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jus A polymerase chain reaction assay adapted
to plum pox potyvirus detection

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Summary

A sensitive, polyvalent assay based on the polymerase chain reaction (PCR) was developed for plum pox potyvirus (PPV) detection. This technique was adapted for a single tube, the chemical denaturation and reverse transcription of the viral RNA followed by the PCR reaction yielding a 243-base-pair product. As few as 10 fg of purified viral RNA, corresponding to approximately 2000 viral particles, were detected in plant extracts. All PPV isolates tested were amplified, and the amplified fragments were analysed by restriction endonuclease digestion. An *RsaI* restriction site polymorphism in the amplified fragments allowed the discrimination of two groups of isolates. In a field indexing trial, the PCR assay proved to be more sensitive than molecular hybridization using ³²P-labelled RNA probes for PPV detection.

Polymerase chain reaction, PCR; Plum pox potyvirus, PPV; Polymorphism

Introduction

Plum pox potyvirus (PPV) is the causal agent of plum pox (Sharka) disease of stone fruit trees (Dunez and Sutic, 1988). Since the virus is unevenly distributed and present sometimes at very low levels in infected trees, the control of the disease by sanitary selection measures requires detection techniques of high sensitivity. Varveri et al. (1988) developed a molecular hybridization assay for the detection of PPV, using ³²P-labelled RNA probes

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corresponding to the carboxy terminal half of the capsid protein gene of PPV strain D, which typifies one of the two viral serotypes (D and M) described by Kerlan and Dunez (1979). This probe proved to be more sensitive than an ELISA during a field indexing trial (Varveri et al., 1988). The polyvalence of the molecular hybridization assay was further improved by using RNA probes corresponding to PPV D non-structural protein genes, which are able to detect both serotypes D and M with the same sensitivity (Wetzel et al., 1990).

Recently, an enzymatic procedure named the polymerase chain reaction (PCR) was described which allows the amplification of very low amounts of target nucleic acids (Saiki et al., 1985, 1988). This technique has been used successfully to detect very low amounts of viral nucleic acids (Larzul et al., 1988; Gama et al., 1988; Yu kai et al., 1989; Kaneko et al., 1989; Carman et al., 1989; Dangler et al., 1989) and of plant viroids (Puchta and Sanger, 1989).

In this study, a PPV detection method is described based on PCR, in which PPV RNA is first reverse transcribed and then amplified from crude plant extracts, without the need for preliminary nucleic acid purification, making the procedure easy and rapid.

Materials and Methods

PPV strains and isolates

Various strains and isolates of PPV collected from infected orchards in Mediterranean countries, and propagated in GF305 peach seedlings under glasshouse conditions were used as templates for the PCR procedure.

PPV D RNA, purified as described by Varveri et al. (1987), and the clone pBPPV1 (Varveri et al., 1988) containing the PPV D target sequence, were used to determine the sensitivity of the PCR technique.

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

Primers

Two oligonucleotides' primer consensus sequences were selected from homologous regions of the three published PPV sequences: PPV D (Teycheney et al., 1989), PPV NAT (Maiss et al., 1989), and PPV Rankovik (Lain et al., 1989). Both non-degenerate primers, synthesized using an Applied Biosystem DNA synthesizer, are 20-mers containing 60% G + C, and result in the amplification of a 243-bp fragment (Fig. 1). They were used without any special purification.

Molecular hybridization

To allow samples to be analysed by molecular hybridization and PCR, each sample was cut into small strips with a sterile razor blade, and the strips were randomly divided into two groups. The strips to be analysed by molecular hybridization were ground in 50 mM citrate buffer, pH 8.3 (w/v = 1/4) containing 20 mM diethylthiocarbamate (DIECA) and 2% (w/v) polyvinylpyrrolidone (PVP K25, Fluka). After centrifugation of the resulting plant sap (10 min, Eppendorf microcentrifuge) the supernatant was diluted tenfold in 12 × SSC 6% formaldehyde (SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7). Using a hybridot apparatus (BRL), aliquots of 10 μ l were then spotted on a nitrocellulose membrane saturated with 20 × SSC.

