

# Serological Characterization of Hungarian Plum Pox Virus Isolates

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Three Hungarian (No.2, 4 and 9), and a Moldavian (K) plum pox virus isolates were compared with a characterized Spanish isolate (5.15) by RT-PCR, ELISA, dot-blot and Western blot analysis. Monoclonal antibodies prepared against the external, intermediate and internal sequences of the coat protein of the Spanish isolate were able to differentiate the four isolates. Hungarian isolate No. 2 proved to be serologically identical to the Spanish isolate, while No. 4 showed appreciable differences and No. 9 could be recognized only by the monoclonal antibodies representing the intermedial and internal parts of the coat protein. K isolate showed a more distant relationship to other isolates. Our experiment provided the first demonstration of the presence of D type isolates in Hungary.

## Introduction

Plum pox virus (PPV) is the most severe and economically important virus of cultivated stone fruit trees in European countries. The virus is a member of the proposed genus Potyvirus, family of Potyviridae (Barnett, 1992). Its flexuous, rod-shaped virions are about 700 nm long and 11 nm in diameter. The single-stranded 9.7 kb. genomic RNA (Lain *et al.*, 1989; Maiss *et al.*, 1989) has a poly(A) tail at the 3' end and is linked to a genome-linked protein at the 5' end (Hari, 1981). The RNA is covered with approximately 200 repeating copies of a single coat protein (CP) of about 30 kD (Ravelonandro *et al.*, 1988).

Potyriviruses present complex serological relationship among members. The CP N-terminal region, located in the surface of the virus particles (Shukla *et al.*, 1988) shows the largest difference in sequence (Shukla *et al.*, 1989). This region of the CP is specially labile in the case of PPV during purification (López-Moya, 1994a), representing a problem in the production of virus-specific antisera. PPV-specific monoclonal antibodies (MAbs) were prepared using different Spanish isolates purified in the presence of protease inhibitors (Ló-

pez-Moya, 1994b). In this paper isolates of PPV from Hungary, Moldavia and Spain were compared using a number of MAbs differing in antigenic sites by enzyme linked immunosorbent assay (ELISA), dot blot and Western blot analysis in order to define the serological relationships among these, geographically distant PPV isolates.

## Materials and Methods

### Virus isolates

The PPV isolates used in this study came from the Plant Protection Institute (Budapest) collection: isolate No. 2 was from apricot (Keszthely), isolates No. 4 and No. 9 from plum (Karcag and Budapest, respectively) and the K isolate was isolated from sour cherry in Kisinov, Moldavia. A previously described Spanish isolate 5.15 from apricot (López-Moya, 1994a) was included in all experiments.

Isolates were maintained by mechanical inoculation in *Nicotiana benthamiana* Domin. plants kept in a growth chamber.

### RT-PCR

A two step reverse transcription-polymerase chain reaction (RT-PCR) protocol was performed with total nucleic acid extraction from infected plant samples. Briefly, 0.1 g leaf tissue was grinded

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in 400 µl of sterile 25 mM Tris-HCl (pH 7.5) buffer containing 25 mM MgCl<sub>2</sub>, 25 mM KCl and 1% sodium dodecyl sulphate (SDS) (López-Moya *et al.*, 1995). After two phenol/chloroform extractions (1:1) the nucleic acids were precipitated with 1/10 vol. of 7.5 M NH<sub>4</sub>-acetate and 2 vol. of 96% ethanol. Pellets were washed with 70% ethanol, vacuum dried and resuspended in 20 µl sterile water.

An aliquot of 3.5 µl sample was adjusted to 20 µl reaction volume with 50 µM Tris-HCl (pH 8.9); 50 mM KCl; 7.5 mM MgCl<sub>2</sub>; 250 µM of each dNTP; 20 units of RNasin and 0.75 µM of the specific downstream oligonucleotide primer (see below). One unit of Avian myeloblastosis virus (AMV) reverse transcriptase was added, and the reaction incubated for 45 min at 42 °C.

