PARTIAL CHARACTERIZATION OF PLUM POX VIRUS (PPV) ISOLATES FROM BULGARIA

*S. Milusheva, **V. Mavrodieva and **L. Levy

Abstract

Samples from Prunus hosts, exhibiting typical Plum pox virus (PPV) symptoms were collected from orchards in Bulgaria and bud-wood grafted, and maintained on GF 305 peach. Using RT-PCR with PPV (3’)NTR primers and DASELISA, samples were confirmed to be infected with PPV. Biological characterization was carried out onto six Prunus genotypes and ten herbaceous species. Limited correlation relevant to virulence of the isolates was noted between the two biological tests. The isolates were confirmed to be PPV-M using serotype-specific ELISA and PPV strain-specific RT-PCR. The 3’-terminal segment (1.4 kb) of each of the isolates RNA was sequenced and compared with sequences available in GeneBank. Isolates showed 97–99% identity of their protein sequences with the PPV-M isolates PS and SK68.

Key words: PPV-M, characterization, biological indexing, RT-PCR, sequencing

Introduction

The shanka disease agent, Plum pox virus, like many other plant viruses has been found in nature in the form of different strains. Currently, four PPV strains have been differentiated on the basis of their biological, immunological and molecular properties: Marcus (PPV-M), Dideron (PPV-D), El Amar (PPV-EA), Cherry (PPV-C) (Kerlan and Dunez 1979, Pasquini and Barba 1997, Candresse et al. 1998). In this respect, detailed information concerning strain characterization of Bulgarian PPV isolates is still limited. The aims of present study were: determina-
tion of strain specificity of seven PPV isolates from Bulgaria and investigation on resemblances and differences in some their biological and molecular properties.

Materials and methods

Plant material

Seven PPV isolates, maintained onto the indicator GF 305 were used: Pl1-87 from plum; Apr35-15 from apricot; P5-34, P9-7, P9-21, P27-62 from peach and SwC8-34 from sweet cherry.

Biological indexing

Biological characterization was carried out by chip-bud grafting onto six Prunus genotypes and mechanical inoculation onto 10 herbaceous species. In the first range of hosts the mentioned isolates were grafted onto cv. ‘Stanley’ (Prunus domestica L.) IVP (in vitro propagated) and rootstocks: Zhalta dzhanka (P. cerasifera Ehrh.), P. mahaleb L. seedlings, Gisela 5 (P. cerasus L. × P. canescens L.) IVP, Damil (P. dawyckensis L.) IVP, Inmil (P. incisa L. × P. serrulata Lindl.) IVP. A minimum of three plants from the same genotype were inoculated with each source of infection.

In the second range of hosts were inoculated mechanically following herbaceous indicator species: Celosia argentea L., Chenopodium amaranticolor Coste et Reyn., C. foetidum Schrad., C. quinoa Willd., Nicotiana benthamiana Domin., N. clevelandii Gray, N. occidentalis Wheeler., Nycandra physaloides Gaertn., Petunia × hybrida Vilm. and Pisum sativum L.

The symptoms were observed and ELISA detected the presence of virus.

Serological assays

The Durviz 5B-IVIA DAS ELISA kit (Agdia) was used for PPV detection according to the manufacturer instructions. Serotype determination (Cambra et al. 1994) was done using: Ab provided by Dr I. Kamenova and Mabs provided by Dr A. Myrta. Two monoclonal antibodies were used: AL specific for PPV-M serotype and 4DG11 specific for PPV-D serotype.

Molecular techniques

IC-RT-PCR

The polyclonal anti-PPV antibody from Bioreba diluted 1:200 in carbonate buffer pH 9.2 was used for immunocapture. Leaf samples were ground 1:10 (w/v) in PBS buffer with 2% PVP and 0.2% DIECA. Sap was clarified by centrifugation and loaded on pre-washed Ab-coated tubes. After at least 16 hours of incubation at 4°C tubes were washed twice with PBST. Twenty five µl of molecular grade dH₂O were added to each tube, incubated for 5 min at 65°C and gently vortex for 30 s.
15 µl of IC sample were used for the RT-PCR. Two pairs of primers were used for molecular identification and characterization of the isolates: the PPV(3’)NTR primers (Levy et al. 1994) that are specific for the 3’non-translated region of the PPV RNA and strain-specific primers (Wetzel et al. 1991, Candresse et al. 1998) based on the coat protein gene sequence. PCR products were analyzed by 1.5% agarose gel electrophoresis in TAE buffer and EtBr staining.

