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An appraisal of different methods for the detection of the walnut strain of cherry leafroll virus

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Summary

Three methods were evaluated for the detection of cherry leafroll virus: ELISA, dot-blot and reverse transcriptional polymerase chain reaction (RT-PCR). Dot-blot and RT-PCR were carried out in crude plant extracts without any further RNA purification. Dot-blot hybridization using a ³²P-labelled DNA probe was as sensitive as previously reported ELISA results for cherry leafroll virus detection. The most sensitive method was RT-PCR, which amplified a specific fragment of 448 bp from the 3' untranslated region of both viral genomic RNAs. RT-PCR was used to detect cherry leafroll virus in infected walnut buds and twigs.

Detection; Cherry leafroll virus; ELISA; Dot-blot; Polymerase chain reaction, reverse transcriptional; Walnut

Introduction

Cherry leaf roll virus (CLRV) is considered as a member of the nepovirus group of plant viruses (Jones, 1985). Nepoviruses have two separately encapsidated genomic RNAs, covalently linked to a small protein at their 5' end and are polyA-tailed at their 3' end. The virion is icosahedral, composed of 60 copies of a single protein about 54 kDa in size (Harrison et al., 1977).

Several strains of CLRV have been described such as cherry, walnut, elm, birch and others, causing a diversity of disease symptoms: leaf rolling, flower

abortion, mosaics, ring patterns, etc. (Swingle et al., 1943; Cropley, 1961; Cooper et al., 1975; Mircetich et al., 1980). CLRV is naturally transmitted through infected seed and pollen (Callahan, 1957; Schimanski et al., 1972; Cooper, 1976; Cooper et al., 1984; Massalski et al., 1984; Massalski et al., 1988) thus providing the virus a rapid rate of natural spread in the usual woody hosts. For the walnut strain natural spread has been estimated in a commercial walnut orchard to result in an increase of the number of infected trees of about 20% per year (Mircetich et al., 1985). The walnut strain of CLRV (wCLRV) is the causal agent of a lethal disease of English walnuts known as blackline. This disease is the result of a hypersensitive reaction at the graft union with North California black walnut rootstock when susceptible English walnut scions become infected (Mircetich et al., 1980; Rowhani et al., 1988).

Different strains of CLRV (walnut, elm, cherry, olive, dogwood and golden elderberry) have been detected using serological methods (Jones et al., 1971; Waterworth et al., 1973; Savino et al., 1981; Rowhani et al., 1988). For the walnut strain, ELISA has been described as a good method for indexing a nursery stock, although it does not seem to be sensitive enough for general surveys in commercial walnut orchards because the virus is erratically and non uniformly distributed in orchard trees (Mircetich et al., 1985).

PCR (Saiki et al., 1985) is a highly efficient and specific method, theoretically capable of synthesizing $>10^6$ copies of product from a single DNA target sequence. The sensitivity of reverse transcription followed by PCR amplification (RT-PCR) (Sambrook et al., 1989) as a method for the detection of wCLRV was evaluated. In this paper, we report the comparison of the performance of three different methods (direct ELISA, dot-blot hybridization and polymerase chain reaction after reverse transcription (RT-PCR)) for the detection of wCLRV, as a test for the possible introduction of new methods for CLRV detection in infected plants. In the walnut strain of CLRV the most 3' end 1642 untranslated nucleotides preceding the polyA tail (UTR) are almost identical in both RNAs (Borja et al., in preparation) (Fig. 2). This allowed us to design primers for RT-PCR specific for both wCLRV RNAs.

Materials and Methods

Virus sources and purification

CLRV-W8 isolate was kindly provided by Dr. A. Rowhani (University of California, Davis). It was maintained and propagated in *Nicotiana tabacum* cv. Xhanti nc., and purified as described by Rowhani and co-workers (Rowhani et al., 1985). Purified virus was resuspended in sterile glycerol/water 50% and stored at -70°C . Walnut twigs were kindly provided by Mrs. Neus Aletá from an experimental field of IRTA (Reus, Spain). The walnut twigs and buds were stored at -20°C for 9 months prior to use because they had been harvested in springtime when the buds were appearing, and the detection was carried out in winter.

