ELSEVIER

Journal of Virological Methods 92 (2001) 165–175

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Integrated RT–PCR/nested PCR diagnosis for differentiating between subgroups of plum pox virus

Marianna Szemes [a], Miklós Kálmán [a], Arben Myrta [c], Donato Boscia [d], Mária Németh [b], Mária Kölber [b], László Dorgai [a,*]

[a] *Bay Zoltán Institute for Biotechnology, Derkovits Fasor 2, H-6726 Szeged, Hungary*
[b] *Plant Health and Soil Conservation Station, Budaörsi út 141-145, H-1118 Budapest, Hungary*
[c] *Istituto Agronomico Mediterraneo, Via Ceglie 9, I-70010 Valenzano BA, Italy*
[d] *Dipartimento di Protezione delle Piante e Microbiologia Applicata, Universita degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola, 165/A, I-70126 Bari, Italy*

Received 28 October 2000; received in revised form 8 November 2000; accepted 9 November 2000

Abstract

An RT–PCR/nested PCR technique was developed for the simultaneous detection and typing of *plum pox virus* (PPV) and its major types — Dideron (D), Marcus (M), El-Amar (EA) and Cherry (C). Degenerated oligonucleotides were synthesized for the general detection of PPV, flanking the coding sequence for the N-terminal portion of the coat protein (CP), within which strain-specific differences were identified. On the basis of these characteristic differences, degenerated primer pairs were designed to differentiate between the four major subgroups of the virus in nested PCR reactions. The validity of the technique was tested on viral strains and cloned cDNAs overlapping the CP region. High specificity was observed with no detectable cross-reactions. The results of general PPV detection with the new primers and those of the PCR-based detection of the 3′ non-coding region of the viral genome correlated with complete coincidence. The PCR typing results correlated well with those of the RsaI-RFLP and serological typing and revealed a surprisingly high incidence of PPV-D in Hungary.

Keywords: *Plum pox virus* (PPV); PCR; Nested PCR; Differential diagnosis

1. Introduction

Plum pox virus (PPV) is one of the most important viruses causing the disease known as sharka (for reviews, see Németh, 1986; Dunez et al. 1994; Németh and Kölber, 1994; Roy and Smith, 1994; Simón et al., 1997; Lopez-Moya et al., 2000). Among its hosts are a number of economically important stone fruit species — plum, apricot, peach, almond and sour cherry. PPV was reported recently to infect also sweet cherry and walnut (Topchiiska, 1991; Crescenzi et al. 1995; Baumgartnerova, 1996). On the basis of the symptoms,

* Corresponding author. Tel.: +36-62-432252; fax: +36-62-432250.
E-mail address: dorgai@bay.u-szeged.hu (L. Dorgai).

0166-0934/01/$ - see front matter
PII: S0166-0934(00)00284-6

serological and epidemiological data and comparisons at protein and nucleic acid levels, four major types of the virus have been identified. Strains Dideron (D) and Marcus (M), originally described by Kerlan and Dunez (1979), are the most important from an economic point of view. PPV-D occurs in Western Europe and several Mediterranean areas, while type PPV-M has been reported mostly from Central, Eastern and South-Eastern Europe (Pasquini and Barba, 1997; Myrta et al. 1998a). Strain E1 Amar is represented so far by a few isolates found only in Egypt (Dunez, 1988; Myrta et al. 1998a; Wetzel et al. 1991b). The fourth strain, infecting sour cherry (PPV-C) was first found in Moldova (Kalashyan and Bilkey, 1989; Kalashyan et al. 1994) and was characterized by Nemchinov et al. (1996). The geographical distribution is not absolute, the domains of PPV-D, M and C strains are increasingly overlapping. PPV has also been found in Chile (Roy and Smith, 1994), India (Thakur et al., 1994) and very recently, the USA. Since PPV is considered to be a quarantine pathogen by IAPSC, NAPPO and EPPO (Roy and Smith, 1994), substantial efforts are being made to control the spread of the disease by means of certification schemes and eradication.

It is of primary importance to ensure the virus-free state of the propagating materials and to prevent the further spread of PPV through international exchange and commerce. One tool needed to achieve this goal is a reliable, rapid and sensitive detection method for the effective identification of PPV strains in biological samples. Substantial efforts have been made to type and characterize different virus isolates, and methods have been developed both for the general detection of PPV and for the differentiation of its strains. These include biological tests, enzyme-linked immunosorbent assays (ELISA) and, more recently, nucleic acid-based molecular assays. ELISA is widely used in routine diagnosis; polyclonal antisera and monoclonal antibodies provide sufficient specificity for practical applications. However, the uneven distribution of the virus in the host tissues and the seasonal variation of its titer pose serious problems for reliable detection throughout the year. To solve this problem, the polymerase chain reaction (PCR), which offers great sensitivity, was first adapted for PPV detection by Wetzel et al. (1991a). Since then, a number of PCR-based protocols have been developed both for the general detection of the virus and for the specific detection of each PPV type. To differentiate between the subgroups of the virus, either specific primers have been used or PCR has been coupled to serological or DNA analytical techniques (Wetzel et al., 1991a; Candresse et al., 1995; Olmos et al., 1997; Poggi-Pollini et al., 1997; Hammond et al., 1998). Immunocapture-PCR, hybridization of strain-specific probes to the PCR product and restriction fragment length polymorphism (RFLP) analysis of the amplified products allow reliable differentiation. These methods, however, cannot be used readily for simultaneous detection of PPV and typing of all four strains as they apply different diagnostic methods and make use of different parts of the viral genome. In some cases, time and labor-intensive processing of the PCR products is also needed.

