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Simultaneous detection and typing of plum pox potyvirus (PPV) isolates by Heminested-PCR and PCR-ELISA

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Abstract

Two techniques for simultaneous detection and typing of plum pox potyvirus (PPV) isolates belonging to the D or M serotypes, heminested PCR (H-PCR) and PCR-ELISA, have been developed. Ten PPV isolates typed using PPV-D and PPV-M specific monoclonal antibodies by ELISA-DASI were used to validate these two methods. The results obtained show a complete coincidence of the nucleic acid-based techniques with the serological data. When serial dilutions of infected plant extracts were assayed, H-PCR and PCR-ELISA were found to be 100 times more sensitive than the more conventional immunocapture-PCR (IC-PCR) assay. Testing of 228 PPV-infected fruit tree samples coming from different hosts and locations indicated that so far only PPV type D appears to be present in Spain and in Chile. Coupled with print-capture sample preparation (Olmos et al., Nucl. Acids Res. 24, 2192–2193, 1996) the increased sensitivity provided by heminested-PCR allowed the detection of PPV targets of D and M types, in wingless individuals of the aphid vector *Aphis gossypii*. © 1997 Elsevier Science Ireland Ltd.

Keywords: PPV; Detection; Amplification; Serotypes; Aphids; Heminested-PCR; PCR-ELISA

1. Introduction

Plum pox virus (PPV), a member of the potyvirus genus, is the causal agent of the Sharka disease, which is thought to be one of the most important diseases of stone fruit trees, because of

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its large agronomic impact and economic importance (Dunez and Sutic, 1988; Smith et al., 1994). This disease is present in most European and Mediterranean countries as well as in Chile (Roy and Smith, 1994). PPV shows an uneven distribution in the infected trees and its titre decreases during the summer and winter periods. These factors cause serious problems for a reliable detection of the virus in their woody hosts. Therefore, sensitive techniques like immunosorbent assays based on the use of monoclonal antibodies (MAb) (Cambra et al., 1994) and various PCR techniques with or without specific capture of the viral target (Wetzel et al., 1991, 1992; Olmos et al., 1996) were developed for the sensitive and specific detection of PPV.

Two major serotypes of PPV, serotype D (Dideron) and serotype M (Marcus) were described on the basis of serological cross-reactivities using polyclonal antisera (Kerlan and Dunez, 1979). More recently, monoclonal antibodies were developed and allowed the discrimination of these two serotypes (Cambra et al., 1994; Boscia et al., 1997). Recent evidence suggests that the two PPV serotypes show significant differences in their epidemiological properties and in their natural host range (Adamolle, 1993; Quiot et al., 1995) so that control measures may have to be adapted to the particular PPV serotype being targeted. The ability of isolates of the M serotype to readily infecting peach under field conditions (whereas the D serotype is far less able do so) is an indication that such isolates have the potential to further deteriorate the PPV situation in Western European countries. Such an analysis has, for example, justified differential growers compensation for PPV eradication actions in France where PPV-M was introduced during the mid 1980s (Smith et al., 1994). These aggressive PPV isolates were detected recently in Italy (Poggi Pollini et al., 1996; D. Boscia, personal communication). As a consequence, efficient and sensitive detection methods allowing simultaneous typing of PPV isolates are needed.

Sequence analysis of PCR fragments corresponding to the C-terminal part of the PPV coat protein gene has allowed the identification of molecular polymorphisms correlated with the serotype of the PPV isolates (Candresse et al., 1995). In particular, a cluster of non-coding, third-base mutations on five consecutive codons appear to show an excellent correlation with the viral serotype. One of the mutations of the cluster corresponds to a RsaI RFLP polymorphism detected previously which was used for the typing of PPV isolates (Wetzel et al., 1991; Bousalem et al., 1994; Candresse et al., 1994). More recently, this region was selected as the target for the first serotype-specific PCR primers for PPV (Candresse et al., 1995). In order to further increase the sensitivity and the reliability of the simultaneous detection and typing of PPV isolates in field samples, we have now developed two new techniques, heminested-PCR (H-PCR, Erlich et al., 1991; Zhang and Ehrlich, 1994) and ELISA-PCR (Allard et al., 1993) allowing simultaneous detection and typing of PPV isolates. H-PCR was used, in parallel to more conventional serotyping assays, to type a large number of PPV field samples of unknown serotype status from Spanish and Chilean orchards. Coupled with a previously developed print-capture or spot-capture technique allowing very efficient and simple sample preparation (Olmos et al., 1996), H-PCR also allowed for the first time the detection of PPV-D and M from single aphid vectors.