Direct-ELISA

W8 polyclonal antiserum (Rowhani et al., 1988) was kindly provided by Dr. A. Rowhani. Direct ELISA was carried out as follows: 200 μ l of purified IgG, diluted 1/1000 in 50 mM carbonate buffer, pH 9.6, was adsorbed onto each well of 96-well polyvinyl microtitre plates (Nunc) for 4 h at 37°C. The unbound IgG was discarded and the wells washed three times (3 min each) with water. 200 μ l CLRV antigens (purified virus, or tobacco tissue diluted 10–1280-fold (w/v) in PBS-Tween (Clark and Adams, 1977)), were bound at 4°C overnight. The initial 10-fold dilution of the tobacco tissue was reached by grinding 1 g of leaves in 10 ml of buffer, and this suspension was diluted further in a serial dilution manner. The coated solid phase was washed three times with water. 200 μ l of alkaline phosphatase-conjugated IgG was added to wells and kept at 37°C for 4 h; followed by three water washes. Retained alkaline phosphatase was detected by adding 200 μ l of freshly prepared chromogenic substrate (1 mg/ml of *p*-nitrophenyl phosphate (Sigma Chemical Co.) in substrate buffer) (Clark and Adams, 1977) and reading the developed colour at 405 nm in a microtitre plate reader (Titertek Multiscan MC).

Dot-blot hybridization

The 3' UTR region from the cDNA of RNA 1, containing the homology region (see Introduction), was cloned in the sites *Sa*I-*Pst*I of the plasmid vector Bluescript KS+ (Stratagene Inc.) to obtain pCL1.15 (Fig. 2). Serial dilutions of purified virus or tobacco sap diluted 10–1280-fold (w/v) in PBS-Tween were dot-blotted. For this purpose, 200 μ l per dot was loaded onto nylon membranes (Hybond N⁺ Amersham Inc.) or nitrocellulose membranes (Schleicher and Schuell Inc.), with the help of a Minifold SRC 960 (Schleicher and Schuell). Blotted nucleic acids were fixed according to suppliers' protocols, i.e. in 50 mM NaOH for 5 min for nylon membranes, or 2 h at 80°C for nitrocellulose membranes. The probe was plasmid pCL1.15, radioactively labelled by random priming using the Multiprime labelling system from Amersham in the presence of [³²P]dCTP (Amersham). The probe was labelled to a specific activity of approx. 5×10^8 cpm/ μ g. Blots were rehydrated in a 2 \times SSC solution (0.3 M NaCl, 0.3 M sodium citrate, pH 7.0), prehybridized for 4 h in a solution containing 5 \times SSC, 5 \times Denhardt's solution, 0.5% SDS, and 20 μ g/ml of single-stranded herring sperm DNA (Sambrook et al., 1989) at 65°C, and hybridized overnight in a solution containing 5 \times SSC, 5 \times Denhardt's solution, 0.5% SDS, 20 μ g/ml of single-stranded herring sperm DNA, and the probe at 65°C. Membranes were washed 4 times in 2 \times SSC, 0.1% SDS at 65°C, and exposed to an X-ray film (AGFA Curix RP2) for 24 h at -70°C with intensifying screens.

Reverse transcription and PCR amplification (RT-PCR)

wCLRV-infected tobacco and CLRV-infected walnut buds were homogenized with a mortar in plant dilution buffer (PDB) (0.4 g/l ml) containing 16.6 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 83 mM KCl, 0.083% Tween 20, 0.0166% SDS in diethyl pyrocarbonate-treated water. Serial dilutions were made in PDB from 10¹ to 10¹⁰ fold (w/v). Walnut twigs were homogenized with a Politron (model pt10/35) in PDB (0.4 g/l ml), and diluted later 10³-fold in PDB.