The ³²P-labelled RNA probes, corresponding to PPV D structural and non-structural protein genes (Varveri et al., 1988; Wetzel et al., 1990), were synthesized using the Riboprobe Gemini System kit (Promega). The hybridizations were performed using the conditions of Melton et al. (1984), as described previously (Varveri et al., 1988; Wetzel et al., 1990). The membranes were exposed to X-ray films for 65 h using intensifying screens.

PCR assays

Plant extracts were prepared by grinding the samples in sterile water (w/v = 1/4); to avoid contamination from one sample to another, each sample was carefully put in a separate plastic bag containing gauze (Bioreba) and ground using a rolling grinder (Meku). The resulting plant sap was collected and centrifuged for 10 min (Eppendorf microcentrifuge). The supernatant was then diluted tenfold with sterile water. Ten μ l from these plant extract preparations were used for the PCR assay. Dilutions of purified nucleic acids were prepared in sterile water or in healthy peach extracts prepared as described above. Disruption of the virus particles was achieved by treating the samples first with 1% Triton X100 for 10 min at 65°C. The denaturation of target nucleic acid templates was then accomplished by adding methyl mercury hydroxide to 10 mM and further incubating the tubes for 10 min at room temperature. Following neutralization of the methyl mercury hydroxide by addition of 20 mM β -mercaptoethanol for 10 min at room temperature, a reverse transcription mixture containing 50 mM Tris-HCl buffer (pH 8.3 at 42°C), 50 mM KCl, 7.5 mM MgCl₂, 0.1 U Inhibit ACE (5 prime 3 prime Inc.), 20 μ M dNTPs, 0.1 μ M primer 1, and 0.5 U avian myeloblastosis virus RTase (Genofit), was added in each tube, to give a final volume of 20 μ l. Reactions were incubated at 42°C for 45 min. Following the reaction, 80 μ l of PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.1% gelatin) containing 1 μ M of both primers, 200 μ M dNTPs and 2 U of *Taq* DNA polymerase (Stratagene) were added to each tube and covered with 100 μ l of mineral oil. The following thermal cycling scheme was used (Perkin Elmer Cetus cycler), for 40 cycles: template denaturation at 92°C (1 min), primer

annealing at 62°C (2 min), DNA synthesis at 72°C (2 min). Amplification reactions were analysed by electrophoresis of 10- μ l aliquots from each reaction mixture on a 6% polyacrylamide gel, in Tris borate EDTA buffer (Maniatis et al., 1982). Bands were visualized either by ethidium bromide or silver staining (Biorad kit). Restriction analysis of the amplified fragments by *AluI* and *RsaI* endonucleases were done on 10- μ l aliquots, which were first twofold diluted in water to adjust the salt concentration. Five units of enzyme were added and the reactions performed for 2 h at 37°C. The gel analysis was performed as described above.

Results

Amplification from cloned PPV-D cDNA

The purified clone pBPPV1 described by Varveri et al. (1988) containing the PPV D target sequence for amplification was used as template for the first PCR assays. Tenfold dilutions of the clone pBPPV1 were prepared in sterile water or in healthy peach extracts, and amplified by PCR. After PAGE, DNA bands were visualized by ethidium bromide staining. An amplified fragment of the expected size was obtained in the assays containing the clone pBPPV1, whereas no DNA band was seen in the buffer and healthy peach controls. The analysis of the amplified fragment by digestions by the restriction endonucleases *AluI* and *RsaI* revealed the presence of *AluI* and *RsaI* restriction sites at the expected positions (Teycheney et al., 1989; Fig. 1). As few as 0.1 fg of purified clone pBPPV1 diluted in sterile water, corresponding to 20 target copies per assay, were detected after amplification. When diluted in healthy peach extracts, 1 fg of purified clone pBPPV1 corresponding to 200 target copies per assay, were