In this study, the development of a set of degenerated PCR primers to be used for the detection of PPV and its four major strains is described. The procedure involves only the simple and very sensitive technology of PCR and it might, therefore, afford practical advantages over the existing protocols. The first RT–PCR reaction detects PPV and a set of four nested reactions carried out on the amplified product of the first reaction allows differentiation between the four known PPV types.

2. Materials and methods

2.1. Origin of plasmids, PPV strains and isolates

Plasmids carrying cloned cDNAs overlapping the coat protein (CP) coding region of PPV-M and PPV-C were kindly provided by Dr L. Palkovics (Palkovics et al., 1993) and Dr L. Nemchinov (Nemchinov et al., 1996), respectively. PPV-EA, in the form of symptomatic GF 305 leaves, was a gift from Dr T. Candresse. PPV-D

originated from Dr M. Barbra and was maintained on GF 305 under greenhouse conditions. All other PPV isolates were from the strain collection of the Plant Health and Soil Conservation Station of the Ministry of Agriculture, Budapest, Hungary. The PPV isolates analyzed in this work were collected from symptomatic trees in 21 orchards in distinct growing regions in Hungary between 1966 and 1995, and were maintained on GF 305 peach indicators under field conditions. Seven isolates from Germany (SK21, SK28 and SK80), France (SK25 and SK26), the Czhech Republic (SK1) and Slovakia (SK190), maintained on GF 305 under vector proof greenhouse conditions, were also used in the experiments.

2.2. RNA preparations

Total RNA was extracted from 100 mg of fresh leaves or an equivalent amount of lyophilized leaves as described by Spiegel et al. (1996) with minor modifications. The tissue was pulverized in liquid nitrogen and mixed with 1 ml of homogenization buffer (200 mM Tris–HCl, pH 8.5; 1.5% SDS; 300 mM LiCl; 10 mM EDTA; 1% Na deoxycholate; 1% Nonidet P 40; 0.5% v/v mercaptoethanol added before use; all solutions were made with DEPC-treated water.) The homogenate was centrifuged (all centrifugations were performed at 4°C, 12 000 rpm, for 15 min) and the supernatant was mixed with an equal volume of 5 M potassium acetate, pH 6.0. After incubation for 15 min on ice, the suspension was centrifuged. The supernatant was transferred to a new Eppendorf tube and the RNA was precipitated with an equal volume of isopropanol (1 h at −20°C). The precipitate was collected by centrifugation and washed with 1 ml of 70% aqueous ethanol on ice. The pellet was dried under vacuum and dissolved in 30 μl of DEPC-treated sterile water.

2.3. Primers

For the general detection of PPV, a 1:1 mixture of the two 5′ primers TCCAACRTTGTTRTRCACCA (M3-5′) and TCCAAYRTAGTKGTSCATCA (M4-5′) was used with the 3′ primer CGYYTRACTCCTTCATACCA (M2-3′). In consequence of the size variation of the known PPV-CP sequences in this region, the expected size of the amplified product was in the range 465–516 bp. For the differentiating reactions, the PPV-D specific primers were GCAGCAACTAGCCCAATAMT (M1-5′) and TGTTCCAAAAGTTTGCRRTTGAGGT (M5-3′); the expected size of the product was 159 bp. The PPV-M specific primer pair, GYGGCAACRACTCAACCAG (M6-5′) and CCTTCCTGYRTTCACCAAAGT (M7-3′), amplifies a 207 bp product. Primers TAGTCACCACTACACAGCAG (M8-5′) and AGGAGGTGTAGTAGTTGTTG (M9-3′) are PPV-EA specific, while GGGAAATGATGACGACGTAACTCT (M10-5′) and CAATTACCCCATACGAGAAT (M11-3′) are specific for PPV-C and amplify a 167 and a 224 bp product, respectively.

For methodological comparison, PPV was also detected by PCR, using the sense GTCTCTTGCACAAGAACTATAACC and antisense GTAGTGGTCTCGGTATCTATCATA primers, which are complementary to the 3′ non-coding region of the virus (Hadidi and Levy, 1994). The antisense primer was also used for cDNA synthesis and for amplification of a segment of the PPV genome for RsaI-RFLP analysis, with primer AGACTACAGCCTCGCCAGAT (Koli-396).

2.4. Reverse transcription and PCR conditions

cDNA synthesis was carried out in a reaction mixture (25 μl final volume) containing 2 μl of freshly prepared RNA for template; 1 pmol of M2 or antisense primer; 20 U of M-MuLV reverse transcriptase (Gibco, Fermentas); 100 μM of each dNTP; 1 mM DTT; 50 mM Tris–HCl, pH 8.3; 75 mM KCl and 6 mM $MgCl_2$. The reaction mixture was incubated at 37°C for 1 h and stored at −20°C.

PPV-specific products were amplified by PCR, using a PTC-100 thermal controller (MJ Research, Inc.) in a 25 μl volume containing 5 μl of cDNA; 100 pmol of each general primer (M2 and M3/M4); 100 μM of each dNTP; 1 U of Taq polymerase; 10 mM Tris–HCl, pH 9.0; 50 mM KCl; 1 mM $MgCl_2$ and 0.1% Triton X-100. Denaturation at 93°C for 3 min was followed by 30

cycles of 30 s at 93°C; 30 s at 55°C and 1 min at 72°C, with a final extension step at 72°C for 5 min. Conditions for the amplification of strain-specific products were the same, except that 100 pmol of specific primers and 1 μl of the 1000-fold diluted first PCR reaction mixture for template were used.