Viral RNA was reverse transcribed (RT) in a final volume of 20 µl containing 12.5 µl of the corresponding dilution, 4 µl dNTP mix (12.5 mM each dNTP, pH 7.0), 0.5 µl 17mer 3' primer (25 pmol) (Fig. 2), 1 µl 50 mM MgCl₂, 1 µl RNase Inhibitor (50 U, Amersham) and 1 µl MRV Reverse Transcriptase (200 U, BRL Inc.). The tube was incubated for 50 min in an Intelligent Heating Block (IHB2024 from CAMBIO) at 37°C to synthesize CLRV ss cDNA. The mixture was then briefly centrifuged and the PCR amplification was performed in a final volume of 100 µl containing 20 µl of the CLRV ss cDNA, 1.5 µl of 17mer 3' primer (75 pmol) (Fig. 2), 2 µl of 17mer 5' primer (100 pmol) (Fig. 2) and 8 µl of 10 × PCR buffer (250 mM Tris-HCl, pH 9.5 (25°C), 500 mM KCl, 100 mM MgCl₂, 10 mg/ml bovine serum albumin, provided). The amplification mix was incubated at 94°C for 5 min. Prior to thermal cycling, 1 U of Taq polymerase (Amersham) was added and the 100-µl samples overlaid with an equal volume of mineral oil (Merck) to prevent evaporation. 35 cycles were programmed. Samples were incubated at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min in each cycle. The extension time during the final cycle was increased to 10 min to complete DNA synthesis on all strands. After amplification, 150 µl of chloroform was added and the aqueous phase recovered. The DNA was precipitated with 10 µl 3 M sodium acetate, pH 5.2 and 250 µl of ethanol at -70°C for 30 min and centrifuged 15 min at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in 5 µl of TE, pH 8 (10 mM Tris-HCl, 1 mM EDTA). Samples were electrophoresed through a 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0, according to Sambrook et al., 1989) containing 0.5 µg/ml ethidium bromide, or through a 5% polyacrylamide gel in TBE buffer (80 mM Tris-borate, 1 mM EDTA, according to Sambrook et al. (1989) and stained with 1 µg/ml ethidium bromide. DNA was visualized on a UV transilluminator (Spectroline TC-302).

Results

Direct ELISA for wCLRV detection

The sensitivity of the classical direct ELISA (Clark and Adams, 1977) was evaluated for the detection of wCLRV, using an alkaline-phosphatase conjugate (Rowhani et al., 1988). Scoring as positive any sample giving a

TABLE 1

Evaluation of the sensitivity of a direct ELISA for the detection of wCLRV

Dilution ^a	Purified virus ^b	Infected tobacco ^b
10	9.9	9.6
2 × 10	10	9.4
2 ² × 10	10	8.9
2 ³ × 10	9.5	4.5
2 ⁴ × 10	6.9	2
2 ⁵ × 10	3.3	1.1
2 ⁶ × 10	3.2	1.2
2 ⁷ × 10	2	1

The assay used alkaline-phosphatase conjugated polyclonal antibodies. The concentration of the undiluted purified virus was 130 µg/ml. Other details described in the Material and Methods section. Background values were 0.012 ± 0.002 .

^aNumbers in this column represent the number of times that the original preparation was diluted.

^bTimes over background of the corresponding ELISA readings. Background was considered as buffer alone for the second column or uninfected tobacco tissue (dilution 10) for the third column. No significant differences were observed between the background values.

colorimetric reading at least three times over the background reading of mock-inoculated tobacco, we were able to detect down to 40 ng of purified virus (Table 1). For wCLRV-infected tobacco tissue, this method allowed us to detect virus in 2.5–1.25 mg of leaf (Table 1), using the same criterion.