Cloning and sequencing

Total RNA from healthy and infected plant leaves was purified using the RNeasy Plant RNA kit from Qiagen according to MacKenzie et al. (1997). The 1.4 kb fragments for sequencing were obtained by RT-PCR of total plant RNA extracts using primers described by Nemchinov et al. (1996) and the Titan one tube RT-PCR system from Roche, following the manufacturer protocol. PCR products were cloned into the pCR 4 Blunt-TOPO vector (Invitrogen) according to the manufacturer instructions. After PCR screening of resulting colonies plasmid DNA was purified using the Wizard Plus DNA Purification system (Promega) and analyzed by EcoRI digest for insert presence. Three clones per isolate were sequenced in a commercial facility. Sequences were aligned and compared using the GeneDoc program version 2.6.002 (Nicholas et al. 1997).

Results

PPV identification

PPV presence in all seven isolates was confirmed by Durviz DAS ELISA (results not shown) and IC-RT-PCR with PPV(3’)NTR primers (Phot. 1). All seven isolates produced the expected 220 bp band thus confirming that PPV was present.

Strain determination

Mabs DAS ELISA

All isolates reacted positive with MabAL specific for PPV-M. None of the isolates reacted with Mab4DG11 specific for PPV-D (data not shown).

Strain-specific RT-PCR

All seven isolates tested produced diagnostic bands with P1-PM primers but not with P1-PD primers. Some non-specific bands were seen as well (Phot. 2).

Sequence analysis

The 3’ 1.4 kb segment containing the C-terminal part of the Nlb gene, entire CP gene and the 3’NTR of each isolate RNA were sequenced. Comparison of the puta-
Phot. 1. Electrophoresis analysis (1.5% agarose) of RT-PCR products of Bulgarian isolates with PPV (3') NTR primers: 1 – P27-62, 2 – P5-34, 3 – P9-21, 4 – P9-7, 5 – P11-87, 6 – Apr35-15, 7 – SwC8-34, 8 – PPV-D positive control, 9 – Prunus tomentosa healthy, 10 – NTC (PCR H2O control), M – DNA ladder (photo by V. Mavrodieva)

Phot. 2. Electrophoresis analysis (1.5% agarose) of PPV strain-specific RT-PCR: 1 – PPV-D control, 2 – PPV-M control, 3 – P27-62, 4 – P5-34, 5 – P9-21, 6 – P9-7, 7 – P11-87, 8 – Apr35-15, 9 – SwC8-34, 10 – Prunus tomentosa healthy, 11 – NTC (PCR H2O control), M – DNA ladder (photo by V. Mavrodieva)
tive protein sequences showed 97–99% identity with PPV-M isolates PS (acc. AJ243957) and SK68 (acc. M92280) (Fig. 1). Most of substitutions were located in the C-terminal part of the Nlb gene and N-terminal part of the CP gene. Based on these differences isolates SwC8-34, P5-34 and P9-21 formed the first group, while isolates P27-62, Pl1-87 and P9-7 formed a second group. Isolate Apr35-15 sequence showed some unique substitutions.

**Biological indexing**

Virulence of the isolates was monitored for three years. Based on susceptibility of the *Prunus* hosts, the isolates could be divided into two groups (Table 1). All isolates infected cv. ‘Stanley’ and Zhalta dzhanka. The isolates Pl1-87 and SwC8-34 composed the first group. They infected *P. mahaleb* and Damil, unlike the isolates belonging to the second group. Neither of the isolates studied infected Gisela 5 and Inmil.