2.5. DASI–ELISA with strain-specific monoclonal antibodies

PPV strains were identified by DASI–ELISA (Cambra et al., 1994), using strain-specific monoclonal antibodies (MAbs). The following MAbs were used — MAbAL specific to PPV-M (Boscia et al., 1997), MAbEA24 specific to PPV-EA (Myrta et al., 1998b), MAbAC and TUV specific to PPV-C (Myrta et al., 2000), MAb4DG5 specific to PPV-D, and the universal MAb5B (Cambra et al., 1994). Infected GF305 leaves were ground in PBS-Tween extraction buffer (1/10 w/v) and two plate wells were filled with plant sap from each sample. ELISA reactions were read with a Titertek Multiskan photometer at 405 nm. Positive samples were those that gave a reading (average of two wells) at least three times higher than that for the healthy control in 1 h.

2.6. Reagents and nucleic acid-related techniques

PCR products or restriction fragments were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining, and images were documented with a gel analysis system (GDS-7600, UVP). RsaI was purchased from Fermentas, while Taq DNA polymerase was from Zenon Biotechnology Ltd., Szeged, Hungary; these enzymes were used as recommended by the manufacturers. All other reagents were purchased from commercial sources and were used without further manipulation.

Oligonucleotides were synthesized on a Cruachem model PS250 synthesizer, using phosphoramidite chemistry, and were purified by denaturing polyacrylamide gel-electrophoresis (10% 19:1 acrylamide:bisacrylamide, 7 M urea).

The amino acid and the nucleic acid sequences were compared by multiple alignment, using the Pileup program of the GCG Sequence Analysis Software Package (Devereux et al., 1984 and the Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711). The accession numbers for the sequences as numbered in Fig. 1 are — 1: aj000340; 2: u27652; 3: x81074; 4: x81083; 5: x81080; 6: x57975; 7: x81077; 8: x81081; 9: x81079; 10: x81078; 11: x81076; 12: x81075; 13: x81084; 14: x16415; 15: x81073; 16: d00424; 17: m21847; 18: l42473; 19: l42472; 20: x57976; 21: x81082; 22: s57404; 23: s57405; 24: x56759; 25: x97398; and 26: x56258.

3. Results and discussion

3.1. Primer design

The N and C-terminal ends of the coat proteins of potyviruses are located on the surface and carry the major antigenic determinants (Shukla et al., 1988). A majority of the PPV-CP epitopes are located in the variable N-terminal region (Candresse et al., 1998b), and synthetic peptides overlapping this region were used to develop monoclonal antibodies for differential diagnosis (Shukla et al., 1988; Crescenzi et al., 1998). The sequences of strain-specific epitopes must be mirrored at a nucleic acid level and we, therefore, attempted to identify blocks of the CP coding regions that are characteristic for the viral subgroups. Twenty-four full-length and two partial PPV CP sequences, obtained from GenBank, were analyzed by multiple alignment. As expected, a majority of the sequence differences were found in the amino-terminal regions of the coat proteins, spanning approximately the first 100 amino acids. A substantial portion of these differences were clustered and found to be characteristic for the serologically characterized PPV-D and M types, respectively, (Fig. 1). The CP sequences of viruses that had not been characterized serologically were included in the PPV-D or M groups according to the presence or absence of the RsaI restriction site used to characterize the PPV D and M types by

RFLP (Wetzel et al., 1991a). These latter sequences also fit nicely into the PPV-D and M groups as concerns other features of the CP sequence including the group-specific differences

