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SHORT COMMUNICATION

Plum Pox Virus in Japanese Plum from Argentina: Serological Detection and Molecular Characterization of an Isolate from cv. Red Beauty

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Abstract

A *Plum pox virus* (PPV) isolate detected in a Japanese plum orchard in Pocito (San Juan, Argentina) was transmitted mechanically to *Prunus tomentosa* and *Nicotiana benthamiana*. DAS-ELISA and DASI-ELISA indicated the virus presence and serological relationship with D-strain isolates; IC-RT-PCR amplified a 1.2-kb fragment of the virus genome encoding the CP-3′ nc region. The analysis of the sequence showed the presence of the DAG motif at the 5′ end of the capsid protein and the Rsa I and Alu I sites at the 3′ end. The phylogenetic relationships and multiple alignment with PPV isolates from NCBI database indicated greatest (+98%) homology with the D strain and close identity with MNAT1 (\AF360579) USA peach isolate. The sequence analysed showed two amino acid mutations towards the 5′ N-terminus of CP (the most variable region) with respect to a consensus of PPV D-strain isolates. This is the first molecular characterization of 3′terminal genome region of PPV isolate to confirm D strain in a Japanese plum from Argentina.

Introduction

Plum pox virus (PPV) (genus *Potyvirus,* family *Potyviridae*) is the cause of sharka disease of stone fruit trees (plum, peach, apricot and cherry); it is considered to be the most important pathogen of *Prunus* trees due to the severe yield losses it induces (Nemeth 1994; Cambra et al. 2006). Sharka disease was first detected in Bulgaria (Atanasov 1932) and was initially restricted to Europe. Later it occurred in the American continent, in the US (Levy 2000; Snover-Clift et al. 2007), Canada (Thompson et al. 2001) and Chile (Acuña 1994). More recently, PPV has been reported in Japan (Maejima et al. 2010). In Argentina, PPV was detected in a Japanese plum orchard in Pocito, San Juan, in 2004 (Dal Zotto et al. 2006; Ortego et al. 2006) and the National Service of Plant Health (SENASA) declared the area within a 1000 m radius from the orchard a quarantine zone (Senasa Resolution Nº 24/05) (SENASA 2007). In a survey conducted in spring 2006, an incidence of 0.17% over 62 230 Japanese plums was reported in the valleys of Ullúm, Zonda and Tulua, San Juan (SENASA 2007).

PPV isolates detected worldwide have been classified into seven strains or groups: Dideron (D), Marcus (M) (Kerlan and Dunez 1979), El Amar (EA) (Wetzel et al. 1991), C (Nemchinov and Hadidi 1996), Winona (W) (James and Vargas 2005), Rec (Glasa et al. 2004) and Turkey (T) (Serçe et al. 2009). Most of the PPV isolates characterized have been assigned to these strains, although they might differ in their biological and epidemiological traits, such as aggressiveness, aphid transmission and symptoms (Cambra et al. 2006). Because PPV is a pathogen included in the A2 list of quarantine pests of the European Plant Protection Organization (EPPO 2004) and ISPM 27 International Standards for Phytosanitary Measures of International Plant Protection Convention (IPPC

2012), reliable detection and control management strategies are crucial for maintaining high-yield fruit tree production for fresh consumption, industrialization and export. Argentina produces and exports stone fruit trees and their fruits, mainly plum and peach (FAOSTAT 2007). We have serologically detected and characterized molecularly a PPV isolate from *Prunus salicina* cv. Red Beauty from Pocito, San Juan, Argentina, with the aim to determine molecular variability and the phylogenetic relationship between this isolate and others reported in GenBank.