DILUTIONS

1 2 2² 2³ 2⁴ 2⁵ 2⁶ 2⁷

(x 10)

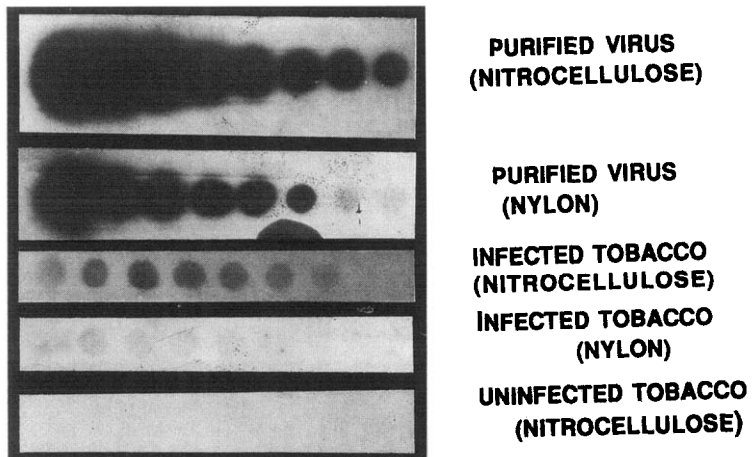


Fig. 1. Evaluation of the sensitivity of a dot-blot hybridization assay for the detection of wCLRV. The probe was radioactively labelled plasmid pCL1.15. The concentration of the undiluted purified virus was 130 µg/ml. Numbers on the top row indicate the dilutions performed on the original undiluted sample. Other details described in the Materials and Methods section.

wCLRV detection by dot-blot hybridization

Virus detection by dot-blot hybridization (Hull, 1984) was performed using a ^{32}P -labelled DNA probe complementary to both viral genomic RNAs (see Discussion). We considered two types of solid supports for hybridization, nylon and nitrocellulose. On nylon membranes, we could detect down to 26 ng of purified virus (Fig. 1), a level of detection comparable to the one achieved by ELISA. Nitrocellulose membranes allowed more sensitivity, although the exact limit was not determined. Using *wCLRV*-infected tobacco tissue in the test, nitrocellulose again performed better in our hands (Fig. 1). Samples representing 2.5 mg of infected leaf could be detected on nylon, compared with figures as low as 0.3 mg on nitrocellulose. A curious effect of hybridization inhibition was observed in the less diluted samples containing plant extracts. Such an effect has already been documented in other instances (Chu et al., 1989).

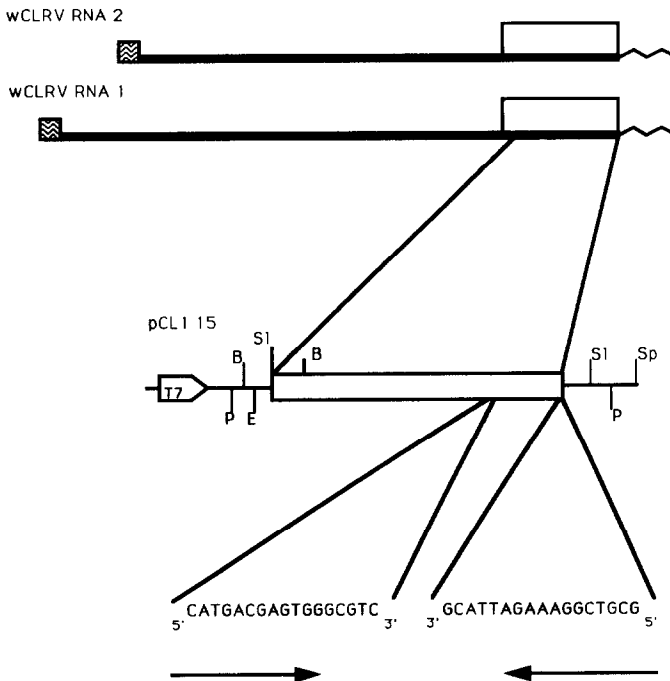


Fig. 2. Primers used for PCR detection of *wCLRV*. *wCLRV* RNA2 (≈ 6.5 kb) and RNA1 (≈ 7.5 kb) (▨ represent the VPg protein and ~ the polyA tail) have a region of high homology (98%), represented by an open box in the figure. cDNA from this region was cloned in the plasmid vector Bluescript KS+ (Stratagene Inc.) obtaining the plasmid pCL1.15. The cDNA insert in the middle of the multiple cloning site and the T7 promoter are represented in the figure. The restriction sites represented in the figure are *Pst*I (P), *Bam*HI (B), *Eco*RI (E), *Sal*I (SI), *Sp*HI (Sp). The region was sequenced (Borja et al., in preparation). Two 17mer oligonucleotides were selected from this sequence: the last 17 nucleotides of the UTR (3' primer), and the 17 nucleotides in positions 1177–1194 (5' primer) of the UTR. The positions and polarity of these 17mers are shown in the figure. Arrows limit the region synthesized by the Taq DNA polymerase.

