

Specific detection of D- and M-isolates of plum pox virus by immunoenzymatic determination of PCR products

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Abstract

Molecular techniques based on the polymerase chain reaction (PCR) can provide rapid and sensitive diagnosis of plum pox virus (PPV), the causal agent of the devastating ‘sharka’ disease of stone fruit trees. The present study compared routine polymerase chain reaction (PCR) procedures against a new system, PCR-ELISA (Boehringer Mannheim), which enables immunoenzymatic detection of PCR products. The results show that this hybridisation system ensures fast and more sensitive detection of PPV associated with stone fruit trees and herbaceous hosts. Strain-specific capture probes were also designed to identify the two major PPV isolates, D and M, without subsequent restriction fragment length polymorphism analysis of the PCR products. Optimisation of all parameters involved in the PCR-ELISA procedure are discussed and its advantages reported. © 1997 Elsevier Science B.V.

Keywords: PCR-ELISA; Microwell capture hybridisation; Plum pox virus; Rapid detection of PPV-D and PPV-M

1. Introduction

Plum pox virus (PPV) causes ‘sharka’, the major viral disease of stone fruit trees in Europe and the Mediterranean region. Since 1991, polymerase chain reaction (PCR) and immunocapture PCR have provided the most sensitive detection assays for PPV. The genome sequence targeted in this

assay is a 243 basepair (bp) fragment in the carboxy-terminal region of the coat protein gene (Candresse et al., 1995). Nucleotide analysis and sequencing of the 3′ non-coding region and restriction fragment length polymorphism (RFLP) analysis of amplified products with restriction enzymes *RsaI* and *AluI* show that isolates of PPV can be classified into four groups: PPV-D, which contains an *AluI* and an *RsaI* recognition sequence; PPV-M; PPV-El Amar, which has so far been found only in Egypt and contains only an

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AluI sequence; PPV-C, which is found only in sour cherry and other cherry species in Moldova and lacks both recognition sequences (Fuchs et al., 1995; Nemchinov and Hadidi, 1996). PPV-D and PPV-M, the two conventional major serotypes (Kerlan and Dunez, 1979), can also be distinguished with serotype-specific PCR primers (Candresse et al., 1995). PPV-D has been reported in most Italian plum and apricot orchards and only sporadically in peach orchards (Crescenzi et al., 1994).

During the spring and summer of 1996, various peach and nectarine cultivars in the Verona and Cesena areas showed severe leaf and fruit symptoms suspected to be caused by PPV infection. Reverse transcriptase PCR (RT-PCR) with conventional primers (Wetzel et al., 1991) and subsequent RFLP analysis showed the consistent presence of strain M in the affected peach trees (Poggi Pollini et al., 1996). Data from more recent field and experimental evidence indicate that the D- and M-isolates exhibit differing epidemiological responses: the M-isolates have a broader experimental range of host plants and spread more rapidly in the field than the D-isolates, rapidly inducing epidemics in peach and apricot orchards, whereas the D-isolates are not readily able to invade peach trees (Fuchs et al., 1995; Quiot et al., 1995). Infection in the nursery could lead to a rapid dissemination of a pathogen, especially if it induces a fast onset of epidemics. It should be noted that severe M-isolates, which have destroyed many orchards in Greece, the former Yugoslavia and Hungary (Polak, 1994) and were previously confined to the eastern European countries, have recently been identified in France and Germany (Candresse et al., 1994).

The PCR-ELISA (Boehringer Mannheim, Germany), the Gen-Eti-K-DNA Enzyme Immunoassay (DEIA; Sorin Biomedica, Italy) and the Captagene GCN-4 (Amrad, Australia) kits that have been recently introduced, enable immunoenzymatic determination of PCR products in the liquid phase without the need for electrophoresis, thereby simplifying the analysis of the results with an ELISA reader. These highly sensitive systems have been used to excellent effect mainly in human medicine (Sakrauski et al., 1994;

Andréoletti et al., 1996). In plant pathology they have been used for the diagnosis of PPV in plum trees and tobacco (Schonfelder et al., 1995), of tomato spotted wilt virus (TSWV) in different *Nicotiana* species (Weekes et al., 1996), of *Polymyxa betae* fungus in sugar beet roots (Mutasa et al., 1996) and of phytoplasmas in various plant species (Poggi Pollini et al., 1997).