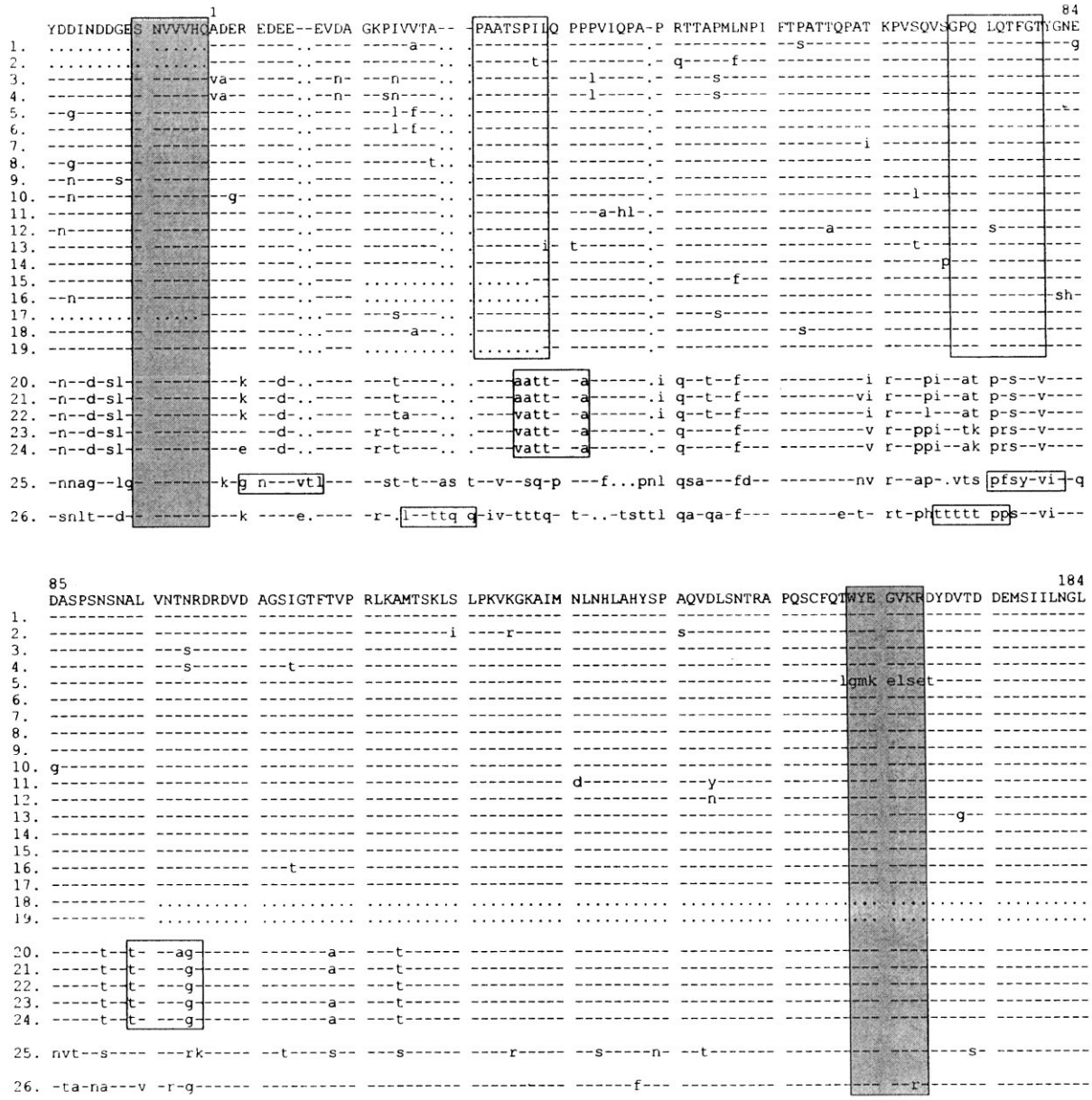

Fig. 1. Alignment of the N-terminal part of the PPV coat protein sequences. The positions of general primers are labeled by shaded boxes and those of the strain-specific primers by open boxes. We classified sequences 1–19 as PPV-D and sequences 20–24 as PPV-M types. Sequence 25 is PPV-C and sequence 26 is PPV-EA. Only differences from the artificial consensus sequence (top line) generated by Pretty are shown; periods stand for deletions or unknown sequences. The completely different amino acid pattern of sequence 5, overlapping with the position of the 3′ general primer, is due to a frameshift mutation; it is similar to other PPV sequences at a nucleic acid level.

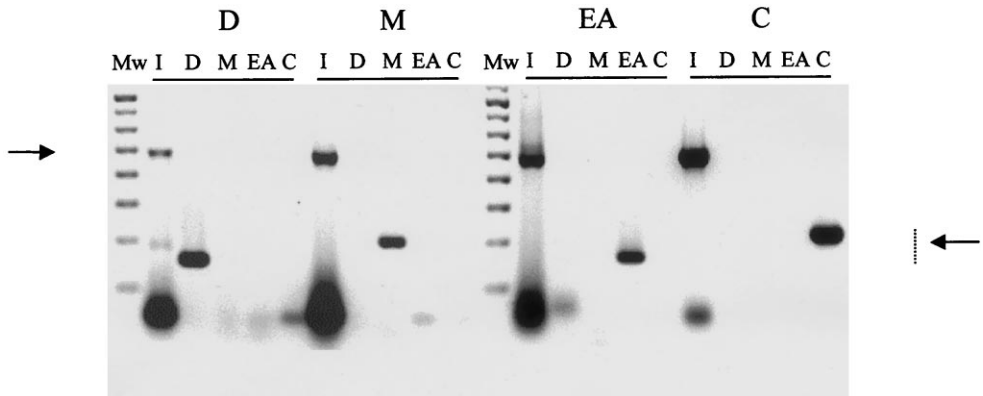

Fig. 2. Detection and typing of PPV with the primers described in this paper. General PCR detection (I) was carried out on PPV-D, PPV-M, PPV-EA and PPV-C-specific templates, followed by all four strain-specific nested reactions on the 1000-fold diluted product of each first reaction. The arrows on the left and right point to the expected products of the general and strain-specific PCR reactions, respectively.

mentioned above. As expected, the primary structures of the PPV-C and EA types differed substantially from each other and from those of the other two groups. In addition to the group-specific differences, other sequence changes were scattered along the whole CP sequence in a non group-specific manner. Again, most of these were found in the amino-terminal region, suggesting that conservation of the C-terminal two-thirds of the coat protein is important for the function.

Regions of group-specific differences between the PPV-D and M types (boxed in Fig. 1) were selected, and oligonucleotides for differentiating primers were synthesized accordingly. When necessary, the oligonucleotides were degenerated so as to be complementary to all known sequences. In order to maximize specificity, the differentiating PCR primers do not necessarily occupy exactly the same positions in the sequences of the four CP types. After analyses of the nucleic acid sequences, the positions of several primers were shifted in order to minimize possible cross-priming. Where possible, we also tried to position the primers so that the amplified products differed in size. We selected two regions for primers for general PPV detection in the viral sequences that flank the region of divergence. The position of the 5′ general primer is immediately upstream of the codon of the first CP amino acid (shaded box in Fig. 1). This region displays moderate differences at a nucleic acid level for the four PPV types. Two sets of degenerated oligonucleotides were, therefore, designed — one is specific for the PPV-D and M types, while the other is specific for the PPV-EA and C types. In the PCR reactions, a 1:1 mixture of the two sets was used. For the position of the 3′ general primer, the first region downstream showing considerable sequence conservation among all known PPV types was selected (pos. 162–168 as numbered in Fig. 1). The primer was mildly degenerated at its 5′ portion so as to accommodate the sequence differences found in the PPV-EA and C types. For the sequences of the primers, see Section 2.3.

