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A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection

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Summary

A highly sensitive assay, based on polymerase chain reaction amplification of cDNA synthesized from the viral RNA of antibody-captured viral particles, has been developed for plum pox potyvirus (PPV) detection. The reaction, called immunocapture/PCR (IC/PCR), yields a specific 243-bp product. The immunocapture step, by allowing the use of large sample volumes and by the viral particle prepurification it achieves, dramatically increases the sensitivity of the assay. As few as 8000 target viral particles per ml of plant extract could be detected by IC/PCR. When compared to direct PCR (Wetzel et al., 1991), molecular hybridization using ^{32}P-labeled cRNA probes and ELISA, this result corresponds to a 250-fold, 625-fold and 5000-fold increased sensitivity, respectively. The high sensitivity of IC/PCR was confirmed during an indexing trial with field samples collected from naturally infected trees. This very powerful technique should have wide ranging applications for the detection of a number of other viruses and pathogens for which specific antisera and sequence data are available.

PCR; Immunocapture; Plum pox virus; Detection; Molecular hybridization; ELISA

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Introduction

Plum pox potyvirus (PPV) is the causal agent of the devastating plum pox (Sharka) disease of stone fruit trees (Dunez and Sutic, 1988). The virus is usually present at very low levels in infected trees where it is, moreover, unevenly distributed. Detection techniques of high sensitivity are therefore required to index trees for the presence of the disease, a key step in the sanitary selection measures which are still the only effective means to fight the disease.

A dot-blot molecular hybridization assay using ^{32}P-labeled cRNA probes corresponding to the carboxy-terminal half of the capsid protein gene of PPV strain D, one of the two viral serotypes described by Kerlan and Dunez (1979), has been developed for PPV detection (Varveri et al., 1988). The polyvalence of the molecular hybridization assay has been further improved by using cRNA probes corresponding to PPV-D non-structural protein genes (Wetzel et al., 1990).

Recently, two different methods based on the polymerase chain reaction (PCR, Saiki et al., 1988) have been described for PPV detection (Korschineck et al., 1991; Wetzel et al., 1991). The single tube protocol we have developed enables the detection of as few as 2000 target viral particles per assay and is clearly more sensitive than molecular hybridization using ^{32}P-labeled cRNA probes (Wetzel et al., 1991). However, the small sample volume used for the PCR analysis (1 μl) limits the sensitivity of the technique in terms of the minimal viral concentration detectable, with an improvement of only 2.5-fold over molecular hybridization and 20-fold over ELISA.

In this study, we describe a modified immunocapture/PCR protocol (Jansen et al., 1990) for PPV detection, in which viral RNA obtained from antibody-captured viral particles is used as the template for cDNA synthesis prior to PCR amplification, allowing much higher sample volumes (250 μl) to be analyzed.

Materials and Methods

PPV strains

PPV strain D, an apricot isolate from southern France, was used for this study. The complete nucleotide sequence of this strain has been determined (Teycheney et al., 1989). The virus was maintained in GF305 peach seedlings. Purified virus solutions were obtained, as described previously (Varveri et al., 1987).

Leaves collected from naturally infected apricot trees, in a small orchard of southern France, were used during an indexing trial of field samples for a comparison between ELISA, molecular hybridization and IC/PCR. Since PPV-D has been isolated near this orchard, the isolate present in the trees is probably closely related to or the same as PPV-D.

Sample preparation

Purified virus solutions were diluted in 50 mM sodium citrate buffer, pH 8.3. Plant extracts were prepared by grinding the leaves (w/v = 1/4) in 50 mM sodium citrate buffer, pH 8.3, containing 20 mM diethyldithiocarbamate (DIECA) and 2% (w/v) polyvinylpyrrolidone (PVP K25, Fluka), in individual plastic bags containing gauze (Bioreba) using a rolling grinder (Meku). The plant sap was then collected and centrifuged for 10 min in an Eppendorf microcentrifuge, resulting in the crude plant extract used for ELISA, molecular hybridization and IC/PCR experiments.

Plant extracts for PCR experiments were prepared by grinding the leaves in sterile water (w/v = 1/4), as previously described (Wetzel et al., 1991).