PCR reaction was preformed in a 10 µl volume, adjusted to 1 Taq reaction buffer, containing 10 mM Tris-HCl (pH 8.3); 1.5 mM MgCl<sub>2</sub>; 250 µl of each dNTP and 0.5 µl of each oligonucleotide primer, corresponding to the nucleotides 8391–8408 in the positive sense and 8900–8883 in complementary sense of the PPV RNA genome (Lain *et al.*, 1989).

Two units of Taq DNA polymerases were added and the reaction incubated in an automatic thermal cycle (Perkin-Elmer 9600) with a cycle profile of 92 °C/45", 42 °C/30", 72 °C/1', for 40 cycles giving the last cycle 10 min final elongation step at 72 °C. The final product of the reaction was analyzed in 10 µl aliquots in 1.0% agarose gel in Tris-borate-EDTA buffer, containing 0.5% ethidium bromide (Sambrook *et al.*, 1989).

### ELISA

Two different ELISA procedures were preformed: in the first protocol, direct ELISA, plates were coated with samples containing the antigen. In the other protocol indirect double antibody sandwich (IDAS ELISA) plates were coated with anti-PPV polyclonal antibody to trap later the antigen. Both procedures used PPV-specific MAbs and anti-mouse horseradish peroxidase conjugate (HRPO) to detect the presence of the antigen in the samples.

Polystyrene plates were coated overnight at 4 °C directly with 100 µl of 1:20 w/v plant extract in 0.05 M sodium carbonate buffer (CB) pH 9.6, or coated previously with 1 µl/ml polyclonal antibody (Sa-

nofi) in CB and then incubated overnight with 1:20 w/v plant extract in 0.15 M NaCl; 0.1 M sodium phosphate buffer (PBS) pH 7.4, containing 0.2% nonfat powdered milk. After each step plates were washed with PBS plus 0.05% Tween-20.

MAbs were added at 0.1–1.0 µg in PBS, followed by goat-anti mouse HRP conjugate, and both incubations performed for 3 h at 37 °C. Reactions were developed adding chromogen/substrate solution containing *o*-phenylene-diamine/H<sub>2</sub>O<sub>2</sub>. Absorbance was measured at 450 nm after addition of 50 µl well of 3 N H<sub>2</sub>SO<sub>4</sub>.

### Dot-blot

Extracts were prepared from fresh plant tissue (1:40 w/v) in Tris-HCl (pH 6.8), containing 30 mM ascorbic acid and 0.2% 2-mercaptoethanol. The mixtures were denatured by heating for 5 min at 100 °C. After the blocking step in 5% skim milk powder in PBS, sheets were incubated first with MAbs at 0.1 µg/ml in PBS and then with peroxidase labeled anti-mouse antibody. The enzyme activity was visualized using 4-chloro-naphtol and H<sub>2</sub>O<sub>2</sub>.

### SDS-PAGE and Western blot

Crude plant extracts (1:20 w/v) were prepared in 0.625 M Tris-HCl (pH 6.8) buffer, containing 30 mM ascorbic acid, mixed with equal volume of Laemmli buffer, containing 2% SDS and 2% 2-mercaptoethanol and denatured by heating for 2 min at 100 °C (Laemmli, 1970). Products were electrophoresed in a 5–12.5%, 1 mm wide discontinuous (SDS-PAGE) gel, and electroblotted (Towbin *et al.*, 1979) onto a nitrocellulose membrane, blocked with 1% skim milk powder in PBS for 30 min and incubated with the MAbs and developed as described above for the dot-blot assays.

## Results

### PCR analysis

No differences between the five PPV isolates were observed in the mobility of the amplified product after RT-PCR. Only a slightly lower relative proportion of the band was observed in the case of the isolate 4 (Fig. 1). The results indicate