The diagnosis of PPV infection in suspected trees and the development of large-scale epidemiological studies have so far been hampered by such factors as the woody nature of the natural hosts and low concentration and uneven distribution of the agent in infected plants. In general, the fundamental criteria for assessing a new diagnostic test are reliability, specificity, sensitivity, ease of use, suitability for mass tests, cost, health hazards to humans and the environmental risks of the substances used (Powell, 1987). The present study specifically tested the PCR-ELISA kit for the diagnosis of PPV-D and PPV-M, without subsequent RFLP analysis, in stone fruit trees, for continuous monitoring of epidemiological conditions.

2. Materials and methods

2.1. Total RNA extraction

Fresh leaf samples with severe symptoms (100 mg) were collected from the peach tree cultivars Redhaven, Elegant Lady, Fayette, Stark Red Gold, Caldesi 2000 and Caldesi 2010 in several orchards near Verona and Cesena and ground in a mortar with liquid nitrogen. Leaf samples from apricot, plum trees and *Nicotiana benthamiana* infected with PPV-D isolates of different geographical origins and from stone fruit trees kept in virus-free conditions were used as PPV-D sources and negative controls, respectively. Total RNA extraction was performed using the RNeasy Plant Total RNA kit (Qiagen, Hilden, Germany), without the use of phenolic compounds, according to the manufacturer's instructions. RNA was eluted with 40 μ l of diethyl pyrocarbonate-treated water in the final step.

2.2. Reverse transcription

A 3 μ l aliquot of RNA preparation was added to a primer annealing reaction mixture containing 100 pmol of antisense primer and sterile H₂O at a final volume of 12.5 μ l. The annealing reaction was then added to 12.5 μ l of a cDNA mixture containing 5 μ l of 5 $\times$ reverse transcriptase buffer, 1 μ l of RNasin (40 units; Promega, Madison, WI), 2.5 μ l of 10 mM dNTPs and 0.5 μ l of Maloney murine leukemia virus reverse transcriptase (200 U/ μ l; Promega). Reactions were mixed briefly and incubated for 45 min at 38°C.

2.3. PCR amplification

After heat denaturation, amplification was carried out in PCR tubes containing the following reaction mixture: 10 μ l of 10 $\times$ PCR buffer, 2 μ l of 10 mM dNTPs, 400 pmol of both sense and antisense primer, 2.5 units of Taq polymerase (Boehringer Mannheim, Mannheim, Germany), 3 μ l of cDNA, and sterile water to a volume of 100 μ l. Sense and antisense primers were used for RT-PCR after Candresse et al. (1995). The reaction mixture was overlaid with 100 μ l of mineral oil and amplified in a DNA Thermal Cycler 480 (Perkin Elmer) with the following parameters: denaturation at 92°C for 1 min, primer annealing at 62°C for 2 min, DNA synthesis at 72°C for 2 min for 40 cycles (Wetzel et al., 1991).

The detection of PCR products was carried out by agarose gel electrophoresis (AGE; 1% in Tris-borate/EDTA buffer) at 5 V/mm with ethidium bromide staining. Post-amplification RFLP analysis was performed by digesting 20 μ l from each sample overnight at 37°C with 1 μ l of *Alu*I or *Rsa*I (Boehringer Mannheim); samples were then electrophoresed in vertical 10% polyacrylamide gels in TBE buffer. Molecular weights were determined using the 1 kb DNA ladder (BRL, Eggenstein, Germany).