Materials and Methods

Virus isolate and diagnostic tests

The virus was obtained from leaves of infected plums cv. Red Beauty from an orchard of 5000 trees in Pocito, San Juan, Argentina, and one leaf sample was inoculated onto 30 tobacco (*Nicotiana benthamiana*) and two Nanking cherry (*Prunus tomentosa*) seedlings. Inoculated plants were maintained in an acclimatized chamber at 25°C and 16-h photoperiod until the onset of symptoms. Samples of plum leaves were collected from 65 symptomatic trees showing irregular edges with chlorotic ring spots. All were analysed by DAS-ELISA (Clark and Adams 1977) using anti-PPV polyclonal antisera (Bioreba, Reinach BL1, Switzerland) and DASI-ELISA with Mab 5B IVIA and Mab 4DG5 IVIA (specific D strain) antisera (Durviz, Valencia, Spain), following the manufacturer's instructions. Absorbance was determined at 405 nm with an ELISA-reader (Dynex MRX II, Chantilly, VA, USA) at 30, 60 and 90 min. A sample was considered positive when its absorbance value was higher than the mean of the healthy controls plus three times the standard deviation (SD). Plum leaves of non-infected trees were used as healthy controls. The positive control was obtained from Bioreba. All serological diagnoses were made following international protocols (EPPO 2004).

IC-RT-PCR, sequencing and phylogenetic analyses

IC-RT-PCR was performed in microtiter strips (immunomodule F8, Nunc™, M.G: Scientific, Inc, Pleasant Prairie, WI, USA) coated with 50 μl of a 1/1000 dilution of anti-PPV (Bioreba) in RNase-free buffer at 37°C and incubated for 4 h; then they were incubated with samples (processed following DAS-ELISA protocols) overnight at 4°C. After each step, the microtiters were washed three times with PBS + Tween 20, RNase-free for 3 min. First strand cDNA was performed in wells with 5 μl of oligo $(dT)_{15}$ primer (Promega, Madison, WI, USA), 5 μl of dNTPs (0.25 mM), 10 μl 5× M-MLV buffer (Promega), 1 μl M-MLV enzyme (0.1 U) (Promega) and diethylpyrocarbonate-treated (DEPC) H_2O to complete a final volume of 50 μl. RT was carried out at 37°C for 30 min. PCR was performed with specific primers that anneal at the 5′ (N-ter) of the CP region and the 3′ end of 3′ nc region of PPV. The primer (5′) CP: CGCG TCACCATGGCTGACGAAAGAGAAGACGAG and the antisense primer (3′) 3′nc: GTCTCTTGCACAACTA TAACC were designed in our laboratory. cDNA (1 μl) was added to a mix of 5 μl of dNTPs (0.25 mM), 5 μl 10× of taq DNA polymerase buffer (Promega), 5 μl MgCl2 (2.5 mM), 5 μl of each primer 3′nc/CP (0.25 uM), 0.3 μl of Taq DNA polymerase (0.25 U-μl) (Promega) in a final volume of 50 μl. The following cycling parameters were used: initial denaturation at 92°C for 1 min, followed by 40 cycles of denaturation at 92°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min and a final extension of 72°C for 5 min. The amplification products were subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide.

PCR products of PPV CP-3′nc from a single sample were purified with GFX-PCR-DNA and Gel band purification kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) from a preparative agarose (1%) gel and ligated into the vector PCR® 2.1 TOPO® Cloning® kit following the supplier's instructions (Invitrogen, Carlsbad, CA, USA). PPV recombinant clones were sequenced by Macrogen Company (Seoul, Korea). The nucleotide and the predicted amino acid sequences were aligned using the CLUSTAL V method from *Lasergene™*, DNAstar (DNAstar Inc., Madison, WI, USA). Phylogenetic analyses were carried out using MEGA 4 software (Tamura et al. 2007). The distance matrices were obtained using CLUSTAL W program with Kimura 2p (Kimura 1980) and evaluated for successive clustering using the Neighbour-Joining algorithm (Saitou and Nei 1987) with a bootstrap of 1000 replicates (Felsenstein 1985).

Results and Discussion

PPV-specific symptoms were observed in the inoculated host plants. Indeed, the virus was successfully transmitted into a Nanking cherry tree, which showed oak-leaf patterns and chlorotic and necrotic spots towards spring (Fig. 1a) and onto 25 eight-leaf stage tobacco seedlings, which developed interveinal chlorosis on young leaves (Fig. 1b). Analyses using DAS-ELISA indicated that PPV was present in 60% of 65