wCLRV detection by the polymerase chain reaction

RT-PCR was carried out in the presence of two primers specific for the wCLRV homology region (Fig. 2). A fragment of 448 nucleotides from both viral RNAs should be amplified (Fig. 2). In Fig. 3 (agarose), the RT-PCR product of 1 μ g purified wCLRV (lane W) and as little as 50 ng of wCLRV-infected tobacco leaf RT-PCR products (IT, lane 4) show a clear band of the expected size (448 bp). The negative controls, consisting of 1 μ g of purified CMV (lane C) and 5 mg of mock-inoculated tobacco leaf (lane UT) showed no product. Grapevine fanleaf virus, which is a virus of the nepovirus group, was also tested as a negative control, and showed no product (data not shown). In order to evaluate the sensitivity of the method, serial dilutions of wCLRV-infected tobacco and wCLRV-infected walnut buds and twigs were reverse-transcribed and amplified by PCR. The PCR products were electrophoresed in a 5% polyacrylamide gel for better resolution of the expected band. In Fig. 3 (polyacrylamide) we could detect a single ethidium bromide-staining product of 448 bp in samples representing 5 μ g of the infected tobacco (IT, lane 4), 5 ng of infected walnut buds (IW, lane 3) and 5 μ g of infected walnut twig (lane T), while no product bands could be seen in the last dilution of the infected walnut buds (lane 4) corresponding to 5 pg, nor in the uninfected walnut (lane UW). While in tobacco we found a direct relationship between the intensity of the band and the total amount of infected leaf, in the case of walnut the less diluted

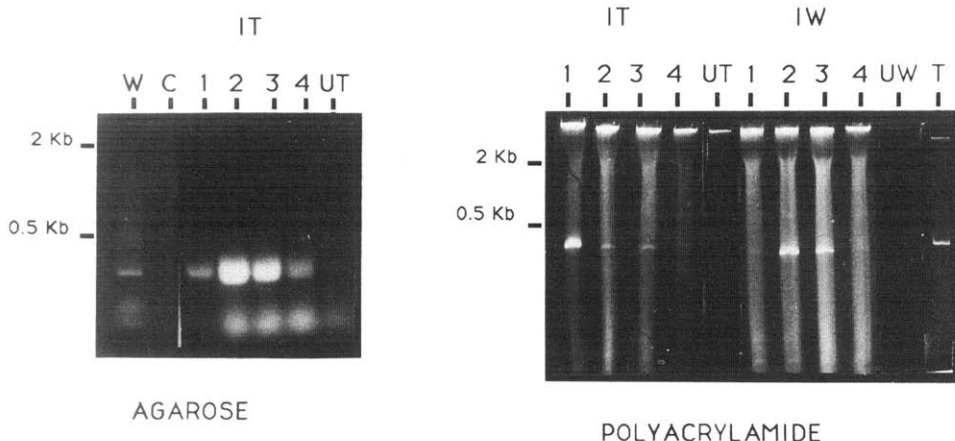


Fig. 3. Evaluation of PCR sensitivity for detection of wCLRV. Agarose and polyacrylamide gels loaded with PCR products. Agarose gel lanes: (W) purified virus, (1) infected tobacco (IT) diluted 2.5×10^1 -fold, (2) 2.5×10^3 -fold, (3) 2.5×10^4 -fold, (4) 2.5×10^6 -fold, (UT) uninfected tobacco. Polyacrylamide gel lanes: (1) infected tobacco (IT) diluted 2.5×10^1 -fold, (2) 2.5×10^3 -fold, (3) 2.5×10^5 -fold, (4) 2.5×10^9 -fold, (UT) uninfected tobacco, (1) infected walnut buds (IW) diluted 2.5×10^1 -fold, (2) 2.5×10^3 -fold, (3) 2.5×10^5 -fold, (4) 2.5×10^9 -fold, (UW) uninfected walnut buds, (T) infected walnut twig diluted 2.5×10^3 -fold. Sizes of molecular weight markers are indicated on the left of the gels. The amount of sample loaded in lane 1 (agarose) was less than in the other lanes, so explaining the absence of the primer zone in the gel.