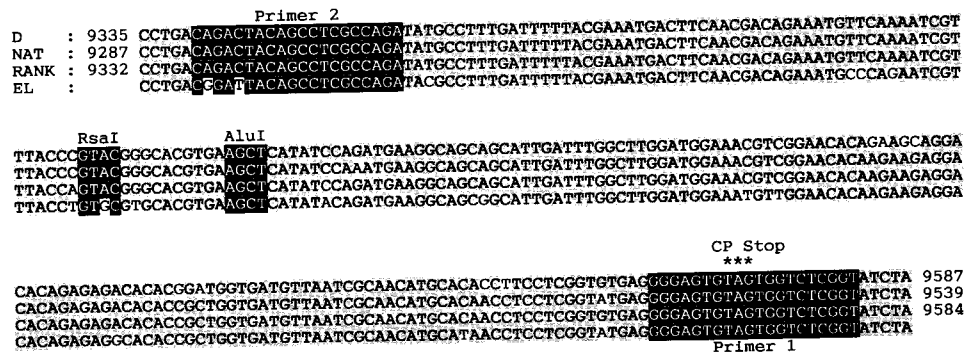


Fig. 1. Alignment of the nucleotide sequences of PPV D (Teycheney et al., 1989), PPV NAT (Maiss et al., 1989), PPV Rankovik (Lain et al., 1989), and PPV El Amar (Wetzel et al., 1991), corresponding to the 243-base-pair amplified fragment. Primer sequences and the restriction sites used in this study are boxed in black. The numbering refers to the position on PPV genome. Shadowed areas correspond to nucleotides found at least twice in the four sequences.

detected after amplification. Silver staining of the gels did not allow the detection of the fragment amplified from lower amounts of DNA (not shown).

Optimization of the PCR assay

To adapt the PCR assay for the detection of PPV, a chemical denaturation and a reverse transcription of the viral RNA were included. As the reverse transcription of the viral RNA occurs under different conditions than the amplification, this step was carried out in a small volume (20 μ l). The produced cDNA was then diluted with amplification buffer and amplified in a volume of 100 μ l. The dilution of the reverse transcription mixture resulted in a decrease of the concentration of $MgCl_2$, avoiding non specific hybridizations of primers which can result from high concentrations of $MgCl_2$. The addition of 1% Triton X100, to disrupt the virion, and of 10 mM of methyl mercury hydroxide to denature the viral RNA, prior to the reverse transcription step, improved the yield of the specific amplified product (data not shown). Increasing annealing temperatures from 52 to 62°C resulted in increased yields of the specific amplified product, whereas decreasing yields were observed at annealing temperatures of 67 and 72°C, probably resulting from lower hybridization of primers to their target sequence. All these observations resulted in the one tube amplification procedure described in Materials and Methods, which was used in all the PCR experiments described in this study.

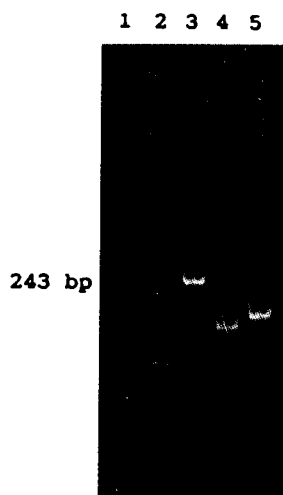


Fig. 2. Ethidium-bromide-stained 6% polyacrylamide gel showing the amplified fragment from PPV strain D genome in plant extract, and the restriction analysis of the amplified fragment. Lane 1: healthy sample; lane 2: molecular weight standards (123 DNA Ladder, B.R.L.); lane 3: undigested PPV amplified fragment; lane 4: PPV amplified fragment digested with *AluI*; lane 5: PPV amplified fragment digested with *RsaI*.

3). Silver staining the gels did not allow the detection of the fragment amplified from lower amounts of RNA (data not shown). The threshold of detection of 2000 viral particles per assay in healthy peach extracts corresponds to a 25-fold lower detection level per assay when compared to molecular hybridization using ^{32}P -labelled RNA probes (Varveri et al., 1988; Wetzel et al., 1990), and to a 5000-fold lower detection level per assay when compared to the ELISA test (Varveri et al., 1988). In terms of detection limit per ml, PCR is still more sensitive than the other techniques, detecting 10 pg of PPV RNA per ml, compared to limits of 25 pg by molecular hybridization, and 200 pg for the ELISA test (corresponding to a detection limit of 4 ng of PPV per ml).