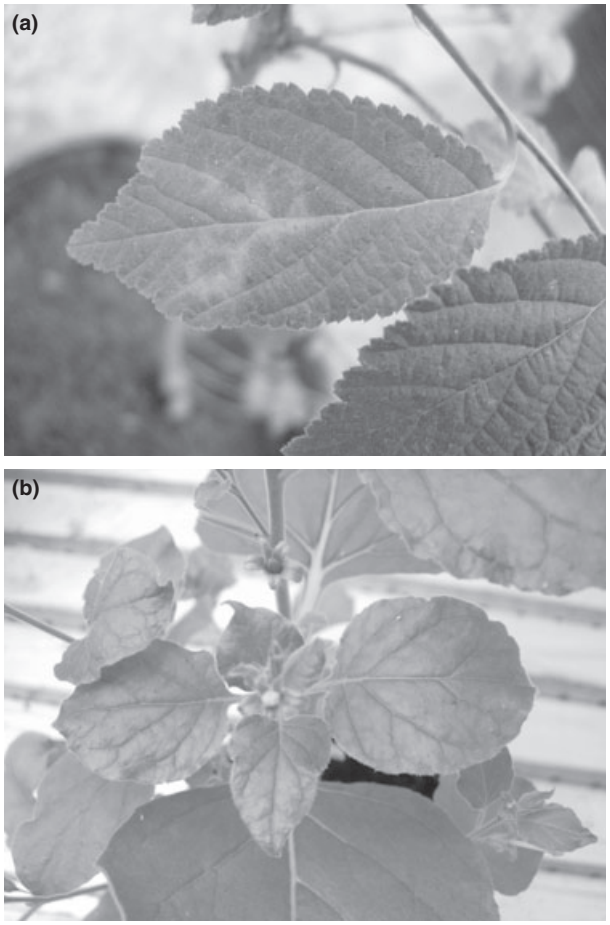

Fig. 1 (a) Oak-leaf pattern on leaves of cherry *Prunus tomentosa* infected with *Plum pox virus* (PPV). (b) Chlorotic areas on tobacco (*Nicotiana benthamiana*) leaves infected with PPV.

plum trees (average Abs.$_{405}$ 1.2), and its presence was also confirmed with DASI-ELISA using 30 samples (average Abs.$_{405}$ 1.5) Mab5B and seven samples (average Abs.$_{405}$ 0.17) with Mab 4DG5. All were positive for PPV and for the PPV D-strain. Then molecular studies were conducted to confirm this result and to characterize an isolate. The IC-RT-PCR amplified a 1220-bp fragment from CP-3′nc region of PPV, which was used for cloning and sequencing. The clones PPV-2 and PPV-8 obtained from a single amplified sample were selected for further sequencing (accession numbers \DQ299537 and \DQ299538, respectively). The final sequence obtained, of 1209 bp (CP-3′nc region) and 330 aa (deduced amino acid sequence) was used for molecular studies. The C-terminus of the CP region exhibited the conserved regions of the restriction sites for Alu I and Rsa I enzymes indicated as present in all the D-strain isolates (Wetzel et al. 1992). In addition, the DAG (asp/ala/gly) motif, which is involved in aphidborne transmission of *Potyvirus* (Lopez-Moya et al. 1999), was found in the 5′N-ter of the CP coding region.

A phylogenetic tree was constructed among 21 isolates of the seven PPV strains and a *Potato virus Y* (PVY) (out-group) using the PPV-2 sequence. The sequences of the different isolates formed well-defined clusters delineated by the main PPV strains (D, M; EA, C, Rec, W and T) (Fig. 2). PPV-2 (Japanese plum host) from Argentina clustered with D-strain