samples showed less intensity in the product bands, the same effect described in the previous subsection. In our hands, wCLRv can be detected in wCLRv-infected tobacco samples representing 2.5–1.25 mg by direct ELISA, 0.3 mg by dot-blot hybridization on nitrocellulose membranes and 5 pg by RT-PCR, therefore RT-PCR is a method for detecting wCLRv approx. 10^9 -fold more sensitive than direct ELISA and approx. 10^8 -fold more sensitive than dot-blot hybridization on nitrocellulose membranes. RT-PCR as a method for detecting wCLRv can also be applied to the detection of virus in walnuts in minute amounts such as 50 ng of bud or 5 µg of twig.

Discussion

Three methods for the detection of wCLRv have been evaluated. It was previously reported that indirect ELISA (I-ELISA), using a peroxidase enzyme conjugate, can detect purified wCLRv at a concentration of 4 ng/ml (Mircetich et al., 1985). Other strains have been detected at concentrations of 6 ng/ml (birch) and 24 ng/ml (*Sambucus racemosa*), respectively, with protein A sandwich ELISA (PAS-ELISA) (Edwards et al., 1985). In our hands, direct ELISA with an alkaline-phosphatase conjugate was less sensitive, as we could only detect purified wCLRv at a concentration of 200 ng/ml (40 ng). The dilution end point of infected sap for ELISA reported previously was 1/640 for wCLRv in infected walnut leaves with I-ELISA, and for birch CLRv in infected *C. quinoa* 1/5000 with PAS-ELISA and 1/1000 with direct ELISA. Our direct ELISA with alkaline-phosphatase conjugate was less sensitive, as the dilution end point of tobacco infected sap for direct ELISA is between 1/80 and 1/160. This reduction in sensitivity could probably be due to partial damage of the wCLRv ELISA reagents (antibodies and conjugates) during the transport. We did not find any inhibition in our direct ELISA, although it has been reported in the less diluted samples of PAS-ELISA (Edwards et al., 1985).

Dot-blot hybridization with a ^{32}P -labelled probe was a good alternative for infected sap. It has been reported that the dilution limit of infected sap for ELISA is lower than for dot-blot (Chu et al., 1989). However, wCLRv was detectable at a sap dilution of 1/640 of tobacco infected sap, a sensitivity similar to the one reported previously for indirect ELISA. The probe used was selected in such a way that both viral genomic RNAs would be detected, as it contained the cDNA from the homology region between both RNAs (Borja et al., in preparation). Nitrocellulose allowed more sensitivity than nylon. Non-radioactive probes (biotin and digoxigenin) were also tested. Dot-blot hybridization with non-radioactive probes was a less sensitive method, and sometimes gave false positives (cross-reaction with mock-inoculated plants) (results not shown). The inhibition observed in the dot-blot with less dilute walnut samples could be due to plant constituents in the sap that would inhibit hybridization. This type of inhibition has been previously described (Chu et al., 1989).

PCR has already been used to detect plant DNA viruses (Rybicki et al., 1990), and RT-PCR to detect plant RNA viruses (Vunsh et al., 1990; Korschineck et al., 1991). Our results are in support of the potential value of RT-PCR as a simple and specific method for the detection of plant RNA viruses in infected sap. Similar tests using a reverse transcription step prior to cDNA amplification have been developed for detection of viroids (Puchta et al., 1989; Hadidi et al., 1990), for several animal RNA viruses including rhinovirus (Gama et al., 1989), HIV (Byrne et al., 1988; Hart et al., 1988; Murakawa et al., 1988), human picornaviruses (Hyypia et al., 1989), and for plant RNA viruses (Vunsh et al., 1990; Korschineck et al., 1991). These reports describe methods that use a previous step for RNA purification. Extraction of undamaged RNA for specific priming of an efficient RT reaction to obtain proper templates for amplification has been described as a major problem (Verbeek and Tijssen, 1990; Chirnside et al., 1990). We have not found it to be necessary in order to obtain high sensitivity for wCLRv detection in tobacco or walnut. This could also be the case for other combinations of plants and plant viruses.