Polyvalence of the PCR assay for PPV detection

The use of the PCR technique for routine PPV detection requires the ability to detect a wide range of PPV isolates. The polyvalence of the PCR assay using the primers P1 and P2 (Fig. 1) was determined with different PPV isolates

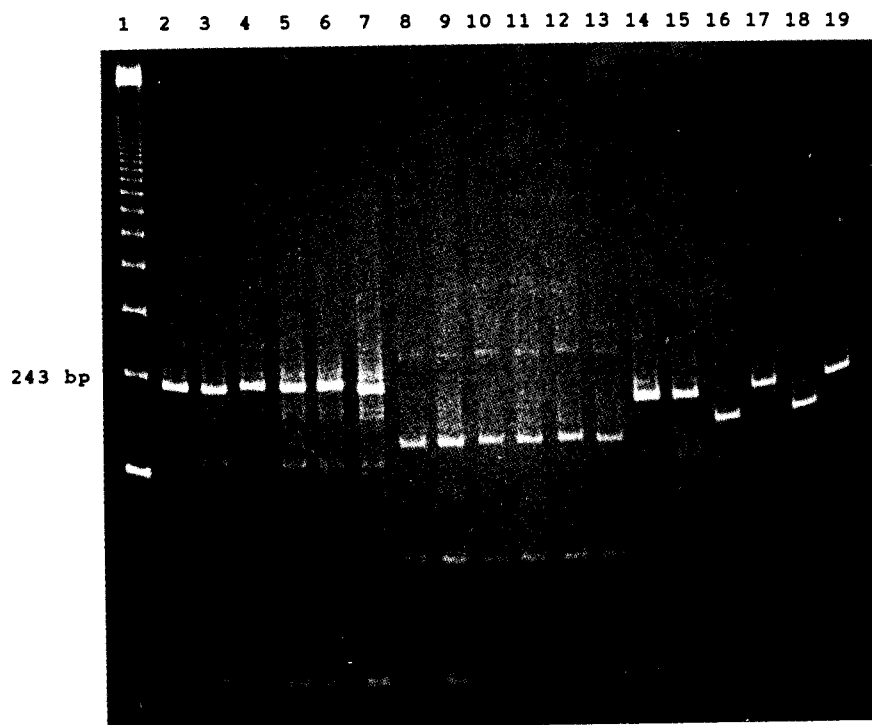


Fig. 4. Ethidium-bromide-stained 6% polyacrylamide gel showing the fragments amplified from different isolates of PPV and the restriction analysis of the amplified fragment. Lane 1: molecular weight standards (123 DNA Ladder, B.R.L.); lanes 2-7: undigested amplified fragments of PPV isolates from Greece (M strain), Egypt (El Amar strain), France (D strain), Cyprus, Spain, Turkey, respectively; lanes 8-13 and 14-19: amplified fragments of PPV isolates from Greece (M strain), Egypt (El Amar strain), France (D strain), Cyprus, Spain, Turkey, respectively, digested with *AluI* and digested with *RsaI*.

collected from infected orchards in various Mediterranean countries and propagated in peach seedlings. These isolates included the two strains typifying the serotypes D and M (Kerlan and Dunez, 1979) and the widely divergent strain El Amar (Wetzel et al., unpublished results). Fig. 4 shows the amplification products and the corresponding restriction fragments obtained upon digestion by the enzymes *AluI* and *RsaI*. All the isolates tested were detected by PCR. Analysis of the restriction profile of the amplified fragments revealed a conserved *AluI* restriction site common to all the isolates tested. In contrast, two groups could be discriminated on the basis of an *RsaI* polymorphism. One group of isolates, with a restriction profile identical to that of purified PPV D RNA, regrouped viruses isolated from France (D strain) and Spain, but also from Italy and Germany (not shown). The second group consisted of strains from Greece (M strain), Egypt (El Amar strain), Cyprus and Turkey. Indeed, the PPV-El Amar nucleotide sequence (Wetzel et al., 1991) corresponding to the 243-bp amplified fragment reveals the presence of an *AluI* restriction site at the expected position, whereas no *RsaI* restriction site is found (Fig. 1). A homology level of 90% was observed between PPV El Amar and the three other sequenced strains of PPV in the region corresponding to the amplified fragment. The low level of divergency found in this region from PPV genome explains the polyvalence of the assay. Two mismatches were found between the primer P2 and the corresponding target sequence on PPV-El Amar, in the 5' half of the primer (Fig. 1). However, these mismatches do not seem to disturb the hybridization of the primer P2 to its target sequence on the PPV El Amar RNA.