Enzyme-linked immunosorbent assay

The Sanofi commercial PPV precoated microplates (Sanofi Phytodiagnostic) were used for the ELISA assays. Aliquots of 250 μl of plant extracts or purified virus solutions were applied to the wells. The limit of detection of the assay was established as previously described (Varveri et al., 1987) and is in the 1 ng/assay range.

Molecular hybridization

Purified virus solutions or plant extracts were diluted (1/1, v/v) in 12 × SSC, 6% formaldehyde (SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Aliquots of 10 μl were then spotted on 20 × SSC-saturated nitrocellulose membranes (Hybond-C Extra, Amersham) using a hybridot apparatus (BRL). Membranes were then air dried and baked in vacuo at 80°C for 2 h.

The ^{32}P-labelled cRNA probes, corresponding to PPV-D capsid protein gene and 3′ non-coding region (pBPPV1, Varveri et al., 1988), and cylindrical inclusion gene (Cl1.2, Wetzel et al., 1990) were synthetized using the Riboprobe Gemini system kit (Promega) in the presence of [α-^{32}P]CTP (800 Ci/mmol, Amersham). The specific activity of the probes was 2.5 × 10^8 cpm/μg.

Hybridizations were performed using the conditions of Melton et al. (1984), as previously described (Varveri et al., 1988; Wetzel et al., 1991). Membranes were then exposed to X-ray films (Kodak X-AR5) for 65 h, using intensifying screens.

PCR experiments

The pair of oligonucleotide primers used in our previous work (Wetzel et al., 1991) were used throughout this study. The primers were selected to hybridize to homologous regions (corresponding to the carboxy-terminal end of the capsid protein gene) of the three published sequences of PPV (PPV-D, Teycheney et al., 1989; PPV-NAT, Maiss et al, 1989; PPV-Rankovik, Lain et

al., 1989). The sequences of the primers are 5'CCCTCACATCACCAGAGC-CA3' (sense primer), and 5'CAGACTACAGCCTCGCCAGA3' (antisense primer), resulting in a 243 bp amplified fragment, as previously described (Wetzel et al., 1991).

The crude plant extracts prepared for the PCR experiments (see above) were serially diluted ten-fold with sterile water. Ten microliters from these preparations were used. Chemical denaturation and reverse transcription of the viral RNA and amplification of the synthetized cDNA were performed in a single tube, as previously described (Wetzel et al., 1991). The following thermal cycling scheme was used (Perkin Elmer Cetus cycler) for 40 cycles: template denaturation at 92°C (20 s), primer annealing at 62°C (20 s) and DNA synthesis at 72°C (40 s). A final 10 min elongation step at 72°C was performed at the end of the 40 cycles. Amplification products were analyzed by electrophoresis of 10 μl aliquots from each reaction mixture on a 1.5% agarose gel, in Tris-borate-EDTA buffer (Maniatis et al., 1982). Bands were visualized by ethidium bromide staining.

Immunocapture/PCR

The Sanofi PPV precoated microplates were used for the IC/PCR experiments. Aliquots of 250 μl of purified virus solutions or plant extracts were applied to the microplates (overnight, 4°C). After washing with PBS buffer, 10 μl of a 1% Triton X100 solution, heated to 65°C, was added to the wells to disrupt (by vortexing) the antibody-bound viral particles and release the viral RNA. The resulting solutions were then transferred to sterile 0.5 ml polypropylene microcentrifuge tubes and processed without further treatment. Chemical denaturation, reverse transcription of the viral RNA and amplification of the synthesized cDNA were then performed, as described above.

In some experiments, to obtain a single tube test, the immunocapture was performed directly in 0.5 ml Eppendorf tubes, according to the protocol of Jansen et al., 1990. In this case, either the commercial goat antiserum, or a rabbit antiserum produced in our laboratory, were used.

Results

Comparison between PCR and IC/PCR for PPV detection

PPV strain D maintained on GF305 peach seedlings was used to compare the detection limit of direct PCR (Wetzel et al., 1991) and IC/PCR. As sample preparation is different between PCR and IC/PCR, the leaves were cut in little strips with a sterile razor blade and randomly divided into two groups to allow identical samples to be analyzed by both techniques. Ten-fold dilutions of the crude plant extracts were prepared in healthy plant extract, and used for amplification as described in Materials and Methods.