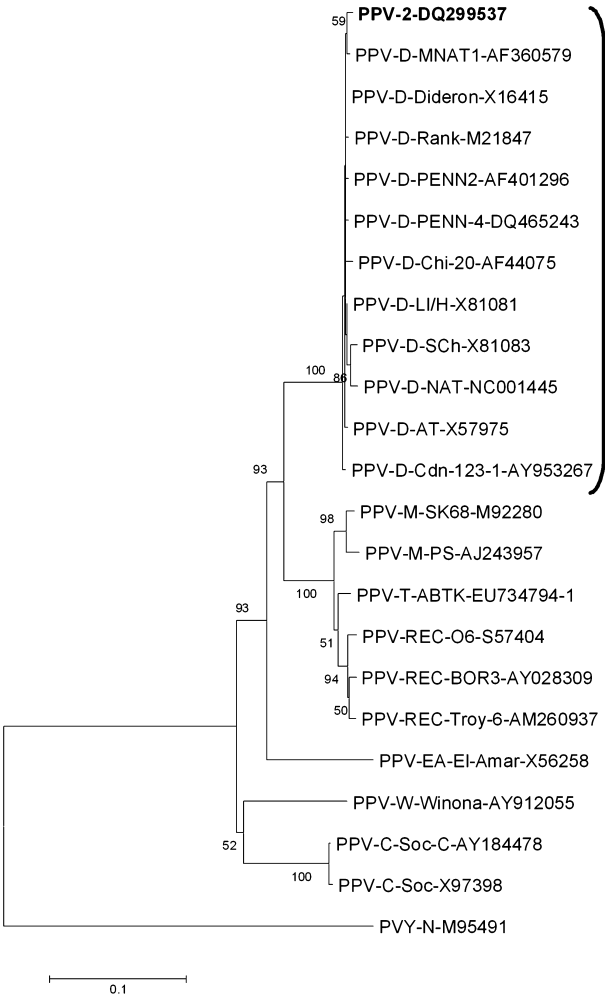

Fig. 2 Neighbour-joining phylogenetic tree of 21 *Plum pox virus* (PPV) isolates from 1220-bp CP-3′nc region. One *Potato virus Y* (PVY) sequence is used as out-group. The optimal tree with the sum of branch length = 0.88145585 is shown. The percentage of replicate trees in which the associated isolates clustered together in the bootstrap test (1000 replicates) is presented next to the nodes. The scale bar represents 0.1 substitutions per site. The evolutionary distances were computed using CLUSTAL W program with Kimura 2p method and the software MEGA-4. The isolate PPV-2 \DQ299537 was included into D-strain isolates. Accession number is next to the name of isolates.

isolates, which formed a monophyletic group (100% bootstrap). Within this group, PPV-2 grouped on the same branch as MNAT1 (\AF3600579) (peach host from USA) with 56% bootstrap. The next close branch within D isolates was D (\X16415) (unknown host), Rank (\M21847) (plum host, original isolate) PENN 2 (\AF401298) (plum host) and PENN4 (\DQ465243) (peach host) (the latter two from USA). Other PPV isolates belonging to strains M, T and REC clustered together, and isolates of strains EA, W and C were the most distant from D isolates. These phylogenetic relationships show that the PPV-2 isolate is associated with PPV isolates from plum and peach hosts, both from North America and Western Europe.

In multiple alignments, the PPV-2 sequence also had high identity with D-strain isolates (of approximately 97–99% in nucleotide and 96–98% in deduced aa sequence) and lower identity (80.9% and 89.5%) with strains C, W, Rec, M, EA, W and T. These results are consistent with those obtained in phylogenetic analyses. The identity found is in agreement with results reported for PPV based on 63 sequences from GenBank, with divergence percentages below 5% in base pairs obtained 'within' race in the nucleotide sequence of the CP region (Candresse and Cambra 2006). In addition, the identity is consistent with the 12–25% of divergence 'among' strains for PPV (Candresse and Cambra 2006) and for other isolates of any single viral species within the genus *Potyvirus* (with CP nucleotide identity above 78%) (Adams et al. 2005).

We also made an alignment with PPV-2 and PPV-8 to obtain a consensus of the D-strain isolates with which they had more than 98.4% nt identity: Cdn-123-1, AT, MNAT1, PENN2, PENN4, Rank and LI/H. The results obtained in the deduced aa sequence showed 97.9–98.8% identity of the whole CP. The analysis of the sequences of PPV-2 and PPV-8 with respect to the consensus mentioned above indicated the greatest variability at the 5′ or N-terminus of the CP region and the lowest variability at the 3′ end. The presence of two aa mutations in the two clones analysed, which are not present in the consensus of the D-strain isolates, was observed at positions 7 and 33 in the 5′ (N-terminus) of the CP, a highly variable domain within the *Potyvirus* genome (Dolja et al. 1994) (Fig. 3). Indeed, CP is one of the genome regions most widely used for characterization of a PPV isolate because of the known intragroup variability occurring among PPV isolates worldwide (Glasa 2009). On the other hand, an isolate (*Prunus* sp. infected with a PPV strain) was found to exhibit variability in different parts of a plant over time (years) of