2. Materials and methods

2.1. PPV isolates and preparation of infected plant samples

Ten PPV isolates, six belonging to serotype D and four to serotype M, were used in this study (Table 1). They were kept in collection under greenhouse at the IVIA, either in a woody indicator (GF305 peach seedlings) or in a herbaceous experimental host, *Nicotiana benthamiana*. In addition, 228 plant samples infected with unclassified PPV isolates collected from different stone fruit trees were analysed. These samples were obtained from various regions of Spain (206 samples) and from Chile (22 samples kindly provided by C. Cereceda of the Servicio Agrícola y Ganadero, SAG, Santiago de Chile).

Table 1
Comparison of the serotyping results of ten PPV isolates using serotype-specific monoclonal antibodies in ELISA-DASI tests, heminested PCR (H-PCR) assays specific for either the D or the M serotypes and D serotype specific ELISA-PCR

PPV isolate	Host	Serotype	ELISA-DASI (MAbs)			H-PCR		D-specifc
			4DG5	4DG11	AL	D	M	ELISA-PCR
3.3 RB	N.b.	D	+	+	−	+	−	+
NAT-FL	N.b.	D	+	+	−	+	−	+
W731-739	N.b.	D	+	+	−	+	−	+
Turquia	GF 305	D	+	+	−	+	−	+
Dideron Canino	GF 305	D	+	+	−	+	−	+
Des-AT	N.b.	D	+	+	−	+	−	+
Ps	N.b.	M	−	−	+	−	+	−
MS89	N.b.	M	−	−	+	−	+	−
O-Grecia	N.b.	M	−	−	+	−	+	−
Sutic Forte	GF 305	M	−	−	+	−	+	−

The propagation hosts for the various PPV isolates are indicated (N.b.: *Nicotiana benthamiana*, GF 305: GF 305 peach seedlings). Plus signs indicate positive reactions or amplifications in any of the tests while minus signs indicate negative reactions.

Extracts of plant material were prepared by grinding plant material 1/20 (w/v) in PBS buffer, pH 7.2, supplemented with 2% (w/v) polyvinylpyrrolidone (PVP-10) and 0.2% (w/v) sodium diethyl dithiocarbamate (DIECA). For sensitivity comparisons, ten-fold serial dilutions of an extract of GF305 inoculated with isolate PPV-D Dideron Canino were prepared in healthy apricot cv. Moniquí extract. Alternatively, samples were analysed using the print-capture or spot-capture PCR (PC-PCR or SC-PCR) techniques described by Olmos et al. (1996).

2.2. ELISA-DASI

Double-antibody sandwich indirect ELISA (DASI) (Cambra et al., 1991) was carried out by coating Nunc Maxisorp immunoplates with 200 μl per well of a solution of 1 μg/ml anti-PPV rabbit immunoglobulins (W1001) in carbonate buffer. PPV-D specific (MAb 4DG5, 0.1 μg/ml and MAb 4DG11, 0.02 μg/ml; Cambra et al., 1994) and PPV-M specific (MAb AL, ascitic fluid diluted 1/40 000; Boscia et al., 1997) monoclonal antibodies were then applied. A monoclonal antibody detecting all PPV isolates (MAb 5B-IVIA, 0.1 μg/ml; Cambra et al., 1994) was used to verify the presence of PPV. Following incubation with plant samples, alkaline phosphatase conjugated goat anti-mouse immunoglobulins (Boehringer Mannheim) were used. The plates were read at 405 nm at 15 min intervals for 1 h without stopping the reactions, in a Titertek Multiscan Plus MK II (Flow) automatic reader. Positive and negative controls (non-inoculated GF-305 peach seedlings or *Nicotiana benthamiana*) were systematically included.