3.2. Specificity of the primers

The primers were tested for the ability to detect and differentiate between the known types of PPV. RNAs prepared from PPV-EA and PPV-D-infected plant samples and also plasmids carrying cloned cDNAs overlapping the CP region of the PPV-M and PPV-C types were used as template. In the first PCR or RT–PCR reaction, the products amplified by the general primers had the expected size. The four nested PCR reactions carried out on diluted products of the first PCR reaction resulted in strain-specific products with no detectable cross-reaction (Fig. 2). These results demonstrate that the integrated PCR system functions in a model situation.

In order to test the specificity and sensitivity of the system under field conditions, RNA was prepared from 40 independent plant samples exhibiting symptoms of PPV infection. Two sets of PCR reactions were carried out in parallel. In the first set, the newly developed primers were used. The second set served as a control, in which PPV was detected as described by Levy and Hadidi (1994). The presence of the virus was detected in 33 of the 40 samples. Among the 33 positives, 16 generated detectable products in both sets of primary PCR reactions. In the remaining 17 samples, PPV was detected only with the differentiating nested PCR reactions. The seven samples that failed to produce any PPV-specific PCR products with our primers also gave negative results with the control primer pair. Of the positives, 18 isolates were characterized as PPV-M and seven as PPV-D type with no ambiguity. Eight samples produced both PPV-D and M-characteristic PCR products; coincidentally, one was always a dominant component. PPV-EA and PPV-C strains were not detected in this set of samples. The results indicate that the specificity and sensitivity of our new primers for general PPV detection are comparable to those of the primer pair that detect the 3′ NTR region of PPV. In addition to their primary purpose, the strain-specific nested PCR reactions also increase the sensitivity, as indicated by the 17 samples, where the presence of PPV was not detected in the first set of reactions. It is very unlikely that the appearance of mixed PCR products in the nested PCR reactions is due to the relaxed specificity of our primers, since the differentiating primers worked with great specificity under laboratory conditions, and 75% of the field samples gave a single, characteristic PCR product with no background. It is more likely that these cases represent mixed infections.

3.3. Correlation between PCR, RFLP and serological typing

The next goal was to determine how the results of PCR typing described above compare with those of serological and RsaI-RFLP typing. ELISA is used widely for PPV detection in practice, and monoclonal antibodies are available to differentiate between the four major viral strains. Excellent correlations were observed between the results of serological and PCR typing of the PPV-D and M strains (Candresse et al., 1998a). Moreover, Bousalem et al. (1994) reported that RsaI-RFLP of the PCR products amplified from the C-terminal part of the PPV-CP coding regions differentiated reliably between the PPV-D and M types, and the results correlated well with the findings of serological tests. In order to examine the correlations between the three independent diagnostic approaches, we analyzed 92 samples by PCR (85 isolates collected in Hungary and seven isolates from abroad). Two overlapping subsets of this collection were also analyzed by RsaI-RFLP as described by Wetzel et al. (1991a), and by ELISA (see Section 2.5). The results are summarized in Table 1.

PCR detected 52 PPV-M and 20 PPV-D single and 14 D/M, 5 M/C and one D/M/C mixed infections. The serological analysis indicated 68 isolates belonging to PPV-M and 21 to PPV-D serotypes among the 89 isolates tested. No PPV-C or PPV-EA, or any mixed infection was detected by ELISA. The results of PCR and the serological typing were identical in 68 cases (77.3%), all of them representing single PPV-D or M infections. Consistent results were obtained for 18 samples (20.5%), that is ELISA detected one of the two or three PPV strains indicated by PCR. In a single case (2.2%), the PCR and ELISA results were contradictory. Sample SK185 was characterized by PCR as a PPV-D strain, while ELISA indicated a PPV-M type. This sample was also analyzed by RFLP, the result of which supported the PCR typing. Forty-six of the 92 samples were analyzed by RFLP. In 42 cases (91.3%), the results of PCR and RFLP typing was identical, including 40 single PPV-M or PPV-D infections and two M/D mixed infections. Four more analyses (8.7%) gave consistent results. The overall correlations between the three independent diagnostic approaches were excellent: 100% for PCR/RFLP and 97.8% for both PCR/ELISA and RFLP/ELISA.

In summary, it is concluded that the exclusively PCR-based typing system we have developed is useful not only for the detection of PPV, but also

Table 1
PCR, RFLP and ELISA typing of PPV isolates[a]