2.4. PCR-ELISA: amplification, incorporation of digoxigenin in the amplified products and selection of isolate-specific probes

Amplification was carried out as described in

Section 2.3, but employing 250 μ M of PCR-ELISA labelling mix (Boehringer Mannheim) containing the four nucleotides, one of which was labelled with digoxigenin (DIG). Using the sequence information reported by Candresse et al. (1995), we designed specific 18-mers for D- and M-isolates to anneal in the 243 bp amplified product to the most variable region between the two strains (five mutations) around the *Rsa*I site. Probe sequences were: 5'-bio-ACGACACCCG-TACGGGCA-3' (for D-isolates) and 5'-bio-AC-AACGCCTGTGCGTGCA-3' (for M-isolates). Primer, probe synthesis and biotinylation at the 5'-end were performed by Life Technologies (UK).

2.5. PCR-ELISA: specific hybridisation of the DIG-labelled amplicons

A 10 μ l aliquot of amplified product, or of appropriate dilutions in sterile water, were added to 20 μ l of denaturation solution and incubated for 10 min at room temperature.

The hybridisation solution (220 μ l) containing the various dilutions of the specific probe (D or M) was added to this solution, and 200 μ l of this mixture were then placed in one well on an ELISA strip, previously coated with streptavidin, and incubated for a variable time. All the reagents, except for the specific probes, were supplied with the PCR-ELISA DIG detection kit (Boehringer Mannheim). As well as the healthy samples, controls were also used which consisted of amplified products diluted in the hybridisation buffer without the specific probe. The following experimental conditions were controlled: (1) duration of incubation: 1, 3, 12 h; (2) incubation temperature: 40, 45, 50°C ($\pm$ 1°C); (3) probe concentration: 10, 30, 50 pmol/ml of incubation buffer; (4) other conditions: no shaking, shaking at moderate speed.

2.6. PCR-ELISA: immunoenzymatic reaction and spectrophotometric determination

After incubation with the specific probe, the wells were washed three times for 3 min in the washing buffer supplied. Then the DIG-specific antiserum bound to horseradish peroxidase (anti-

Table 1
Detection of PPV-D and PPV-M by PCR and PCR-ELISA

Source	No. positive by PCR and RFLP analysis		No. positive by PCR-ELISA	
	PPV-D	PPV-M	PPV-D	PPV-M
Peach trees with symptoms (15) ^a	0/15 ^b	15/15	0/15	15/15
Apricot trees (8)	6/8	2/8	6/8	2/8
Plum trees (8)	8/8	0/8	8/8	0/8
<i>N. benthamiana</i> (5)	5/5	0/5	5/5	0/5
Healthy apricot, peach and plum trees (10)	0/10	0/10	0/10	0/10

^a Number of samples examined.

^b Positive/tested.

DIG-POD), diluted 1/100 as suggested in Boehringer's instructions, was added to the conjugate buffer. Incubation time was 1 h or 3 h at 37°C with moderate shaking. After three washings, 200 µl of ABTS substrate were added to each well, followed by incubation in the dark at 37°C for 30 min; the reading was taken with an ELISA reader at 405 nm.

2.7. Quantification of PCR-ELISA sensitivity

To determine the sensitivity of the microwell hybridisation assay, dilution series (from 200 ng to 100 pg determined by spectrophotometric readings) of PCR products amplified from samples affected by PPV-D or -M were analysed for comparison by AGE as well as by capture hybridisation.

3. Results

3.1. Analysis of the extraction protocol and of the RT-PCR-amplified products

The RNA extraction protocol, based on the commercially available RNeasy kit, produced high-quality RNA suitable for use in RT-PCR. A 243 bp product could always be detected clearly by AGE from all infected samples. As expected, there were no products when extracts from uninfected plants were used (data not shown).

3.2. Reliability of the PCR-ELISA method

The results recorded with the PCR-ELISA method were in complete agreement with those from conventional RT-PCR analysed by AGE, followed by RFLP with *Aha*I and *Rsa*I (Table 1). Our method proved to be highly reliable: the infected samples always showed very high absorption, visible even with the naked eye, as compared to the control samples from healthy plants and amplified products used without probes. No cross-reactions were ever found between D- and M-isolates, and a clear discrimination between positive and negative results was achieved; the background in all the reactions was always less than 0.20 optical density units (Table 2). The values reported in Table 2 refer to optimum and identical method conditions for the two probes: 3 h incubation with the probe, moderate shaking, a temperature of 40°C, probe concentration of 30 pmol/ml incubation buffer, 1 h incubation with antiserum and moderate shaking.