2.3. Immunocapture-PCR (IC-PCR)

Clarified plant extract, 100 μl (centrifuged 5 min at 13 000 rpm), were submitted to an immunocapture undertaken directly in the tubes used for the reverse transcriptase PCR (RT-PCR) reaction as described previously (Wetzel et al., 1992). The coating of the immunocapture tubes was carried out using 2 μg/ml of immunoglobulins purified from a rabbit polyclonal antiserum (W1001). The one-step RT-PCR protocol described by Candresse et al., 1995 was used. Briefly, 25 μl of RT-PCR mix 10 mM Tris–HCl pH 8.8, 50 mM KCl, 1.5 mM·$MgCl_2$, 0.3% Triton X-100 (w/v), 1 μM of each primer, 250 μM dNTPs, 0.25 units of AMV-RT (Boehringer Mannheim) and 0.5 units of *Taq* DNA polymerase (Boehringer Mannheim) were added directly to all washed capture tubes. The cDNA synthesis and amplification were carried out in a

TECHNE PHC3 cycler at 42°C for 45 min followed by a denaturation phase at 92°C for 2 min and 40 cycles of amplification (92°C for 30 s, 62°C for 30 s and 72°C for 1 min). The PPV-specific primers used were as described previously (Wetzel et al., 1991). Following amplification, 10 μl of PCR products were analysed by 3% agarose gel electrophoresis in TBE buffer, stained by ethidium bromide and finally visualised under UV light. In most cases, the preliminary capture phase of IC-PCR was also used to prepare samples before amplification using the heminested-PCR or the PCR-ELISA procedures.

2.4. Heminested-PCR (H-PCR)

For the first amplification, two modifications of the RT-PCR process (Wetzel et al., 1992) were introduced: only 0.1 μM of each external primers (P1, P2) were used and 30 cycles of the thermal cycling scheme described above were performed. For the second amplification, 0.5 μl of the products obtained during the first round of amplification were added to the reaction mix 10 mM Tris–HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM of external primer P1, 0.5 μM of internal primer (nested primer) PD (5′CTTCAACGACACCCGTACGG3′) or PM (5′′CTTCAACAACGCCTGTGCGT3′), 250 mM dNTPs and 0.5 U of Taq polymerase in a final volume of 25 μl. The amplification process began with a denaturation phase of 5 min at 92°C, followed by 35 cycles of amplification, with a temperature profile of 30 s at 92°C, 30 s at 62°C and 1 min at 72°C. PCR products were analysed as described above. In some cases, the samples preparation before amplification was carried out following the print-capture PCR (Olmos et al., 1996) protocol, avoiding the extract preparation.

2.5. PCR-ELISA

The RT-PCR products were labelled with digoxigenin during the amplification process. For this purpose, the dNTPs mixture was modified to include: 250 μM dATP, 250 μM dCTP, 250 μM dGTP, 237.5 μM dTTP and 12.5 μM DIG-dUTP (Boehringer Mannheim). A one-step RT-PCR was carried out as described above. The amplification products were analysed using the PCR-ELISA detection kit supplied by Boehringer Mannheim, according to the protocol supplied with the kit. The 5-biotinylated PPV-D specific capture probe was 5′CCGTACGGGTGTCGTTGAAGTC3′ and the probe-target hybridisation temperature was fixed at 53°C.

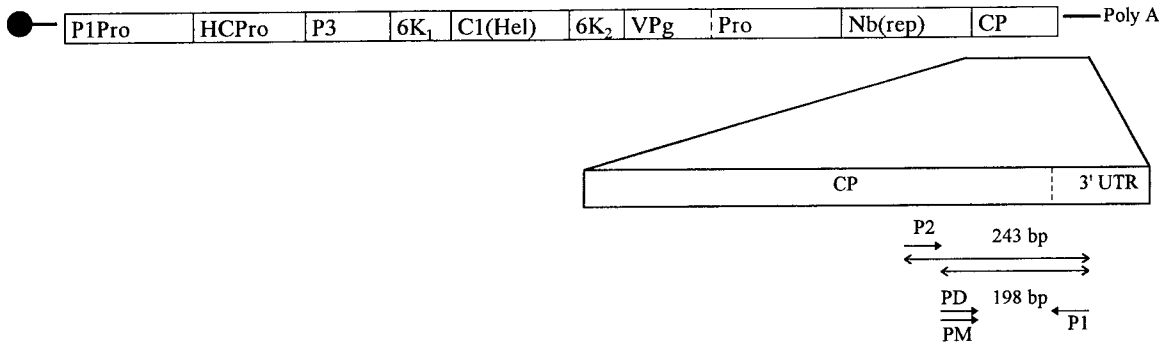
2.6. Heminested-Spot capture-PCR (H-SC-PCR) analysis of individual aphids