The isolates could be divided into three groups on the base susceptibility of herbaceous plants (Table 2). All isolates studied infected *N. benthamiana, N. clevelandii, N. occidentalis, P. × hybrida, P. sativum, C. foetidum, C. quinoa* and *C. amaranthicolor*. The isolates P9-21 and SwC8-34 infected *N. physaloides* and formed the first group. The

<table>
<thead>
<tr>
<th>Biological groups</th>
<th>Susceptible genotypes</th>
<th>Unsusceptible genotypes</th>
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<tbody>
<tr>
<td>First biological group</td>
<td>‘Stanley’, Zhalta dzhanka, <em>Prunus mahaleb</em>, Damil</td>
<td>Gisela 5, Inmil</td>
</tr>
<tr>
<td>P1-87, SwC8-34</td>
<td></td>
<td></td>
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<tr>
<td>Second biological group</td>
<td>‘Stanley’, Zhalta dzhanka</td>
<td><em>Prunus mahaleb</em>, Damil, Gisela 5, Inmil</td>
</tr>
<tr>
<td>Apr35-15, P5-34, P9-7, P9-21, P2762</td>
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</table>

Table 1: Results from the biological indexing onto *Prunus* genotypes
isolate P5-34 did not infect *N. physaloides*, but infected *C. argentea* and formed the second group. The isolates P9-7, P27-62, Pl1-87, Apr35-15 infected neither *N. physaloides* nor *C. argentea*. Those isolates presented the third group (Table 2).

### Discussion

The results obtained demonstrated that all seven isolates of PPV belonged to strain M. Sequence comparison showed 97–99% identity with PPV-M isolates PS and SK-68. The PPV-M is considered to be more virulent and aggressive than other strains and its isolates are characterized by a broader experimental range of host plants (Fuchs et al. 1995). The isolates we studied, varied in their virulence to *P. mahaleb* and Damil (*P. dawyckensis*). Obtained results revealed that two of the isolates Pl1-87 and SwC8-34 infected *P. mahaleb*. The visual symptoms were exhibited two years after inoculation on a few leaves. Probably these isolates have replicated very slowly in *P. mahaleb*. These results are in contrast with the data obtained by Desvignes et al. (1998) concerning capability of PPV-M to replicate in *P. mahaleb*. Damil inoculated with the mentioned both isolates manifested very weak symptoms and DAS ELISA test confirmed the presence of the virus.

Isolates virulence onto susceptible and non-susceptible *Prunus* genotypes showed limited correlation with isolates virulence onto herbaceous hosts. The isolates P9-7, P27-62, and Apr35-15 infected one and the same range of experimental *Prunus* genotypes and herbaceous varieties. The isolate SwC8-34 related to the groups with higher virulence in both biological trials.

PPV isolates studied formed two groups based on sequence analysis of the nucleic acid and putative protein sequences of the 3’ 1.4 kb fragment of the genome. Isolate Apr35-15 showed some unique sequences. Studies of other parts of the genome of these isolates are underway.

No significant correlation between groups formed on virulence data to *Prunus* and herbaceous hosts, and based on sequence data was noted.

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<tr>
<td>First biological group</td>
<td><em>N. benthamiana, N. clevelandii, N. occidentalis, N. physaloides, P. × hybridra, P. sativum, C. foetidum, C. quinoa, C. amaranticolor</em></td>
<td><em>C. argentea</em></td>
</tr>
<tr>
<td>Second biological group</td>
<td><em>N. benthamiana, N. clevelandii, N. occidentalis, P. × hybridra, P. sativum, C. foetidum, C. quinoa, C. amaranticolor, C. argentea</em></td>
<td><em>N. physaloides</em></td>
</tr>
<tr>
<td>Third biological group</td>
<td><em>N. benthamiana, N. clevelandii, N. occidentalis, P. × hybridra, P. sativum, C. foetidum, C. quinoa, C. amaranticolor</em></td>
<td><em>N. physaloides, C. argentea</em></td>
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Table 2

Results from the biological test onto herbaceous species
Acknowledgements

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Literature


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