George). In another experiment, different isolates of GFLV producing three symptom types (fanleaf deformation, vein banding, and yellow mosaic) were analyzed in samples prepared from young leaves and shoot tips of infected grapevines.

Detection of GFLV in different grapevine tissues. GFLV-infected tissues were sampled from young leaves and shoot tips collected from field-grown vines, young leaves and shoot tips from dormant cuttings forced in water in the laboratory, cambial tissue and phloem scraped from dormant cuttings, and root tissue from forced dormant cuttings.

Sensitivity of PCR for detecting GFLV. To determine the sensitivity of PCR for detecting GFLV from grapevine leaves, weighed amounts of leaf tissue from GFLV-infected vines were mixed with healthy leaf tissue to make the following ratios of infected to healthy tissue: 1:0, 1:10, 1:50, 1:200, and 0:1. Extracts for PCR were prepared from each according to method 4. In addition, purified GFLV RNA was diluted in water 40, 8, and 1.6 ng/ml and 320, 64, 12, and 2.4 pg/ml for detection by PCR.

RESULTS

Detection of GFLV by PCR and the effect of the sample preparation method. GFLV was readily detected by PCR from unencapsidated RNA and from GFLV-infected *G. globosa* regardless of sample preparation procedure. When infected grapevine tissue was sampled, results were negative with methods 1, 2, and 3. The addition of healthy grapevine extracts prepared for methods 1, 2, and 3 to GFLV-virion RNA also inhibited PCR detection. This suggested the presence of substance(s) inhibitory to reverse transcriptase and/or *Taq* DNA polymerase (Amplitaq, Perkin-Elmer Cetus) in grape tissue.

These inhibitors could sometimes be neutralized when the final sample extract from method 3 was frozen overnight and thawed the next day and had approximately 2 mg of activated charcoal added to 10 μl of extract. However, positive detection was inconsistent with this approach. Other additives, such as 2% nicotine or 2% PVP 40 added to the extraction buffers used for methods 1–3 did not increase the sensitivity of detection of GFLV.

Method 4 produced consistently positive results with both GFLV-infected *G. globosa* and with infected grapevine tissue (Fig. 1). Subsequent experiments were conducted with method 4 for sample preparation.

Detection of different GFLV isolates and the effects of different cultivars. GFLV from infected *V. vinifera* cvs. Chenin Blanc, French Colombard, and Thompson Seedless as well as *V. rupestris* cv. St. George (Fig. 2) was detected by PCR. No differences were observed between cultivars. These infected vines included the three symptom types reported for GFLV.

Detection of GFLV in different grapevine tissues. When different GFLV-infected grapevine tissues (e.g., leaves, bark scrapings, and roots) were extracted by method 4 and compared for PCR detection of GFLV, GFLV was detected in samples from all three sources (Fig. 3).

Sensitivity of PCR for GFLV. PCR detection of GFLV was successful when infected samples were diluted with healthy tissue at all dilutions tested (Fig. 4). When leaf samples from 200 vines were collected, bulked as one sample, and used in PCR amplifica-

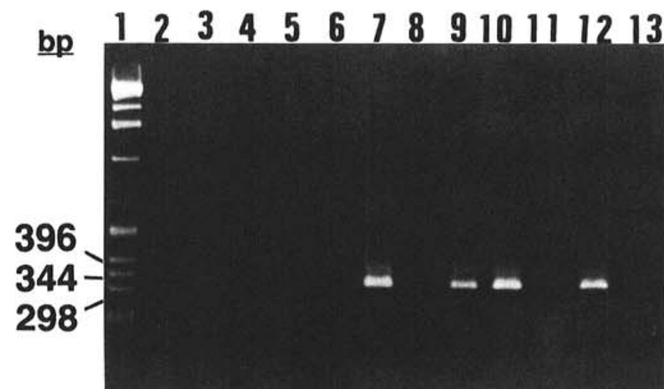

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products with different sample extraction methods, contrasting the results obtained with grapevine fanleaf virus (GFLV)-infected *Gomphrena globosa* and *Vitis vinifera*. Lane 1, DNA ladder; lanes 2–5 and 10–11, tissue extracted by method 3; and lanes 6–9, tissue extracted by method 4. Lanes 2 and 6, healthy; lanes 3 and 7, GFLV-infected *G. globosa* tissue; lanes 4 and 8, healthy; lanes 5 and 9, GFLV-infected *V. vinifera* tissue; lane 10, healthy *G. globosa* and lane 11, healthy *V. vinifera* extracts were added to the reaction containing 10 pg of pure GFLV-RNA, respectively. Lane 12, GFLV-RNA control, 10 pg; and lane 13, buffer control.