Adult wingless *Aphis gossypii* fed for 2–5, 15–20 or 60 min on symptomatic detached leaves from GF305 peach seedlings infected with PPV-D isolate Canino or fed on non-inoculated GF305, were individually squashed on Whatman 3 MM paper. The round bottom of plastic Eppendorf tubes was used to facilitate the complete disruption of each aphid. The membranes with aphid-squashes were stored at 4°C until use. Similarly *A. gossypii* fed for 2 h on GF305 peach seedlings infected with various PPV isolates (belonging to D or M serotype) or fed on non-inoculated GF305 were individually squashed as indicated previously. The spots from individual aphids were then used for PPV detection according to the PC-PCR/SC-PCR protocol described by Olmos et al., 1996 followed by a second round of amplification using the heminested-PCR protocol described above. Amplification products were analysed in parallel after the first and second rounds of amplification.

3. Results

3.1. Optimisation and evaluation of Heminested-PCR and PCR-ELISA for the detection and typing of PPV isolates

The external primers used were able to amplify all PPV isolates tested. In order to optimise the heminested-PCR amplification reactions, various parameters such as the annealing temperature (55, 60, 62 and 65°C) and number of cycles (20, 25, 30, 35 and 40) were evaluated using PPV-infected GF305 peach seedlings as targets. For the first