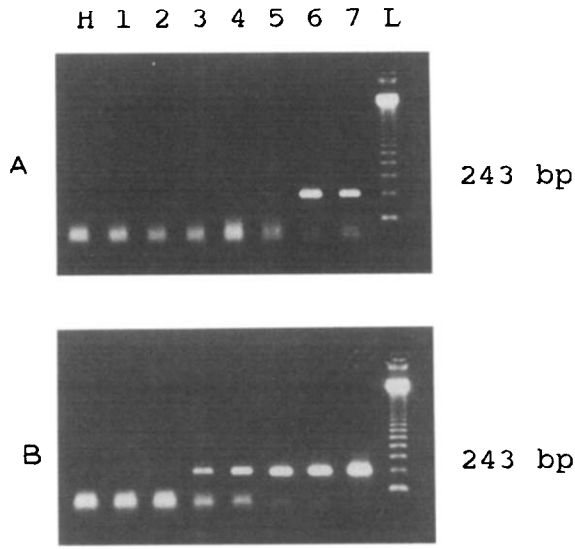

Fig. 1. Comparison between PCR and IC/PCR for PPV detection in infected peach samples. Crude plant extracts and 10-fold dilutions prepared in healthy plant extract were obtained as described in Materials and Methods and used for PCR (A) and IC/PCR (B). The amplification products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. L, molecular weight standards (123 bp DNA ladder, BRL); Lanes 7–1: PCR products from crude plants extracts undiluted and diluted 10, 10^2, 10^3, 10^4, 10^5, 10^6-fold, respectively; H, healthy peach control.

An amplified fragment of the expected size was obtained from infected material by both PCR and IC/PCR, whereas no fragment was amplified from healthy controls (Fig. 1). Restriction analysis of the amplified fragments by the endonucleases *Alu*I and *Rsa*I revealed the presence of restriction sites at the expected positions (Teycheney et al., 1989), further confirming the specificity of the amplification reactions.

Whereas an amplified fragment was detected after PCR of infected samples diluted only up to 10^2-fold, IC/PCR proved able to detect PPV sequences in the same samples even after a 10^4-fold dilution, indicating an at least 100-fold higher sensitivity. A similar sensitivity was obtained (results not shown) using immunoglobulin-coated Eppendorf tubes (Jansen et al., 1990) to perform the initial immunocapture phase. The whole process, from IC to reverse transcription and then to PCR, can thus be performed in a single tube.

Sensitivity of IC/PCR for PPV detection

Purified virus solutions obtained as described previously (Varveri et al., 1987) were used to determine the maximal sensitivity of IC/PCR for PPV detection. Ten-fold dilutions of the purified virus solution were prepared and used in the IC/PCR experiments, as described in Materials and Methods. The

TABLE 1

Limit of detection of purified PPV using different techniques. The purified virus solutions were obtained as previously described (Varveri et al., 1987). The detection limits by ELISA and molecular hybridization using ^{32}P-labelled cDNA probes (MH/cDNA) are those of Varveri et al. (1987). The detection limits by molecular hybridization using cRNA probes (MH/cRNA) are those of Varveri et al. (1988) and Wetzel et al. (1990).

	Sensitivity (pg of virus per assay)	Sample volume (μl)	Sensitivity (pg of virus/ml)
ELISA	1000	250	4000
MH/cDNA	100	10	10000
MH/cRNA	5	10	500
PCR[a]	0.2	1	200
IC/PCR	0.2	250	0.8

[a]The detection limit by PCR was determined from purified viral RNA (Wetzel et al., 1991) obtained as previously described (Varveri et al., 1987).

results are shown in Table 1, together with compiled results obtained previously using various other techniques (Varveri et al., 1987, 1988; Wetzel et al., 1990, 1991).