The fact that the RT-PCR assay is sensitive, non-radioactive, rapid and requires small amounts of target RNA could make protocols of this type useful alternatives to conventional ELISAs and dot-blot hybridization for the rapid detection of wCLRv. wCLRv detection by gel electrophoresis analysis of the PCR-amplified cDNA products (Fig. 2) was superior to ELISA or even to assays with radioactive probes. We considered an assay as positive when the size of the amplified major product was 448 bp and negative when a major product of this size was absent. No false positives were found, not surprisingly since the primers used were wCLRv-specific. Controls of plants infected with cucumber mosaic virus or grapevine fanleaf virus (a nepovirus, like CLRv), multipartite RNA viruses, did not show any amplified product. Amplification of a fragment of 2-kb was also carried out under the same conditions, but it was discarded because it was less sensitive (data not shown). The inhibition observed in the RT-PCR of less dilute walnut samples could be due to plant constituents in the sap of walnuts that would inhibit one or both reactions. The amplification was specific for viral RNA and extremely sensitive: the endpoint dilution shown in Fig. 3 was approx. 10^9 -fold lower than that routinely obtained by ELISA (Table 1), and 10^8 -fold lower than the one obtained by dot-blot hybridization with a radioactive probe (Fig. 1), which is as sensitive as the detection predicted for plant RNA viruses (Vunsh et al., 1990), but without previous RNA purification. We believe that this is an important practical simplification for routine diagnosis schemes. Phenol extraction procedures of crude plant extracts are time consuming and phenol handling also requires caution due to its toxic and burning properties. Both considerations are especially important when many samples are to be examined. In cases where high sensitivity needs to be combined with relatively high numbers of samples, simplifications in the scheme of analysis allow increase in speed. This could be, for instance, the case for detection of CLRv in pollen samples, where 2×10^4

germinating grains/sample are needed using immunological methods (Masalski et al., 1988).