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                Capture probe 3'ctgaagttgCtgTggGcaTgcC5'-Biotin
                              ::::::::::::::::::::
                           PD cttcaacGacAccCgtAcgG
     9340                     ::::::::::::::::::::                    9583
PPV-D cagactacagcctcgccagataT/18bp/atgacttcaacGacAccCgtAcgGgca/153bp/aggggagtgtagtggtctcggt PPV-D
      T  D  Y  S  L  A  R  Y /6 aa/ M  T  S  T  T  P  V  R  A /51 aa/ R  G  V  *
PPV-M cagactacagcctcgccagataC/18bp/atgacttcaacAacGccTgtGcgTgca/153bp/aggggagtgtagtggtctcggt PPV-M
      ::::::::::::::::::::    ::::::::::::::::::::          ::::::::::::::::::::
   P2 CAGACTACAGCCTCGCCAGA>    PM cttcaacAacGccTgtGcgT>    <CCCTCACATCACCAGAGCCA P1
```

Fig. 1. Position on the PPV genome of the various PCR primers used in this study. The sequence of the relevant PPV-D serotype consensus genomic region is shown with its numbering according to the complete genomic sequence. The corresponding region of PPV-M consensus is given, together with the amino acid sequence encoded. The sequence of the primers is shown in upper case. P1 and P2 are the conserved sequence external primers. PD and PM are the serotype-specific internal primers used in the heminested-PCR procedure. The sequence of the PPV D-specific capture probe used in the ELISA-PCR procedure is also shown. Nucleotides showing consensual divergence between the two serotypes are highlighted in bold.

round of amplification using the PPV-specific external primers the best results were obtained with an annealing step at 62°C and 30 cycles of amplification. For the second round of amplification, using one external primer and one serotype-specific internal primer, these parameters were found optimal at 62°C and 35 cycles, respectively (results not shown). The size of the amplified fragments was respectively, 243 and 198 bp for the first and second round of amplification (Fig. 1).

Post-PCR ELISA detection of digoxigenin-labelled PCR products obtained using the PPV-specific primers was possible according to the instructions of the kit supplier (Boehringer Mannheim). A PPV-D specific capture probe was designed to hybridise to the same variable region targeted by the serotype specific primers used in the H-PCR system (Fig. 1). The temperature of the hybridisation reaction between the capture probe and the labelled PCR products was optimised by systematically varying it in two degree increments in the 37–55°C range. The best results were obtained at 53°C, a temperature at which efficient detection of the homologous targets was still observed while eliminating completely reactions with targets belonging to the heterologous serotype (Table 1).

H-PCR and ELISA-PCR were then compared with serotype-specific monoclonal antibodies used in an ELISA-DASI system for the typing of ten PPV isolates. As shown in Table 1 and in Fig. 2, both nucleic acids based techniques provided unambiguous typing results which, in addition, showed a perfect correlation with the serological typing. In the case of H-PCR, Fig. 2 shows that clear bands were obtained for all isolates using the homologous internal primer while no cross-amplifications could be detected. For ELISA-PCR, all D serotype isolates showed optical densities (OD) of between 3.4 and 2.0, 1 h after substrate addition. In contrast, both negative controls (kit negative, buffer amplification control and healthy plant control) and PPV-M infected samples, had ODs of less than 0.05.

3.2. Comparison of the sensitivity of IC-PCR, H-PCR and ELISA-PCR for the detection of PPV in woody plant samples

Table 2 shows the results obtained using the various techniques. The amplification products obtained us. IC-PCR were detected as a clear band upon agarose gel electrophoresis up to the $1{:}10^3$ (corresponding to a 1: 20 000 plant material weight to grinding buffer volume dilution factor). The H-PCR amplification products were readily detected up to the $1{:}10^5$ dilution (one plant material weight in 2×10^6 grinding buffer volume). Similarly, Table 2 shows that the same dilution of $1{:}10^5$ was also detected readily as positive in the ELISA-PCR assay, irrespective of the time point at which the OD was measured following the addition of the substrate. Both techniques appear therefore to have sensitivities about one 100-fold greater than IC-PCR.

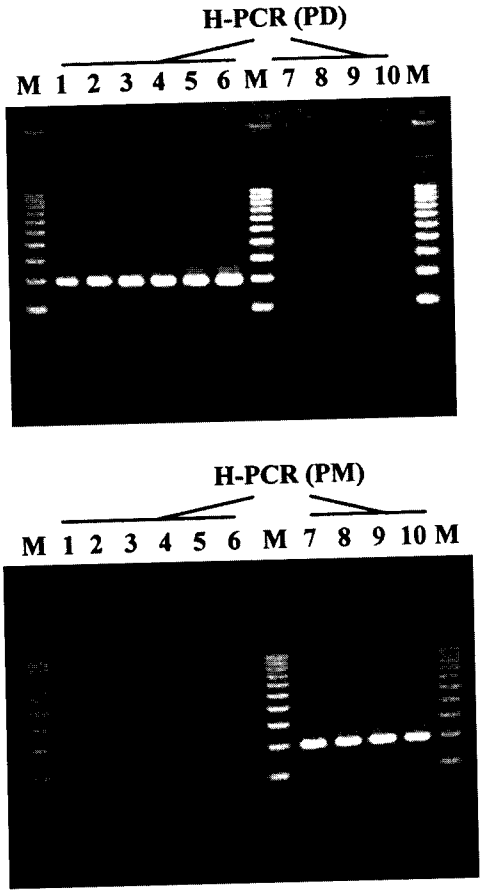