Detection of PPV by molecular hybridization and PCR during a field indexing trial

To test the ability of the PCR technique for use in routine detection of PPV, a comparative detection assay using molecular hybridization with ³²P-labelled RNA probes and PCR was performed. The samples were collected from infected trees in a naturally infected orchard of southern France. Since a grinding buffer permitting an optimal detection of PPV by both techniques has not been found, each sample was cut into small strips. These strips were then divided randomly into two groups to provide maximal homogeneity between them, allowing identical samples to be analysed by both techniques. A good correlation was obtained between molecular hybridization and PCR, all the samples positive by molecular hybridization (18–56) also being positive by the PCR technique. In addition, whereas an identical detection level was obtained between molecular hybridization and ethidium staining of the amplified fragments, the silver staining allowed the detection of the viral amplified fragment in 11 additional infected samples (19%), indicating the higher sensitivity of PCR for PPV detection. Fig. 5 represents samples analysed by molecular hybridization using the probe pBPPV1 (Varveri et al., 1988) and by PCR. No non-specific reactions by molecular hybridization nor band

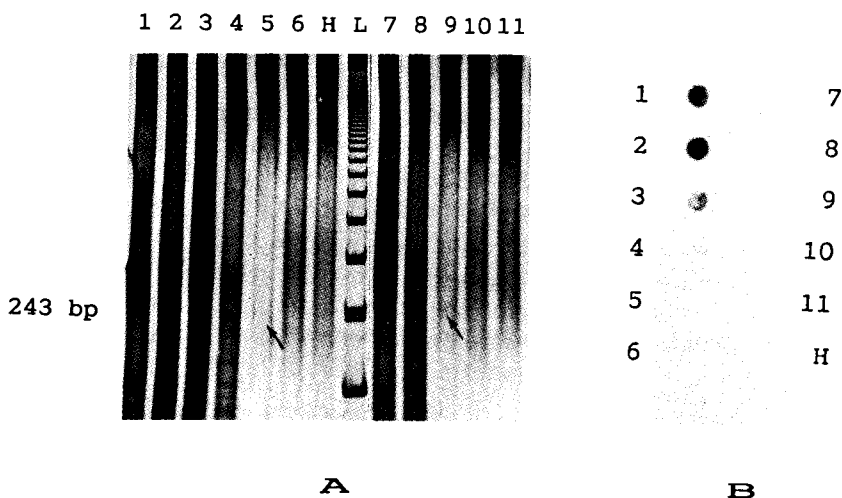


Fig. 5. Comparison of molecular hybridization and PCR in a PPV field indexing assay. **A:** silver-stained 6% polyacrylamide gel showing the product of amplification of samples (1-11). H: healthy sample; L: molecular weight standards (123 DNA Ladder, B.R.L.). **B:** analysis of the same samples (1-11) by dot blot molecular hybridization using the probe pBPPV1 (Varveri et al., 1988); H: healthy sample. The arrows indicate amplified fragments detected after PCR in samples judged negative by the molecular hybridization assay.

corresponding to the viral amplified fragment were seen with healthy material. Results similar to those obtained with probe pBPPV1 were obtained with probes corresponding to non-structural viral protein genes (data not shown). The analysis of the amplified fragments revealed a restriction profile typical of PPV strain D (data not shown). Occasionally, additional bands of unknown origin were observed in both healthy and infected samples, probably resulting from non specific hybridization of primers to plant genomic DNA or cDNA.