isolate code	source species	year of isolation	PCR typing	RsaI RFLP typing	ELISA typing	isolate code	source species	year of isolation	PCR typing	RsaI RFLP typing	ELISA typing
SK1*	plum	1972	M	M	M	SK77	plum	1974	M/(C)	M	M
SK2	plum	1974	M	nt	M	SK80*	plum	1980	(D)/M	nt	M
SK3	peach	1974	M	nt	M	SK82	plum	1973	M	nt	M
SK4	plum	1974	M	nt	M	SK83	myrobalan	1979	(D)/M	nt	M
SK6	apricot	1966	(D)/M	M	M	SK84	myrobalan	1979	M	M	M
SK12	plum	1973	M	M	M	SK91	apricot	1982	(D)/M	nt	M
SK13	plum	1973	M	nt	M	SK181	peach	1986	(D)/M	nt	M
SK18	Prunus sp.	1970	M	nt	M	SK185	peach	1986	D	D	M
SK21*	plum	1971	M	nt	M	SK186	peach	1986	M/(C)	M	M
SK22	peach	1972	M	nt	M	SK188	peach	1986	(D)/M	nt	M
SK23	apricot	1972	M	nt	M	SK190*	plum	1991	D	nt	D
SK25*	apricot	1973	M	nt	M	SK191	plum	1991	M	M	M
SK26*	apricot	1973	(D)/M	nt	M	SK192	apricot	1993	M	M	M
SK28*	plum	1971	D/M	nt	M	SK193	peach	1993	D	D	D
SK33	plum	1986	M	M	M	SK194	peach	1993	(D)/M	Nt	M
SK35	plum	1986	M	nt	M	SK195	peach	1991	D	D	D
SK36	plum	1973	M	M	M	SK196	peach	1991	(D)/M	D/M	nt
SK37	plum	1986	M	M	nt	SK197	peach	1991	(D)/M	D/M	D
SK40	peach	1973	M	M	M	SK198	peach	1991	M	nt	M
SK41	apricot	1971	M	M	M	SK199	peach	1991	M	M	M
SK42	plum	1986	M	M	M	SK201	peach	1991	D	D	D
SK43	apricot	1968	M	M	M	SK203	myrobalan	1991	D	Nt	D
SK44	apricot	1968	M	M	M	SK204	almond	1991	M	M	M
SK45	plum	1986	M	M	M	SK205	w. apricot	1991	M	M	M
SK46	plum	1986	M	M	M	SK206	peach	1991	M	M	M
SK47	plum	1986	M	nt	M	SK207	almond	1991	M	M	M
SK48	greengage	1974	M	nt	M	SK208	apricot	1991	M	M	nt
SK50	plum	1986	M	nt	M	SK209	peach	1991	D/M	Nt	D
SK52	apricot	1978	M/C	nt	M	SK210	peach	1991	D	Nt	D
SK53	apricot	1978	M	nt	M	SK211	peach	1991	D	Nt	D
SK54	peach	1976	(D)/M	nt	M	SK212	apricot	1991	D	Nt	D
SK55	peach	1976	M	nt	M	SK233	peach	1992	D	D	D
SK56	peach	1976	M/C	nt	M	SK236	peach	1992	D	D	D
SK58	peach	1976	M/(C)	M	M	SK237	peach	1994	M	Nt	M
SK59	peach	1976	M	M	M	SK238	peach	1994	D	Nt	D
SK60	plum	1986	M	M	M	SK239	peach	1994	D	D	D
SK61	peach	1975	M	M	M	SK240	peach	1994	D	Nt	D
SK64	apricot	1979	(D)/M/C	nt	M	SK241	peach	1994	D	D	D
SK63	myrobalan	1978	M	M	M	SK242	plum	1991	D	Nt	D
SK65	plum	1986	M	M	M	SK243	peach	1995	D	Nt	D
SK67	plum	1986	D/(M)	nt	M	SK244	peach	1995	D/M	M	M
SK68	plum	1986	M	M	M	SK245	plum	1995	M	nt	M
SK70	plum	1982	M	nt	M	SK246	plum	1995	M	nt	M
SK72	plum	1978	M	M	M	SK247	apricot	1995	D	D	D
SK74	plum	1979	M	M	M	SK248	plum	1995	D	nt	D
SK76	plum	1974	M	M	M	SK249	w. almond	1995	D	nt	D

[a] *Not Hungarian isolates.

for differentiating between its four major strains. As exemplified by the analysis of field samples, the specificity and sensitivity of our general PPV detection are comparable with those of the method that detects the 3′ non-translated region (3′ NTR) of the virus genome. It has been noted (Hadidi and Levy, 1994) that primers designed to amplify the N-terminal coding region of the viral CP are not as specific as primers designed to amplify the 3′ NTR, and these could potentially detect non-PPV type viruses. The general primers have not yet been tested against other viruses, so it is conceivable that some cross-reaction might occur. However, the Potyvirus CP sequences available in the GenBank have been checked and all of them contained sequences different enough at the primer binding sites to prohibit effective amplification. It is also noted that our 3′ general primer is complementary to a CP coding region that is conserved in all known PPV sequences. In our opinion, the use of this primer for both cDNA synthesis and PCR confers sufficient specificity. Should possible cross-reactions be a major concern, the specificity of our general PPV detection can be increased further by including an immunocapture step and/or using a 3′ NTR primer for cDNA synthesis.

Both the specificity and the sensitivity are increased further by the differentiating nested PCR reactions, which appear to be specific for their cognate PPV strains. The typing of over 100 field samples (see above, and data not shown) always resulted in unambiguous strain determinations. Double and even triple mixed infections, not revealed by RsaI-RFLP and ELISA typing, were also detected. The signal amplification power of the coupled RT–PCT/nested PCR allowed accurate typing in cases when PPV itself was not detectable in the first RT–PCR reaction. Since our strain-specific primers do not overlap the sequence variation that results in the diagnostic restriction site, their application for typing saves the RsaI site for RFLP as an independent diagnostic approach.

The very high correlations between the results of PCR, serological and RFLP typing indicate that the PCR method described in this study differentiates reliably the major PPV strains and could be used in practice. However, the exceptional isolate for which the molecular and serological typing results were contradictory demonstrates that the genetic background of the natural PPV strain variation is more complex than is reflected by our present knowledge. Neither molecular nor serotyping at their present state reflects adequately the full spectrum of this complexity; nor do we know how it is associated with economically important viral phenotypes, such as host specificity and virulence.