Fig. 2. Typing of PPV isolates using serotype-specific internal primers in the heminested-PCR procedure (H-PCR). H-PCR amplification products (198 bp) obtained from PPV isolates using specific internal primers (D or M). The PPV D isolates (tracks 1–6) were amplified only when the homologous internal primer (PD) was used. Similarly, the PPV M isolates (tracks 7–10) were amplified only using PM as internal primer. Tracks M, 100 bp DNA Ladder molecular marker from Advanced Biotechnologies Ltd. Tracks 1–10, PPV isolates from IVIA collection, RB3.3, NAT-FL, W731-739, Turquía, Dideron Canino, Des-AT, Ps, Ms89, O-Grecia, Sutic Forte, respectively.

3.3. Typing of PPV-infected field samples using H-PCR and ELISA-PCR

The results summarised in Table 3 show a perfect correlation between the various techniques for both PPV detection and serotype assignment, with a single exception. Four Japanese plum samples from the region of Valencia (Spain) gave a negative reaction with the 4DG5 D-specific monoclonal antibody while reacting positively by the D-specific H-PCR assay. These four samples were found negative with the AL M-specific monoclonal and later confirmed negative in a M-specific H-PCR assay (result not shown), thus, demonstrating that they do not correspond to the M serotype. These results indicate that the H-PCR technique can be used to routinely screen field samples for PPV infection with the added advantage of allowing simultaneous typing of the isolates being detected. The results also indicate that the M serotype of PPV appears so far to be absent from the Spanish and Chilean orchards.

3.4. Detection of PPV targets in viruliferous aphids using Heminested-Spot Capture-PCR

PPV targets from isolate PPV-D Dideron Canino were detected by H-SC-PCR after 2–5 min acquisition period in three of 40 assayed individuals *A. gossypii* (7.5% amplified from the total of aphids assayed). After 15-20 min and after 60 min acquisition period, PPV PCR targets were amplified in 4/40 (10%) and in 8/40 (20%),

Table 2
Comparison of the respective sensitivities of ELISA-PCR, heminested PCR (H-PCR) and immunocapture-PCR (IC-PCR) for the detection of plum pox virus in serial dilutions of infected GF305 peach seedlings

	Dilutions[a]					Controls[b]			
	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	a	b	c	d
OD 15'[c]	0.91	0.82	0.81	0.87	0.04	0.04	0.05	0.03	0.04
OD 30'[c]	1.85	1.73	1.80	1.82	0.04	0.04	0.05	0.04	0.04
OD 45'[c]	2.52	2.37	2.38	2.36	0.05	0.04	0.05	0.04	0.04
H-PCR	+	+	+	+	−	[d]	−	[d]	−
IC-PCR	+	+	−	−	−	[d]	−	[d]	−

Plus signs indicate the presence of an amplified material band visible after ethidium bromide staining following agarose gel electrophoresis. Minus signs indicate the absence of detectable amplified material.
[a] Serial ten-fold dilutions of PPV infected GF305 peach extracts were prepared in healthy apricot extract. Dilution 1:1 corresponds to one weight of plant sample homogenized in 20 volumes of grinding buffer.
[b] Controls: (a) distilled water assayed in the ELISA detection system; (b) distilled water used as negative control of the amplification procedure; (c) extract of PPV infected apricot, amplificated without DIG-dUTP; (d) extract of healthy GF 305 peach seedlings used as negative control in the amplification procedure.
[c] OD x' indicate optical densities at 405 nm × min after the addition of substrate in the ELISA-PCR assay. The values given are the average of two wells.
[d] Not tested.

respectively, individual aphids from the total assayed.

Table 4 shows the results of four different experiments in which *Aphis gossypii* were allowed to feed for 2 h on the leaves of PPV-infected or of control, non inoculated GF305 peach seedlings. Gel electrophoresis analysis revealed a PPV-specific amplification product in some of the insects analysed by SC-PCR (18 out of 55 aphids tested), but not in control aphids which had been allowed to feed on healthy control plants. A second round of heminested-PCR was then performed on the amplification products derived from the SC-PCR procedure. In this way, H-SC-PCR allowed the detection of the virus in a larger number of the same aphid extracts (31 out of 55, see Table 4). Again, no signal was obtained from the control aphids which had been kept on healthy plants (Fig. 3). No differences were observed between the final percentage of PPV-D or M targets (58.3 and 54.8%, respectively) amplified by H-SC-PCR from the total of aphids assayed, while by SC-PCR differences were observed (50.0% and 19.3%, respectively for PPV-D or M targets).

4. Discussion