Optimisation of the parameters used is summarized in Table 3. Incubation temperature was a particularly critical factor because above 45°C the final absorption value markedly dropped (from 1.50 to 0.69 with PPV-D + D-probe, and from 1.59 to 0.71 with PPV-M + M-probe); the decrease of probe concentration (to 10 pmol/ml incubation buffer) and the absence of shaking had little effect on the final outcome. The increase in probe concentration from 30 to 50 pmol/ml incubation buffer and of incubation times (overnight

Table 2
Results obtained with the PCR-ELISA method

Source	Absorption at 405 nm (OD) with probe		Absence of probe
	PPV-D	PPV-M	
Peach trees (15) ^a	0.12 (0.09–0.17)	1.60 (1.20–1.81)	0.11 (0.08–0.15)
Apricot trees (PPV-D) (6)	1.50 (1.25–1.72)	0.14 (0.10–0.17)	0.10 (0.08–0.14)
Apricot trees (PPV-M) (2)	0.13 (0.10–0.16)	1.53 (1.50–1.56)	0.10 (0.09–0.11)
Plum trees (8)	1.50 (1.21–1.73)	0.13 (0.10–0.16)	0.10 (0.08–0.12)
<i>N. benthamiana</i> (5)	1.56 (1.42–1.68)	0.12 (0.10–0.15)	0.11 (0.08–0.14)
Healthy samples (10)	0.13 (0.08–0.16)	0.14 (0.09–0.16)	0.11 (0.07–0.13)

^a Mean value; minimum and maximum values in parentheses.

^b Number of samples tested.

with capture probes and 3 h with antiserum) did not significantly improve absorption values.

3.3. Comparison of detection by PCR-ELISA and gel electrophoresis

To determine the sensitivity of the PCR-ELISA method, titration experiments were carried out comparing conventional PCR analysis by AGE and by capture hybridisation with the parameters determined in Section 3.2. As shown in Table 4, the detection limit for both PPV-D and PPV-M in

gel electrophoresis was at a concentration of about 6 ng. The cut-off value in the PCR-ELISA test was fixed at 0.33 OD units (twice the highest value with uninfected samples) with an equivocal zone of $\pm 30\%$ (Sakrauski et al., 1994). This approach provided clear discrimination between positive and negative results, without borderline cases: at about 500 pg of PCR products from both isolates, a positive signal was still detectable.

Table 4
Detection limit of PCR products by gel electrophoresis and PCR-ELISA

Source	Gel electro-phoresis ^a	PCR-ELISA ^b	
		PPV-D	PPV-M
200 ng ^c	++ ^d	1.50	1.60
100 ng	++	1.49	1.60
50 ng	++	1.43	1.50
25 ng	++	1.40	1.44
12.5 ng	+	1.20	1.23
6.25 ng	+	1.00	1.02
3 ng	—	0.82	0.91
1.50 ng	—	0.74	0.73
750 pg	—	0.59	0.60
500 pg	—	0.51	0.52
250 pg	—	0.23	0.25
100 pg	—	0.11	0.12

^a Detection was similar for both D- and M-isolates.

^b Absorption values in OD units (mean of four samples); values at least three times higher than the highest healthy control absorption value (0.16) were considered as positive.

^c Determined by spectrophotometric readings.

^d ++, +, relative band intensities; —, absence of detection.

Table 3
Optimisation of the main parameters for using the PCR-ELISA kit

Hybridisation conditions	Absorption at 405 nm (OD) ^a	
	PPV-D	PPV-M
	+ D-probe	+ M-probe
Temperature		
40°C ^b	1.50	1.59
45°C ^b	1.01	1.13
50°C ^b	0.69	0.71
No shaking ^b	1.32	1.37
Probe concentration		
10 pmol/ml ^c	1.27	1.19
50 pmol/ml ^c	1.52	1.62

^a Each value is the mean of three samples.