An identical detection level per assay was obtained for PCR and IC/PCR, both techniques detecting as little as 0.2 pg of purified virus per assay (i.e., 10 fg of purified viral RNA, corresponding to 2000 target copies, Wetzel et al., 1991). However, when expressed in terms of minimal virus concentration detected (pg of purified virus per ml), IC/PCR detects 0.8 pg of purified virus per ml (which corresponds to 8000 target particles per ml), compared to 2×10^6 particles per ml (200 pg/ml) for direct PCR. This result indicates that, for the detection of PPV in crude plant extracts, IC/PCR is 250 times more sensitive than PCR (Wetzel et al., 1991) and 625 times and 12 500 times more sensitive, respectively, than molecular hybridization using ^{32}P-labeled cRNA probes (Varveri et al., 1988; Wetzel et al., 1991) and cDNA probes (Varveri et al., 1987). Similarly, IC/PCR is 5000 times more sensitive than ELISA (Varveri et al., 1987).

Comparison between IC/PCR, molecular hybridization and ELISA for PPV detection in field samples

To confirm the greater sensitivity of IC/PCR and to test its ability for routine detection of PPV, a comparative detection assay was carried out between ELISA, molecular hybridization using ^{32}P-labelled cRNA probes and IC/PCR using field samples. The samples were collected from naturally infected apricot trees in southern France. As in previous experiments (Varveri et al., 1988; Wetzel et al., 1990), two kinds of samples were used: samples collected in parts of the trees bearing symptoms (noted 'symptom +') and samples from symptomless parts of the trees (noted 'symptom −'). Previous results have shown that 'symptom +' samples usually contain a high enough concentration of viral particles to be indexed as infected by DAS-ELISA or molecular hybridization but that a high proportion of 'symptom −' samples is usually