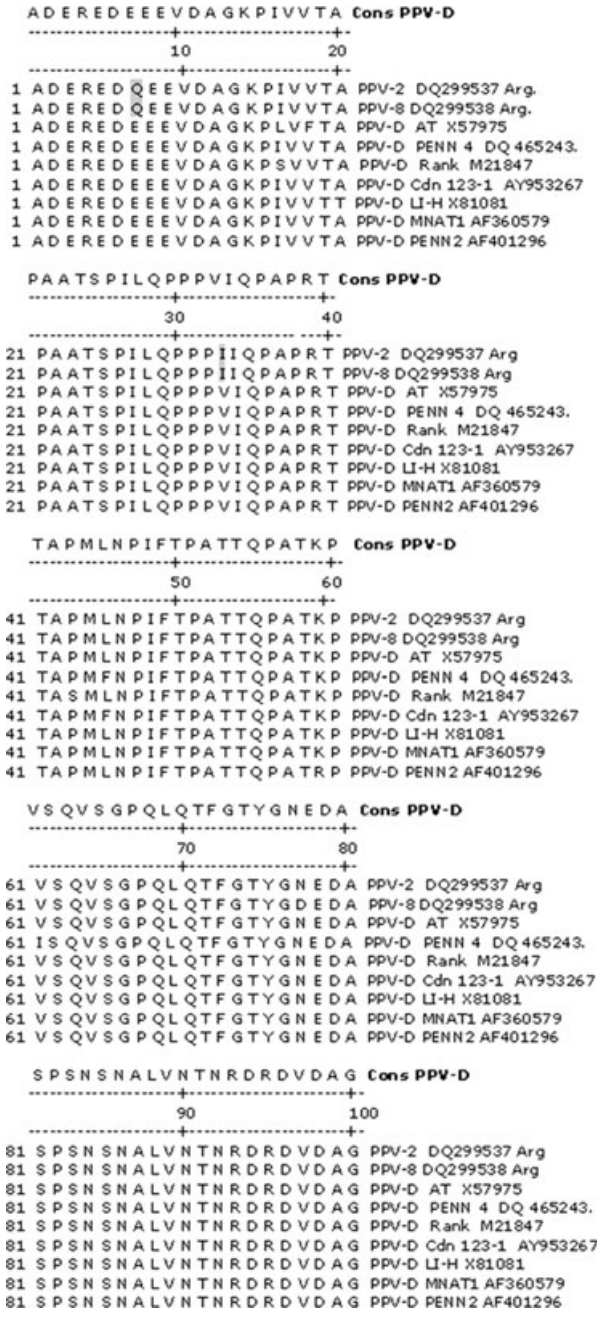

Fig. 3 Consensus of *Plum pox virus* (PPV) D-strain isolates. The PPV-2 \DQ299537, PPV-8 \DQ299538, PENN2 (\AF401296); MNAT1 (\AF360579); PENN4 (\DQ465243); LI/H (\X81081), Rank (\M21847), Cdn 123-1 (\AY953267) and AT (\X57975). N-terminus of CP is shown. The mutations at position 7, Q × E (Glutamine × glutamic) and 33, I × V (isoleucine × valine), amino acids observed in both cloned sequences with respect to the PPV-D consensus are indicated in dark.

infection, generating distinct populations that evolve independently in different tree organs (Jridi et al. 2006). Furthermore, recent studies of PPV genetic diversity based on partial sequences involving the

N-terminus of the CP region found a greater divergence within D-strain isolates (Glasa et al. 2012). In these studies, serological and molecular results allowed us to confirm the isolate as D strain of PPV. Nevertheless, this isolate presents two aa mutations at N-terminus of CP compared with a consensus of D-strain isolates. This characteristic correlates only to a single isolate from Japanese plum cv. Red Beauty obtained in the Pocito orchard. A similar observation has been reported for other South American PPV isolates (Reyes et al. 2003; Fiore et al. 2010). Our study provides the basis for future research of new isolates from the same region to explore if the variability occurs in other plants of the same cultivar and/or in other varieties grown in this area. Such studies have already been initiated in the area because PPV is a threat to fruit production in Argentina.

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