^b Probe concentration 30 pmol/ml, incubation time 3 h.

^c Hybridisation temp. 40°C, moderate shaking, incubation time 3 h.

4. Discussion

The results indicate that the PCR-ELISA kit is particularly reliable and specific since the amplified products from infected plants were hybridised with highly specific homologous probes and the assessment of the results did not reveal any problems. The specificity of the method is also highlighted by the absence of cross-reactions between the two groups (D and M) of isolates (Tables 1 and 2).

PCR-ELISA can be used for direct diagnosis of PPV-D and PPV-M isolates without subsequent and expensive RFLP analysis of the PCR products. This high specificity was also demonstrated for the detection of several phytoplasma 'species' in trees and shrubs with different capture probes (Poggi Pollini et al., 1997). Furthermore, the specificity of a product obtained with PCR can often be determined only after hybridisation with a specific probe, regardless of the primers used. Thus it is necessary to use a hybridisation method that is simple, non-isotopic and detectable with inexpensive instruments. The PCR-ELISA kit fully meets these requirements (Mantero et al., 1991).

The PCR-ELISA procedure can properly discriminate two isolates of a pathogen by using specific capture probes with a 27% level of divergence (five nucleotides on 18). PPV isolates have been classified into four groups, one of them being represented by the Egyptian isolate El Amar which has never been reported in Europe. This isolate shares a very high sequence homology and cannot be distinguished from M-isolates either by serotype-specific primers, designed in the most variable region between the two serotypes, or by conventional PCR followed by RFLP analysis (Candresse et al., 1995). Our specific capture probes were also designed in the same region, although the lower level of divergence between PPV-M and PPV-El Amar in this genomic part (only two nucleotides) as well as in other regions could hamper the use of PCR-ELISA to distinguish properly between these two isolates. However, a recent study designed to differentiate two organisms with high sequence similarity—apple proliferation and plum leptonecrosis phytoplas-

mas—showed that a two-base difference in the PCR products can be reliably detected by oligonucleotide hybridisation in the presence of tetramethylammonium chloride (Malisano et al., 1996). The ability to differentiate very similar targets by adding a tetramethylammonium chloride wash solution to the PCR-ELISA procedure after the hybridisation step is now under evaluation.

Preliminary tests to check the sensitivity of the method have demonstrated that PCR-ELISA is about ten times more sensitive than electrophoretic determination of PPV-amplified products (Table 4). This increase in sensitivity is comparable to the results reported in the medical and plant pathology fields (Sakrauski et al., 1994; Poggi Pollini et al., 1997). Other researchers have demonstrated the superior sensitivity of the DEIA (DNA enzyme immunoassay) procedure over gel electrophoresis detection of PCR products from PPV-infected plants (Schonfelder et al., 1995).

The PCR-ELISA kit is simple to use and eliminates the need for the use of hazardous chemicals during the electrophoresis procedures, especially if RFLP analysis of the amplified products is necessary. It is amenable to the processing of large numbers of samples and the results can be easily read and recorded using the same simple equipment as that used in ELISA tests. At present, its biggest drawback is certainly the cost, even if it is easy to envisage a reduction linked to the excellent results achieved and the use of these methods on an ever wider scale in human medicine (Schonfelder et al., 1995).

The development of rapid methods for RNA extraction may also help in the definition of rapid and simple routine protocols. The RNA extraction procedure using the commercially available Qiagen RNeasy kit, based on spin-column matrices, enables a rapid and efficient RNA yield. This method, which eliminates the use of hazardous chemicals, was recently described as an efficient procedure for the extraction of high-quality RNA and subsequent detection by RT-PCR of several viruses in woody plants (MacKenzie et al., 1997).

PPV is considered of quarantine significance by IAPSC, NAPPO and EPPO, and considerable

efforts—such as certification schemes, eradication programmes and development of rapid, sensitive and massive detection techniques—are being made everywhere to limit the spread of ‘sharka’ disease (Roy and Smith, 1994). The procedure described above can be useful as a highly sensitive and specific monitor of epidemiological conditions to prevent early PPV infections in nursery fields.

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