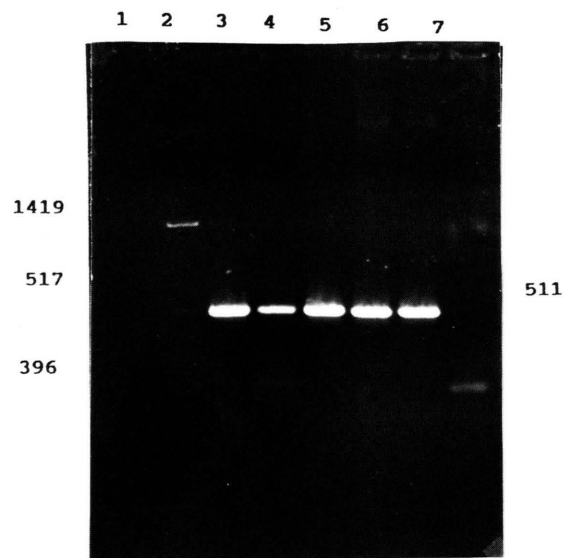


Fig. 1. Analysis of RT-PCR amplification of PPV-RNA from *N. benthamiana* infected samples. Lane 1, *Hinf*I digested pUC 18 as size markers. Lanes 2,3,4,5 and 6: samples of plant infected with PPV isolates 2,4,9, K and 5.15. Lane 7: healthy plant used as negative control. Electrophoresis was performed on a 1% agarose gel stained with ethidium bromide.

an equivalent size for N-terminal region of all the isolate CPs.

ELISA and Dot blot assay

Three previously described PPV-specific MABs (López-Moya *et al.*, 1994b) directed against superficial (1EB6), intermediate (4DG11) and internal (3C6) CP regions were used in both ELISA and dot-blot procedures. MABs 1EB6 and 4DG11 reacted well both in DAS and indirect ELISA procedures, while MAB 3C6 exhibited a higher reactivity in the indirect ELISA assay, indicating that the internal region is better exposed when the virions are partly degraded in the protein adsorbed in the plates (López-Moya *et al.*, 1994b). Results are summarized in Table I.

While in IDAS-ELISA isolates 2,4 and 5.15 were detected by the three MABs, isolate 9 and K failed to be detected by 1EB6. Isolates 2 and 9 were detected with 4DG11, but 4 was not. Serological properties of the isolates can clearly discriminate them. Similarities among isolates were found in indirect ELISA, where the MAB 3C6 recognized all virus isolates, indicating that

Table I. Reactivity of three Mabs to five PPV isolates in IDAS-ELISA<sup>a</sup> or in indirect ELISA<sup>b</sup>.

Mabs	Healthy	2	4	9	k	5.15
1EB6 <sup>a</sup>	– <sup>c</sup>	+	+	–	–	+
4DG11 <sup>a</sup>	–	+	–	+	+	+
3C6 <sup>b</sup>	–	++	++	++	++	++

<sup>a</sup> Micotitre plates were coated with polyclonal antibody, incubated with extracts from healthy or PPV infected plants before Mab incubation.

<sup>b</sup> Plates were coated with extracts from healthy or PPV infected plants.

<sup>c</sup> Reactivity below 2 times (–), above 10 times (+) or above 20 times (++) the readings obtained with uninfected plant extract.

the same serological reactions can be obtained for dot blot analysis (Fig. 2).

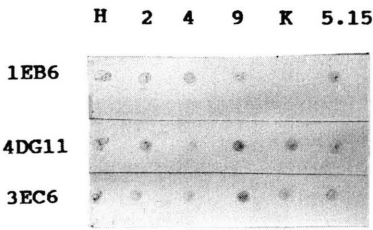


Fig. 2. Dot blot with three Mabs (1EB6,3DG11 and 3C6) of extracts from plants infected with 4 Hungarian isolates (2,4,9 and K) and isolate from Spain (5.15). H, sample from healthy plant.

Western-blot analysis

Isolates 2, 9, K and 5.15 showed in SDS-PAGE a similar apparent CP size around 36kDa, as shown in Fig. 3 in a Western blot analysis with 3C6 MAB. Isolate 4 exhibited a larger CP, around 38 kDa. These results confirmed the differences between isolate 4 and the others, in terms of the electrophoretic mobility of the CP.

Similar mobilities of CPs were observed using Western blot with other MABs, with the same exceptions in reactivity as previously described in ELISA or dot-blot procedures.