A heminested-PCR (H-PCR) protocol and serotype-specific primers were developed which allow the simultaneous detection and typing of plum pox virus isolates. In this protocol, all PPV isolates are amplified during the first round of PCR due to the use of broad specificity detection primers (Wetzel et al., 1991; Candresse et al., 1994 and 1995). During the second round of heminested amplification, one of the external primers is combined with one of two possible serotype-specific internal primers. Although the internal primers were initially designed to use in single round, serotype specific PCR (Candresse et al., 1995), they appear to function equally effectively in H-PCR. The two serotype specific primers target a region showing a cluster of five highly conserved mutations discriminating the two main serotypes of PPV (see Fig. 1). When annealed to an heterologous isolate, each 20-mer internal primer will show five closely spaced mismatches (positions 1, 4, 7, 10 and 13 starting from the primers 3' end, see Fig. 1). In particular, this configuration includes a potentially mismatched

Table 3
Comparison of the detection and typing of PPV isolates using either ELISA-DASI with a general (5B) or with PPV-D (4DG5) or PPV-M (AL) specific monoclonal antibodies or a PPV-D specific heminested PCR (H-PCR) procedure

Host	Country	ELISA			D-specific
		5B	4DG5	AL	H-PCR
Peach	Spain	131	131/131	0/131	131/131
	Chile	6	6/6	0/6	6/6
Nectarine	Spain	25	25/25	0/25	25/25
	Chile	nt	nt	nt	nt
Apricot	Spain	10	10/10	0/10	10/10
	Chile	4	4/4	0/4	4/4
Plum	Spain	6	6/6	0/6	6/6
	Chile	nt	nt	nt	nt
Japanese plum	Spain	34	30/34	0/34	34/34
	Chile	12	12/12	0/12	12/12
Total		228	224/228	0/228	228/228

The host plant and country of origin of the various samples are indicated, together with the number of samples testing positive on the number of samples assayed.
nt, not tested.

nucleotide at the 3′ end of the primer, which should insure optimal discrimination between the two serotypes (Kwok et al., 1990), as indeed was observed. Although the region targeted by the PCR procedure (C-terminal region of the coat protein gene) and the immunodominant region of the virus responsible for serotype determination (N-terminal region of the CP, Candresse et al., manuscript in preparation) are physically separated, the correlation between the two typing technique appears to be excellent (only four out of 228 field samples showing an unexplained discrepancy). The possibility that some PPV isolates may behave differently in the two assays, either as the result of mutations or as the result of recombination (Revers et al., 1996) should however be kept in mind.

In parallel to the H-PCR procedure, a post-PCR ELISA procedure was developed allowing both the detection and the typing of an amplified product labelled with digoxigenin during synthesis. The results obtained clearly demonstrate that both H-PCR and ELISA-PCR can be used to discriminate between the D and M serotypes of PPV in both herbaceous (*N. benthamiana*) and woody (GF305 peach seedlings) plant samples. In this case, only a PPV-D specific capture probe has been designed so far. The clear results obtained in H-PCR using the PPV-M specific primer indicate, however, that the development of a PPV-M specific capture probe should pose no major challenge. Evaluation of the sensitivity of the H-PCR and ELISA-PCR procedures indicate that both techniques are approximately 100-fold more sensitive than the current most sensitive assay, immunocapture-PCR (Wetzel et al., 1992; Candresse et al., 1994). Given the known limit of sensitivity of IC-PCR, this would translate in a detection limit of about 20 viral particles per assay for both the H-PCR and ELISA-PCR tests. The two techniques developed have complementary advantages: while ELISA-PCR offers the possibility to automatise post-PCR treatment of samples and to eliminate the need for gel electrophoresis and consequently the use of ethidium bromide, H-PCR is cheaper and, depending on the circumstances, it may be possible to carry out the second round of serotype-specific amplification only on a limited number of samples shown to be infected after the first amplification. In order to limit the impact of contamination, H-PC-PCR using direct tissue blotting in paper before amplification (Olmos et al., 1996) can be successfully used. It may also be possible, in the future, to modify the

Table 4
Detection of PPV-PCR targets in individual aphids (*A. gossypii*) by heminested-print capture-PCR (H-PC-PCR)

	Source plant	1st. round H-PC-PCR	H-PC-PCR
Exp. No. 1	PPV-RB (D)	5/12	7/12
	Healthy	0/6	0/6
Exp. No. 2	PPV-3.3 RB/GF (D)	7/12	7/12
	Healthy	0/6	0/6
Exp No. 3	PPV-Ms79 (M)	4/12	9/12
	Healthy	0/6	0/6