Discussion

In this study, a single tube method which permits PPV RNA chemical denaturation, reverse transcription and amplification is described, each step only requiring the addition of reagents. Nucleic acid purification is not needed, PPV RNA being amplified directly from crude plant extracts. The specific product of amplification corresponds in size (243 bp) to that predicted by published sequences of PPV (Teycheney et al., 1989; Maiss et al., 1989; Lain et al., 1989).

The threshold of sensitivity obtained with this protocol (2000 viral particles amplified per assay in plant extracts) is several orders of magnitude greater than the theoretical sensitivity of PCR (Saiki et al., 1985). This can be attributed to several factors. The first is the low efficiency of the reverse transcription step, since tenfold higher amounts of purified RNA than the

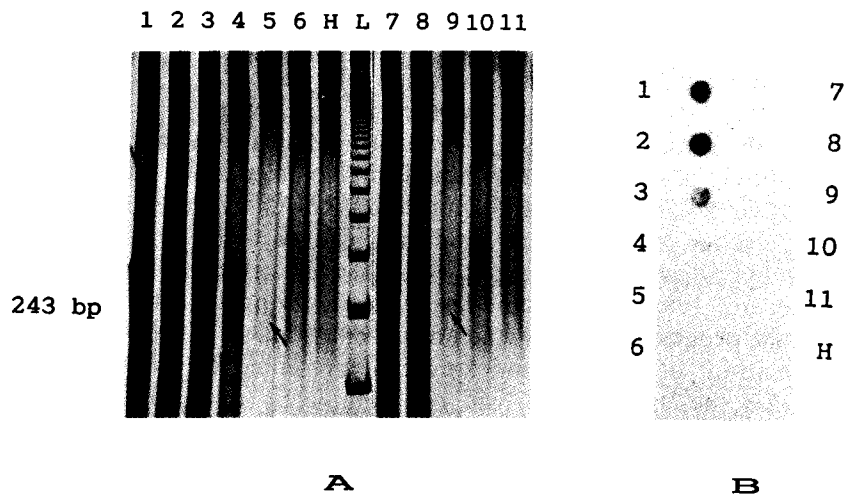


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corresponding purified DNA are required for detection. The amplification directly in plant extracts also decreases the efficiency of synthesis of the amplification product, since only tenfold greater amounts of nucleic acids can be detected in plant extracts, as compared to sterile water.

Even with these problems, the resulting sensitivity and the ease of implementation of this protocol should enable its use for the detection of other plant viruses, or for the study of cellular RNAs. The use of this technique for PPV detection under routine conditions proved to be more sensitive than molecular hybridization (Varveri et al., 1988; Wetzel et al., 1990) or ELISA (Varveri et al., 1988). The major challenge in trying to detect woody plant viruses is the problem caused by the uneven distribution of the virus in infected trees. The result of the field indexing trial presented in this study tends to indicate that the use of PCR will not completely overcome this problem. However, the net gain in detection sensitivity achieved with PCR will probably translate in a reduction of the number of individual samples taken from a tree to produce the composite sample traditionally indexed.

The polyvalence of the PCR assay was demonstrated by the amplification of isolates of PPV from several countries. The restriction analysis of the amplified fragment of these isolates revealed two groups of isolates, based on the presence or absence of a *Rsa*I restriction site. It is of interest to note that the two D and M serotypes (Kerlan and Dunez, 1979) fall into different groups of isolates. It is also of interest to notice that the isolates coming from oriental Europe showed a profile similar to PPV M, whereas those coming from occidental Europe showed a profile similar to PPV D. However, these isolates have not been serologically classified. It is therefore too early to try to establish a correlation between the restriction profile of the amplified fragments and the serological classification. Work toward the direct sequencing of the amplified fragments is currently in progress, and will allow us to further evaluate the relationships between nucleotide sequence and geographical origin of PPV strains. Further efforts will also be directed at the improvement of the experimental procedure, to obtain a maximal efficiency for the synthesis of the amplified products. As the sensitivity of the PCR procedure is also dependent on the sensitivity of the final detection technique of the amplified fragments, evaluation of biotin labelled probes for dot blot molecular hybridization detection of the amplified products is also in progress.

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