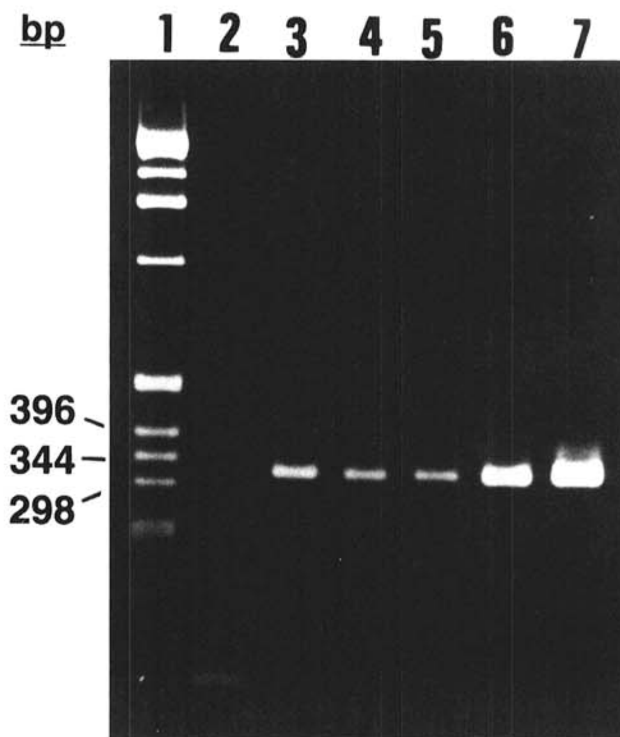

Fig. 2. Agarose gel electrophoresis of polymerase chain reaction products from leaf samples of selected grapevine cultivars and species infected with several isolates of grapevine fanleaf virus (GFLV): lane 1, DNA ladder; lane 2, healthy *Vitis vinifera* cv. Thompson Seedless; lane 3, *V. rupestris* cv. St. George infected with GFLV 100; lane 4, *V. vinifera* cv. Chenin Blanc infected with the vein-banding isolate GFLV 105; lane 5, *V. vinifera* cv. Thompson Seedless infected with the yellow mosaic isolate GFLV 107; lane 6, *V. vinifera* cv. French Colombard infected with fanleaf isolate GFLV 103; and lane 7, GFLV RNA control, 10 pg.

tion, we could easily detect one GFLV-infected vine mixed with 200 healthy ones. Results also indicated that GFLV-RNA could be used; the level of detection was 128 fg of RNA (Fig. 5).

DISCUSSION

The goal of this work was to develop a method of PCR that could successfully detect GFLV in grapevine tissue. The tremendous sensitivity of PCR offers well-appreciated advantages for detection of plant pathogens such as GFLV. However, this technique would be limited in application if grapevines could not be sampled directly. Standard sample extraction procedures for PCR failed to detect GFLV in grapes, although these methods worked well for detection of GFLV in *G. globosa*. Because grapevine tissues contain substantial amounts of phenolic compounds and polysaccharides that frequently interfere with nucleic acid manipulations, this result was expected. In addition, as reported by Demeke and Adams (3), some plant polysaccharides have direct inhibitory effects on PCR amplification.

An extraction method for grapevine tissue that has eliminated this problem is reported in this paper. The results obtained by this method were consistent and reproducible. Detection of GFLV was readily achieved in all cultivars tested and for all GFLV isolates tested. The test was extremely sensitive, allowing for detection of 128 fg of GFLV RNA from less than 4 mg of infected leaf tissue.

The sensitivity and convenience of the GFLV PCR method reported in this paper make it attractive for both research and service programs. The sensitivity of this PCR protocol should open up new areas of GFLV research, allowing detailed studies of vector biology and host-resistance mechanisms. Grapevine virus testing associated with certification and clean-stock programs could be greatly improved by a technique that is reliable throughout the growing season. GFLV titer in infected vines fluctuates significantly throughout the year. Previous reports (7,18) indicated that the serodiagnostic test ELISA could not be reliably used to detect GFLV in infected field-grown vines during the summer. Detection of GFLV in infected grapevines in the field by PCR was possible during the growing season (A. Rowhani, C. Chay, D. A. Golino, and B. W. Falk, *unpublished data*), and this method could be adopted to complement or substitute for ELISA.

GFLV is one of many virus diseases that affect grapevines, a crop of tremendous worldwide importance as fresh fruit, raisins, juice, and wine. Control of this virus disease in grapes depends on the ability of researchers to detect and eliminate the agent through grapevine clean-stock programs. As sequence data becomes available for other grapevine viruses, the sample extraction method described in this paper may facilitate the application of PCR technology to these viruses as well.