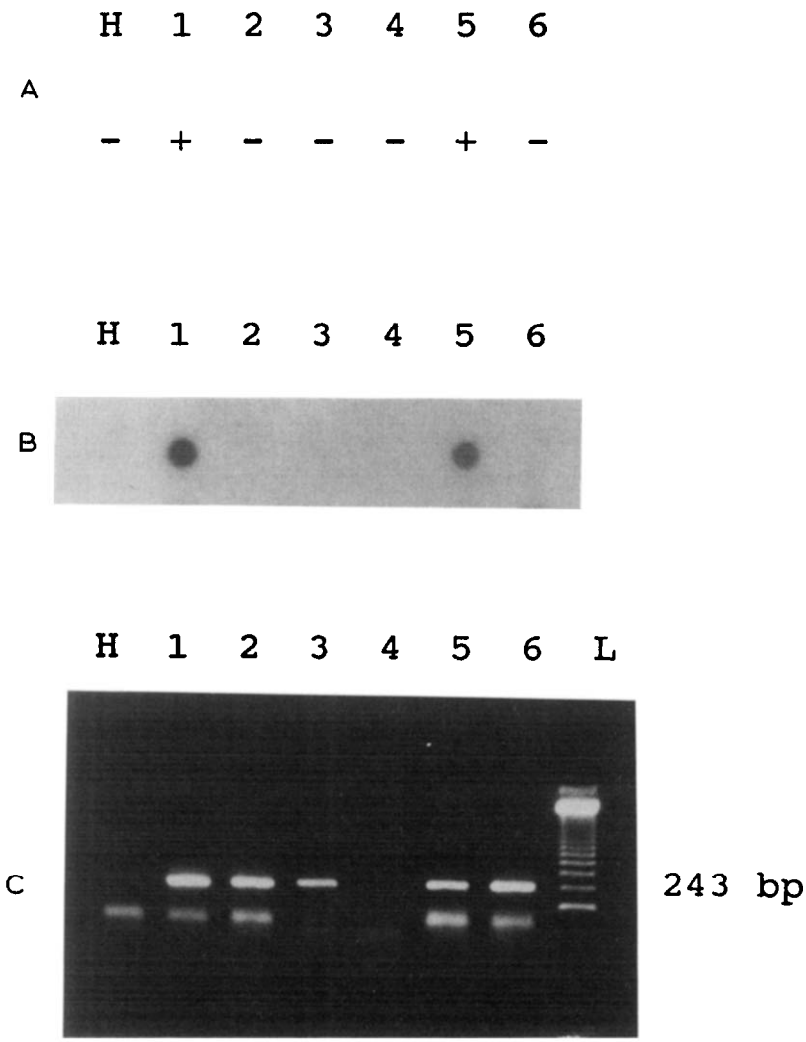

Fig. 2. Comparison between ELISA, molecular hybridization using ^{32}P-labeled cRNA probes and IC/PCR for PPV detection in six apricot field samples. The samples were prepared and analyzed by the three techniques, as described in Materials and Methods. A, ELISA analysis of samples (1–6) and of a healthy control (H). The threshold of detection was determined, as previously described (Varveri et al., 1987). +, indicates samples judged infected, −, samples indexed negative. B, molecular hybridization analysis using probe pBPPV1 (Varveri et al., 1988) of the same samples (1–6) and of a healthy control (H). C, IC/PCR analysis of the same samples (1–6) and of an healthy control (H). The amplification products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. L, molecular weight standard (123 bp DNA Ladder, BRL).

indexed negative, even though all samples are collected from trees known to be infected (i.e., showing distinct symptoms in at least one part of their canopy) (Varveri et al., 1988; Wetzel et al., 1990).

The same grinding buffer being used for ELISA, molecular hybridization

and IC/PCR, the same plant extracts were analyzed by the three techniques. Because, apart from the controls, all samples were collected from trees known to be infected, all negative reactions with the samples should be regarded as 'false negatives'.

As an example, Fig. 2 presents the results of the parallel analysis by ELISA, molecular hybridization using probe pBPPV1 (Varveri et al., 1988), and IC/PCR, of six of the field samples. As can be seen, no non-specific reactions were seen with the healthy control but three additional samples are indexed positive by IC/PCR, as compared to the ELISA and molecular hybridization results.

The results obtained are summarized in Table 2. It should be stressed that false positive reactions were not observed with any of the techniques when analyzing the healthy controls. A good correlation was obtained between ELISA, molecular hybridization and IC/PCR. All samples which were positive by ELISA (28/50) were also positive by molecular hybridization and IC/PCR. Molecular hybridization using probe pBPPV1 (Varveri et al., 1988) detected the virus in seven additional samples (35/50), confirming its higher sensitivity, when compared to the ELISA test (Varveri et al., 1988; Wetzel et al., 1990). Identical results to those obtained with probe pBPPV1 were obtained with probe Cl1.2 (Wetzel et al., 1990; not shown). Using IC/PCR, the virus could be detected in 45/50 of the samples, demonstrating the very high sensitivity of IC/PCR for PPV detection. Restriction analysis of the amplified fragments using the endonucleases *Alu*I and *Rsa*I revealed a restriction profile typical of PPV-D, confirming the specificity of the amplification reactions. IC/PCR detected the virus in all the 'symptoms +' samples (18/18), and in 84% (27/32) of the 'symptoms −' samples. By comparison, ELISA and molecular hybridization detected the virus in 47% (15/32) and 62% (20/32), respectively, of these samples (Table 2). These results clearly demonstrate the superior ability of IC/PCR to detect low concentrations of virus in infected field samples as compared to other detection techniques.

TABLE 2

Comparison of different techniques for PPV detection in infected field samples. The samples were collected from naturally infected apricot trees. Two types of samples were collected: samples taken from parts of the trees showing symptoms (symptom +) and samples taken in symptomless parts of the trees (symptom −). The various samples were analyzed in parallel by ELISA, molecular hybridization (MH) using the ^{32}P-labeled cRNA probe pBPPV1 (Varveri et al., 1988) and IC/PCR, as described in Materials and Methods.

	Number of samples	ELISA		MH		IC/PCR	
		+	−	+	−	+	−
Symptom +	18	13	5	15	3	18	0
Symptom −	32	15	17	20	12	27	5
Total	50	28	22	35	15	45	5
% Positive		56		70		90	

'+', samples indexed positive; '−', samples indexed negative.