Discussion

PPV genome encodes one large polypeptide of 350 kDa, which is processed into several functional proteins, produced in equimolar amounts. Most final proteins revealed a high degree of sim-

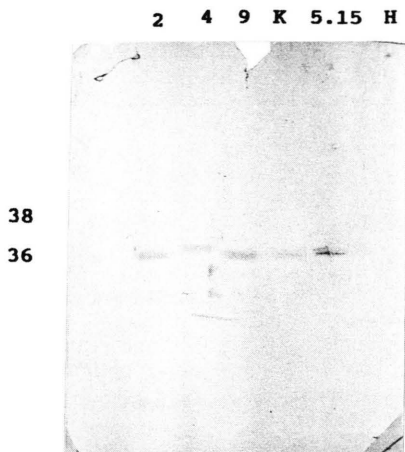


Fig. 3. Western blot with monoclonal antibody 3C6 of extracts from plants infected with PPV isolates 2, 4, 9, K and 5.15. H, sample from healthy plant. Isolate 4 shows a CP with a larger size, around 38 kDa.

ilarities among members of the group. Consequently, not all proteins were suitable for classification and identification virus species and strains by conventional ELISA (Adamolle *et al.*, 1994). Two serological groups of PPV isolates were described by Kerlan and Dunez (1979): a severe PPV-M (Marcus) strain and a milder PPV-D (Dideron) strain. The major differences between PPV strains were found in the N termini of the CP. This region has been shown to be exposed on the surface of the particle. On the basis of monoclonal antibodies raised against the different regions of the PPV-CP, strains could be differentiated and classified (Cambra *et al.*, 1994; Candresse *et al.*, 1994; Deborré *et al.*, 1994; López-Moya *et al.*, 1994a; Asensio *et al.*, 1995).

Monoclonal antibodies recognized different epitopes of CP reacted differently with the isolates used in our experiments, indicating that different serotypes of PPV occurred in Hungarian stone-fruit plantations. It was possible to characterize and compare Hungarian isolates different from the Spanish isolate 5.15, which was the original isolate used for MAb production and previously characterized (López-Moya *et al.*, 1994a). This isolate showed serological identity with Hungarian isolate 2 both in DAS-ELISA and IDAS-ELISA.

Experiments described earlier (Asensio *et al.*, 1995) that MAb 4DG11 reacted only with the D type isolates. In our experiments all Hungarian isolates gave a positive reaction with this MAb indicating that all of them could be classified as D type. Until now only one Hungarian PPV isolate (SK68, isolated by M. Németh) was compared serologically with other European isolates. In these experiments SK68 was classified as a typical M type (Deborré, 1994).

Tests with 1EB6 MAb demonstrated slight differences between isolates 2 and 4. Only the MABs representing the most conserved sequences of the internal epitopes of CP were able to recognize isolate 9, indicating the distant relationship of this isolate to others. K isolate reacted only with the MABs of internal and intermedial epitopes, especially in dot blot experiments, demonstrating the unique nature of this isolate. The isolate is probably the same sour cherry isolate which was considered as an unusual strain of PPV (Deborré *et al.*, 1994; Nemchinov *et al.*, 1994).

The results of the present study showed that MABs raised against Spanish PPV strains were suitable for detection and differentiation of Hungarian PPV isolates. Until now, only one Hungarian isolate (SK68), belonging to the M type, was characterized by complete nucleotide sequence (Palkovics *et al.*, 1993). The isolates used in our experiments demonstrated the presence of isolates of D serotype, too. The great variability of the isolates was probably due to the geographically short distance from the origin of the virus.

Our results suggest that the spread of other, occasionally more virulent PPV strains in the Carpathian Basin should be considered. Isolates of different serotypes did not show significant differences in test plants. The usefulness of the test plant detection method underlines the need of using different diagnostic methods. Comparison of serological methods based on MABs, electrophoretic mobility of CP, immunocapture RT-PCR showed complete correlation and confirmed the existence of the two major serogroups (Bousalem *et al.*, 1994). Survey of dominance and distribution of these serotypes needs a more detailed study.



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