Exp. No. 4	PPV-Cont.RB (M)	2/19	8/19
	Healthy	0/9	0/9

The results of four different experiments using different strains of PPV are given. The aphids were allowed to feed on PPV-infected or on healthy GF305 peach seedlings two hours before being processed by H-PC-PCR. A gel analysis was performed to assess the results of the first round of PCR amplification before proceeding with a second round of H-PC-PCR. The numbers given are the number of aphids giving positive amplifications signals (amplified DNA band detectable after ethidium bromide staining) divided by the total number of aphids tested.
The serotype of the PPV isolate used is indicated in parentheses.

internal primers in order to conduct single-tube, single- mix nested PCR without having to open the tube again (Ulrich et al., 1993).

When considering diagnostic applications on plant samples and, in particular, woody plants, pre-PCR sample preparation should be considered carefully. It is well known that plants are frequently rich in inhibitors of the RT or PCR reactions (Candresse et al., 1997). Our results indicate that both the H-PCR and the ELISA-PCR are compatible with two efficient and very simple sample preparation techniques, immunocapture (Wetzel et al., 1992) and print-capture or spot-capture (Olmos et al., 1996), further increasing their range of application to routine diagnosis. The results obtained during the indexing of over 200 field samples of unknown status are further evidence of the reliability of these techniques and also demonstrate the absence of PPV-M isolates from Spanish and Chilean orchards so far. The methods described here are two powerful tools which should find applications in PPV epidemiological studies and in PPV eradication or control efforts.

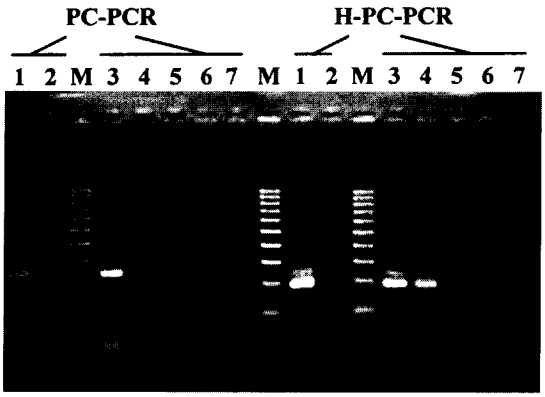

Fig. 3. Detection of PPV in *A. gossypii* using the print-capture PCR (PC-PCR) and heminested PCR (H-PCR) procedures. PC-PCR amplification products (243 bp) and H-PC-PCR products (198 bp) obtained from the same samples after a second round of amplification. PPV-infected GF305 peach seedlings (track 1), sample from healthy GF305 peach seedlings (track 2), *A. gossypii* wingless individuals fed on donor PPV-infected plant (tracks 3, 4 and 5), and *A. gossypii* individuals fed on GF305 healthy plant (tracks 6 and 7). Tracks M, 100 bp DNA Ladder molecular marker from Advanced Biotechnologies Ltd.

Several reports have demonstrated the feasibility of detecting persistently or semi- persistently transmitted viruses in their aphid vectors using PCR assays (López-Moya et al., 1992; Hadidi et al., 1993; Singh et al., 1995). More recently Singh et al. (1996), detected potato virus Y (PVY) in extract of single aphids. Given the increase in sensitivity afforded by H-PCR we decided to evaluate the possibility of PCR-based detection and typing of PPV in *A. gossypii* following the spot capture-PCR system (Olmos et al., 1996) directly from aphids squashed in paper.

Non-persistently transmitted virus can be acquired by the aphids in short feedings of a few minutes (Shukla et al., 1994). However, our results show that longer acquisition periods increased the number of aphids where PCR-targets were amplified. For this reason we used a 2 h acquisition period, for our other assays.

The detection of PPV amplifiable material in wingless individuals of the aphid vector *A. gossypii* raises several questions. Although H-SC-PCR clearly allowed a larger number of aphids to be detected as PPV positive (in 56.3% of the assayed individual aphids), amplification signals were also obtained after a single round of spot-capture PCR (in 32.7% of the assayed aphids). These results are in contrast to the fact that *A. gossypii* is thought to be a poorly efficient vector of PPV (Avinent et al., 1994; Labonne et al., 1995). It should also be stressed that, so far, we have no information on a potential correlation between the ability of individual aphids to transmit PPV and their PCR status. *A. gossypii* being a rather poor vector, it is highly unlikely that all the PCR-positive aphids would have transmitted PPV to test plants if given an opportunity. The availability of non-aphid transmissible isolates of PPV (Maiss et al., 1989) offers the opportunity to use the techniques described here to further our understanding of the relationships between PPV and its insect vectors. Also the success in amplifying PPV PCR targets from pre-squashed aphids opens new possibilities for epidemiological studies.

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