Any diagnosis is only as good as the information it is based on. The strain-specific primers were developed on the basis of group-specific peptide patterns in the N-terminal part of the PPV CPs. These patterns were identified by comparing a fairly large but limited number of CP sequences, and the primers were slightly degenerated so as to accommodate the known sequence variations. In all the positive cases tested, the presence of one or more PPV strain was detected, indicating that the identified patterns are present in field isolates of the virus. It is possible, however, that patterns not identical to those described in this study might be present in still unsequenced strains. In this case, the degree of degeneracy of the primers could be increased in order to accommodate the new sequence information.

An unexpected outcome of this study is the observation of a surprisingly high incidence of the PPV-D strain in Hungary. A striking feature is the temporal distribution of the PPV-D type. It was detected by PCR in nine of 50 isolates (18%) collected up to 1986, only one of which contained this type as a single infecting virus. ELISA did not indicate the presence of PPV-D in this subset of isolates. This is perhaps not surprising since most of these isolates had PPV-M as a major and PPV-D as a minor component, as indicated by the relative amounts of the PCR products. However, a sudden increase in the frequency of the PPV-D strain occurred among the 35 isolates collected between 1991 and 1995. Among these isolates, PPV-D was detected in 23 cases by PCR (65.7%) and in 20 cases by ELISA. The slight difference was due to two isolates where PCR detected a mixed D/M infection, and ELISA indicated the presence of PPV-M type alone, and to one isolate

which was not analyzed serologically. This huge increase in the incidence of PPV-D coincides with the political and economic changes in Hungary, and suggests that PPV-D was 'imported' into Hungary by uncontrolled propagating materials in this period, during which health regulations were not enforced as strictly as earlier. The results are in good agreement with earlier reports of an increasing PPV-D incidence in Hungary among isolates collected after 1995 (Pribék and Gáborjányi, 1997; Pribék et al., 1998)

Acknowledgements

We thank Dr T. Candresse, Dr L. Palkovics and Dr L. Nemchinov for the PPV-EA infected sample and for the PPV-M and PPV-C CP plasmids, respectively. The authors also thank Andrea Vörös and Aranka Bálint for their excellent technical assistance. M. Szemes has received a Ph.D. fellowship grant from Bay Zoltán Foundation for Applied Research.

References

Baumgartnerova, H., 1996. First findings of plum pox virus in walnut trees. Acta Virol. 40 (1), 59–60.

Boscia, D., Zeramdini, H., Cambra, M., Potere, O., Gorris, M.T., Myrta, A., Di Terlizzi, B., Savino, V., 1997. Production and characterization of a monoclonal antibody specific to the M serotype of plum pox potyvirus. Eur. J. Plant Pathol. 103, 477–480.

Bousalem, M., Candresse, T., Quiot-Douine, L., Quiot, J.B., 1994. Corrélation entretrois techniques permettant de différencier les isolates du plum pox potyvirus. EPPO Bull. 24, 651–656.

Cambra, M., Asensio, M., Gorris, M.T., Pérez, E., Camarasa, E., Garcia, J.A., Moya, J.J., Lopez-Abella, D., Vela, C., Sanz, A., 1994. Detection of plum pox potyvirus using monoclonal antibodies to structural and non-structural proteins. EPPO Bull. 24, 569–577.

Candresse, T., Macquaire, G., Lanneau, M., Bousalem, M., Quiot-Douine, L., Quiot, J.B., Dunez, J., 1995. Analysis of plum pox virus variability and development of strain-specific PCR assays. Acta Hortic. 386, 357–369.

Candresse, T., Cambra, M., Dallot, S., Lanneau, M., Asensio, M., Gorris, M.T., Revers, F., Macquaire, G., Olmos, A., Boscia, D., Quiot, J.B., Dunez, J., 1998a. Comparison of monoclonal antibodies and polymerase chain reaction assays for typing of isolates belonging to the D and M serotypes of plum pox potyvirus. Phytopathology 88 (3), 198–204.

Candresse, T., Rafia, P., Dunez, J., Cambra, M., Asensio, M., Navrátil, M., Garcia, J.A., Boscia, D., Pasquini, G., Barba, M., 1998b. Characterization of plum pox virus coatprotein epitopes using fusion proteins expressed in E. coli. Acta Hortic. 472 (2), 461–469.

Crescenzi, A., Nuzacci, M., Piazzola, P., Levy, L., Hadidi, A., 1995. Plum pox virus in sweet cherry. Acta Hortic. 386, 219–225.

Crescenzi, A., d' Aquino, L., Nuzzaci, M., Comes, S., Piazzolla, P., Ostuni, A., Bavoso, De, Stradis, A., 1998. Synthetic peptides in diagnosis of plum pox potyvirus. Acta Hort. 472 (2), 491–494.

Devereux, J., Haeberly, P., Smithies, O., 1984. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acid Res. 12, 387–395.

Dunez, J., 1988. Plum pox disease of stone fruit in Egypt. Report of a mission to Egypt. PCP/EGY/6756.

Dunez, J., Ravelonandro, M., Candresse, T., 1994. Plum pox: advances in research on the disease and its casual agent, and possible means of control. EPPO Bull. 24, 537–542.

Hadidi, A., Levy, L., 1994. Accurate identification of plum pox potyvirus and its differentiation from Asian prunus latent potyvirus in Prunus germplasms. EPPO Bull. 24, 633–643.

Hammond, J., Pühringer, H., da Camara Machado, A., Laimer da Camara Machado, M., 1998. A broad-spectrum PCR assay combined with RFLP analysis for detection and differentiation of plum pox virus isolates. Acta Hortic. 472 (2), 483–490.