Discussion

An immunocapture/PCR PPV detection method is described, in which viral RNA from immunocaptured viral particles is used as the template for reverse transcription followed by PCR amplification. This protocol proved to be 250 times more sensitive than the PCR protocol described previously for PPV detection (Wetzel et al., 1991). This 250-fold increase in sensitivity of IC/PCR, as compared to direct PCR, results, probably, from two effects: increased sample size and reduced interference from plant substances. The theoretical gain from these combined factors can be calculated to be 2500 since the inhibitory effects have been estimated to reduce the sensitivity of direct PCR by a factor of 10 (Wetzel et al., 1991). On the other hand, it is unlikely that all the particles in the 250 μl sample are trapped on the ELISA plates and that recovery of the RNA from the trapped particles is 100% efficient, which might explain why the actual gain is only 250-fold. The end result, detection by IC/PCR of as few as 8000 viral target particles per ml of crude plant extract constitutes nevertheless a large improvement over preexisting techniques.

Korschineck et al. (1991) developed a PCR PPV membrane detection assay based on the incorporation of biotinylated nucleotides in the amplification products and on their enzymatic detection on a membrane using streptavidin-alkaline phosphatase conjugates. For detection of PPV in *Prunus*, the samples used are semi-purified RNA preparations obtained by chromatography on Qiagen columns. It is quite difficult to compare their results with our own, either on direct PCR or on IC/PCR since these authors have not precisely quantitated the sensitivity of their assay using purified virus preparations. The improvement over an ELISA assay is reported to be at least 16-fold, which would put their assay in the same sensitivity range as our direct PCR assay. In addition, we believe that any of our assays, by bypassing any kind of tedious sample preparation, provide for a much easier and less expensive detection of PPV.

The use of IC/PCR for PPV detection under routine conditions, during the indexing of field samples collected from naturally infected apricot trees, proved the increased sensitivity of the technique, when compared to molecular hybridization using ^{32}P-labeled cRNA probes (Varveri et al., 1988; Wetzel et al., 1990) or ELISA (Varveri et al., 1988). Despite the low titres and the uneven distribution of the virus in the infected trees, IC/PCR detected the virus in most of the infected samples analyzed (90%). The 5000-times higher sensitivity of IC/PCR, when compared to ELISA, which is the technique currently used, demonstrates clearly the great potential of IC/PCR for the routine detection of PPV. Indeed, taking into account the respective detection rates of ELISA (56%) and IC/PCR (90%) during this small study, it can be shown that analysis of a single sample by IC/PCR gives approximately the same efficiency of detection as analysis by ELISA of a composite sample of five individual samples collected from a single tree (which is the current standard indexing protocol). Thus IC/PCR opens the way to 'single sample' indexing and

probably, although we have not tested it, to large composite sample indexing, two techniques which would greatly simplify current PPV detection protocols. Further improvements in sensitivity could probably be obtained through silver nitrate staining of the PCR products or through their detection by molecular hybridization with internal probes. At the moment, the major limitation to the routine use of PCR or IC/PCR for routine indexing of PPV is probably the increased cost, since rough calculations show these techniques to be 5- to 10-times more expensive than ELISA. However, since the hands-on time is not very different between these techniques, and since the full potential of reduced sampling has not yet been evaluated, it is probably too early to compare ELISA indexing with IC/PCR indexing in terms of a detailed cost/benefit analysis.

Since the primers used in this study allow the detection by PCR of a wide range of PPV isolates (Wetzel et al., 1991), the sensitivity of the IC/PCR method is also of much interest for the characterization and epidemiology of PPV isolates from different geographical origins, the samples often having very low viral titers. Further efforts are now directed towards the direct sequencing of the amplified fragments, to allow epidemiological studies of PPV isolates at the molecular level.

In addition, we believe that this technique has wide ranging applications for the detection of other viruses or pathogens for which specific antibodies are available. The sample cleaning/concentration achieved by the immunocapture step should prove useful for the detection of pathogens that are present either at too low concentrations or in samples containing interfering substances. The test, in the format currently used, necessitates the transfer of the disrupted particles from the microtiter plate to an Eppendorf tube. This tedious and error-prone step can, however, be eliminated, by performing the immunocapture directly in an Eppendorf tube coated with the specific antiserum, as was originally described by Jansen et al. (1990). Alternatively, it is possible to envisage running the PCR directly in the IC microplates. Hence, addition of an immunocapture step, while greatly increasing the sensitivity of the assay, can be achieved while keeping a single tube format and could lend itself to automation.

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