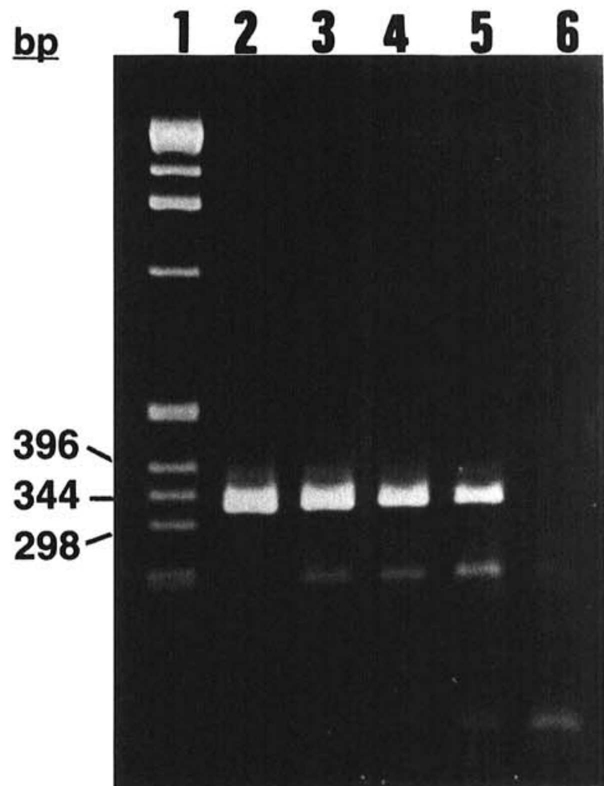

Fig. 4. Ability of polymerase chain reaction to detect grapevine fanleaf virus (GFLV) when a leaf sample from a single infected grapevine (cv. Thomson seedless, isolate GFLV 107) is diluted by samples from healthy vines: lane 1, DNA ladder; extracts prepared from lane 2, undiluted infected leaf sample; lane 3, one infected leaf extracted per 10 healthy leaf samples; lane 4, one infected leaf per 50 healthy leaf samples; lane 5, one infected leaf per 200 healthy leaf samples; and lane 6, healthy grape tissue.

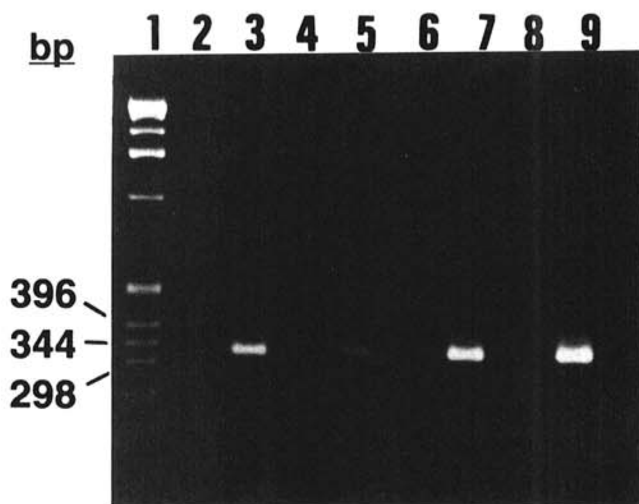

Fig. 3. Influence of tissue source in detectability of grapevine fanleaf virus (GFLV) by polymerase chain reaction: lane 1, DNA ladder; lane 2, leaf tissue from healthy *Vitis vinifera*; lane 3, leaf tissue from *V. vinifera* infected with GFLV 104; lane 4, bark scrapings from healthy *V. vinifera*; lane 5, bark scrapings from *V. vinifera* infected with GFLV 104; lane 6, root tissue from healthy *V. vinifera;* lane 7, root tissue from *V. vinifera* infected with GFLV 104; lane 8, buffer control; and lane 9, GFLV-RNA control, 10 pg.

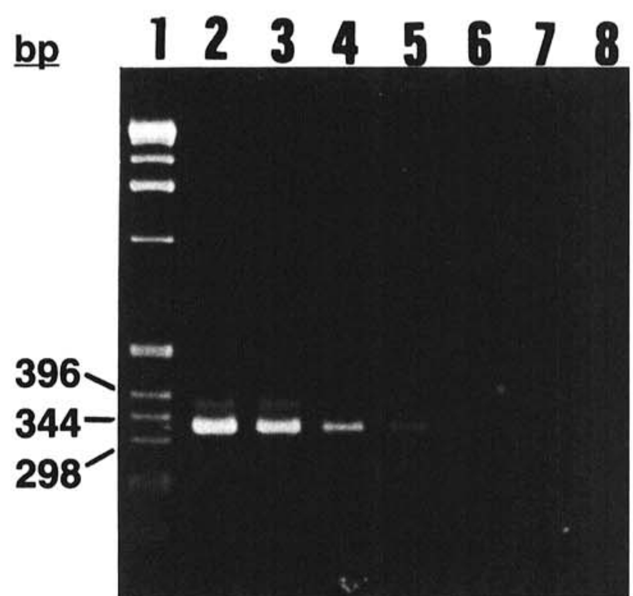

Fig. 5. Sensitivity of polymerase chain reaction for detection of purified grapevine fanleaf virus (GFLV) RNA. Lane 1, DNA ladder; lanes 2–8, fivefold serial dilutions of GFLV RNA in water from 40 μg/ml to 2.4 pg/ml, beginning at 80 pg (lane 2).

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