Kalashyan, Yu.A., Bilkey, N.D., 1989. Plum pox virus in cherry. Plant Virology: Conference of Czechoslovak Plant Virologists, Praha, 276–306.

Kalashyan, Yu., Bilkey, A., Verderevskaya, T.D., Rubina, E.V., 1994. Plum pox virus on sour cherry in Moldova. EPPO Bull. 24, 633–643.

Kerlan, C., Dunez, J., 1979. Differenciation biologique et serologique du souches du virus de la sharka. Annales de Phytopathologie 11, 541–550.

Levy, L., Hadidi, A., 1994. A simple and rapid method for processing tissue infected with plum pox potyvirus with specific 3′ non-coding region RT–PCR assays. EPPO Bull. 24, 595–604.

Lopez-Moya, J.J., Fernandez-Fernandez, M.R., Cambra, M., Garcia, J.A., 2000. Biotechnological aspects of plum pox virus. J. Biotechnol. 76, 121–136.

Myrta, A., Di Terlizzi, B., Boscia, D., Çaglayan, K., Gavriel, I., Ghanem, G., Varveri, C., Savino, V., 1998a. Detection and serotyping of Mediterranean plum pox virus isolates by means of strain-specific monoclonal antibodies. Acta Virol. 42 (4), 251–254.

Myrta, A., Potere, O., Boscia, D., Candresse, T., Cambra, M., Savino, V., 1998b. Production of a monoclonal antibody specific to the El Amar strain of Plum Pox Virus. Acta Virol. 42 (4), 248–251.

Myrta, A., Potere, O., Crescenzi, A., Nuzzaci, M., Boscia, D., 2000. Properties of two monoclonal antibodies specific to the cherry strain of plum pox virus. J. Plant Pathol. (in press).

Nemchinov, L., Hadidi, A., Maiss, E., Cambra, M., Candresse, T., Damsteegt, V., 1996. Sour cherry strain of plum pox potyvirus (PPV): molecular and serological evidence for a new subgroup of PPV strains. Mol. Plant Pathol. 86 (11), 1215–1221.

Németh, M., 1986. Virus, Mycoplasma and Rickettsia Diseases of Fruit Trees. Martinus Nijhoff Publishers, Dordrecht, Budapest.

Németh, M., Kölber, M., 1994. History and importance of plum pox in stone-fruit production. EPPO Bull. 24, 525–536.

Olmos, A., Cambra, M., Dasi, M.A., Candresse, T., Esteban, O., Gorris, M.T., Asensio, M., 1997. Simultaneous detection and typing of plum pox potyvirus (PPV) isolates by heminested-PCR and PCR–ELISA. J. Virol. Methods 68, 127–137.

Palkovics, L., Burgyán, J., Balázs, E., 1993. Comparative sequence analysis of four complete primary structures of plum pox virus strains. Virus Genes 7 (4), 339–347.

Pasquini, G., Barba, M., 1997. Plum pox potyvirus strains: an overview. Proc. Middle Eur. Meeting '96 on Plum Pox 1996, 168–171.

Poggi-Pollini, C., Giunchedi, L., Bissani, R., 1997. Specific detection of D- and M-isolates of plum pox virus by immunoenzymatic determination of PCR products. J. Virol. Methods 67 (2), 127–133.

Pribék, D., Gáborjányi, R., 1997. Hungarian plum pox virus isolates represent different serotypes. Acta Phytopathol. Enthomol. Hungarica 32 (3-4), 281–288.

Pribék, D., Palkovics, L., Gáborjányi, R., 1998. A hazai szilvahimlo vírus izolátumok genetikai polimorfizmusának bizonyítása RT–PCR módszerrel. Növényvédelem 34 (11), 601–605.

Roy, A.S., Smith, I.M., 1994. Plum Pox situation in Europe. EPPO Bull. 11, 515–523.

Shukla, D.D., Stike, P.M., Tracy, S.L., Gough, K.H., Ward, C.W., 1988. The N and C termini of the coat proteins of potyviruses are surface located and the N terminus contains the major virus specific epitopes. J. Gen. Virol. 69, 1497–1508.

Simón, L., Sáenz, P., García, J.A., 1997. Plum pox virus. In: Monette, P.L. (Ed.), Filamentous viruses of woody plants, pp. 75–86.

Spiegel, S., Scott, S.W., Bowman-Vance, V., Tam, Y., Galiakparov, N.N., Rosner, A., 1996. Improved detection of Prunus necrotic ringspot virus by the polymerase chain reaction. Eur. J. Plant Pathol. 102, 681–685.

Thakur, P.O., Bharaway, S.W., Garg, I.D., Khosla, K., Sharma, D.R., 1994. Plum pox virus on stone fruits from India. A new record. Plant Dis. 9, 100–102.

Topchiiska, M., 1991. Detection of plum pox virus by ELISA in Prunus spp. at the different stages of their development. Abstracts of the XVth International Symposium on Virus and Virus-like Diseases of Temperate Fruit. Crops Vienna (Austria) 8–13 July, 1991 p.50.

Wetzel, T., Candresse, T., Ravelonandro, M., Dunez, J., 1991a. A polymerase chain reaction assay adapted to plum pox potyvirus detection. J. Virol. Methods 33, 355–366.

Wetzel, T., Candresse, T., Ravelonandro, M., Delbos, R.P., Mazyad, H., Aboul-Ata, A.E., Dunez, J., 1991b. Nucleotide sequence of the 3′-terminal region of the RNA of the E1 Amar strain of plum pox potyvirus. J. Gen. Virol. 72 (7), 1741–1746.