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References

- Byrne, B.C., Li, J.J., Sninsky, J. and Poiesz, B.J. (1988) Detection of HIV-1 RNA sequences by in vitro DNA amplification. *Nucleic Acids Res.* 16, 4165.
- Chirnside, E.D. and Spaan, W.J.M. (1990) Reverse transcription and cDNA amplification by the polymerase chain reaction of equine arteritis virus (EAV). *J. Virol. Methods.* 30, 133-140.
- Chu, P.W.G., Waterhouse, P.M., Martin, R.R. and Gerlach, W.L. (1989) New approaches to the detection of microbial plant pathogens. *Biotechnol. Genet. Eng. Rev.* 7, 45-110.
- Clark, M.F. and Adams, A.N. (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. *J. Gen. Virol.* 34, 475-483.
- Cropley, R. (1961) Cherry leaf-roll virus. *Ann. Appl. Biol.* 49, 524-529.
- Edwards, M.L. and Cooper, J.I. (1985) Plant virus detection using a new form of indirect ELISA. *J. Virol. Methods* 11, 309-319.
- Gama, R.E., Knappenberger, R.E., Hughs, P.J., Bruce, C.B. and Stanway, G. (1988) Polymerase chain reaction amplification of rhinovirus nucleic acids from clinical material. *Nucleic Acids Res.* 16, 9346.
- Hadidi, A. and Yang, X. (1990) Detection of pome fruits viroids by enzymatic cDNA amplification. *J. Virol. Methods* 30, 261-268.
- Hart, C., Schochetman, G., Spira, T., Lifson, A., Moore, J., Galphin, J., Sninsky, J.J. and Ou, C.Y. (1988) Direct detection of HIV RNA expression in seropositive persons. *Lancet* ii, 596-599.
- Harrison B.D. and Murant, A.F. (1977) *Nepovirus group, Descriptions of Plant Viruses No. 185, Commonwealth Micrological Institute/Association of Applied Biology.*
- Hull, R. (1984) Rapid diagnosis of plant virus infections by spot hybridization. *TIBS* 4, 88-91.
- Hyypia, T., Auvinen, P. and Maaronen, M. (1989) Polymerase chain reactions for human picornaviruses. *J. Gen. Virol.* 70, 3261-3268.
- Jones, A.T. and Murant, A.F. (1971) Serological relationship between cherry leafroll, elm mosaic and golden elderberry viruses. *Ann. Appl. Biol.* 69, 11-15.
- Jones, A.T. (1985) *CLRV Descriptions of Plant Viruses No. 306, Commonwealth Micrological Institute/Association of Applied Biology.*
- Korschineck, I., Himler, G., Sagl, R., Steinkellner, H. and Katinger, H.W.D. (1991) A PCR membrane spot assay for the detection of plum pox virus RNA in bark of infected trees. *J. Virol. Methods* 31, 139-146.
- Massalski, P.R., Cooper, J.I., Hellen, C.U.T. and Edwards, M.L. (1988) The effect of cherry leaf roll virus infection on the performance of birch pollen and studies on virus replication in germinating pollen. *Ann. Appl. Biol.* 112, 415-425.
- Mircetich, S.M., Sanborn, R.R. and Ramos, D.E. (1980) Natural spread, graft transmission, and possible etiology of walnut blackline disease. *Phytopathology* 75, 962-968.
- Mircetich, S.M., Rowhani, A. and Ramos, D.E. (1985) *Blackline disease: walnut orchard management. The Regent of the University of California, Division of Agriculture and Natural Resources, 142-152.*

- Murakawa, G.J., Zaia, J.A., Spallone, P.A., Stephena, D.A., Kaplan, B.E., Wallace, R.B. and Rossi, J.J. (1988) Direct detection of HIV-1 RNA from AIDs and ARC patient samples. *DNA* 7, 287-295.
- Puchta, H. and Sanger, H.L. (1989) Sequence analysis of minute amounts of viroid RNA using the polymerase chain reaction (PCR) *Arch. Virol.* 106, 335-340.
- Rybicki, E.P. and Hughes, F.L. (1990) Detection and typing of maize streak virus and other distantly related geminivirus of grasses by polymerase chain reaction amplification of a conserved viral sequence. *J. Gen. Virol.* 71, 2519-2526.
- Rowhani, A. and Mircetich, S.M. (1988) Pathogenicity on walnut and serological comparisons of cherry leafroll virus strains. *Phytopathology*. 78, 817-820.
- Rowhani, A., Mircetich, S.M., Dheperd, R.J. and Cucuzza, J.D. (1985) Serological detection of cherry leafroll virus in English walnut trees. *Phytopathology* 75, 48-52.
- Sabino, V. and Gallitelli, D. (1981) Cherry leafroll virus in olive. *Phytopathol. Mediterr.* 20, 202-203.
- Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 487-491.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, New York.
- Swingle, R.V., Tilford, P.E. and Irish, C.F. (1943) A graft transmissible mosaic of american elm. *Phytopathology* 33, 1196-1200.
- Verbeek, A. and Tijssen, P. (1990) Polymerase chain reaction for probe synthesis and for direct amplification in detection of bovine coronavirus. *J. Virol. Methods* 29, 243-256.
- Vunsh, R., Rosner, A. and Stein, A. (1990) The use of the polymerase chain reaction (PCR) for the detection of bean yellow mosaic virus in gladiolus. *Ann. Appl. Biol.* 117, 561-569.
- Waterworth, H.E. and Lawson, R.H. (1973) Purification, electron microscopy, and serology of the dogwood ringspot strain of cherry leafroll virus. *Phytopathology* 63, 141-146.