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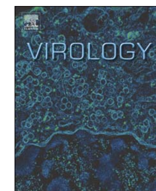

New highly divergent Plum pox virus isolates infecting sour cherry in Russia

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ABSTRACT

Unusual *Plum pox virus* (PPV) isolates (named Tat isolates) were discovered on sour cherry (*Prunus cerasus*) in Russia. They failed to be recognized by RT-PCR using commonly employed primers specific to the strains C or CR (the only ones that proved able to infect sour cherry) as well as to the strains M and W. Some of them can be detected by RT-PCR using the PPV-D-specific primers P1/PD or by TAS-ELISA with the PPV-C-specific monoclonal antibody AC. Phylogenetic analysis of the 3′-terminal genomic region assigned the Tat isolates into the cluster of cherry-adapted strains. However, they grouped separately from the C and CR strains and from each other as well. The sequence divergence of the Tat isolates is comparable to the differences between the known PPV strains. They may represent new group(s) of cherry-adapted isolates which do not seem to belong to any known strain of the virus.

1. Introduction

Plum pox virus (PPV; genus *Potyvirus*; family *Potyviridae*) is a causal agent of sharka, economically the most important viral disease of stone fruits affecting their yield and quality (Cambra et al., 2006). The PPV genome is typical of potyviruses and consists of a single stranded positive sense RNA 9.8 kb in length with a 5′-terminal viral genome-linked protein (VPg) and a 3′ poly (A) tail. A long open reading frame (ORF), flanked with the 5′- and 3′-non-coding regions (NCR), is translated into a 355 kDa polyprotein processed by virus-encoded proteases to 10 functional proteins, namely P1, HcPro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and coat protein (CP). A short overlapping ORF, called PIPO, was predicted within the P3 cistron and can be expressed by ribosomal frameshifting as a product fused with the N-terminal region of P3. Based on genomic sequence differences and phylogenetic analysis, nine PPV strains (D, M, C, EA, W, Rec, T, CR, and An) are recognized to date (Garcia et al., 2014 and the references therein).

PPV is able to infect most, if not all, *Prunus* species (Llácer and Cambra, 2006; James and Thompson, 2006; Polak, 2006). At the same time, PPV strains may differ in their host range. Among nine known strains only two, C and CR, have been proven able to infect sour cherry (*P. cerasus*) (James et al., 2013; Garcia et al., 2014). Both strains seem to have a common origin. Their adaptation to sour cherry is probably attributed to the unique amino acids located mainly in the P1, NIa and CP and conserved among PPV-C and PPV-CR isolates (Glasa et al., 2013). Apart from sour and sweet cherries as natural hosts, PPV-C is capable of infecting a number of other *Prunus* species experimentally (Nemchinov et al., 1998; Barba et al., 2011). The host range of the recently discovered strain CR remains to be determined. The CP gene of PPV-C and PPV-CR consists of 996 nucleotides encoding the corresponding protein of 332 amino acids. The known isolates of PPV-CR and some isolates of PPV-C contain the D96E mutation in the universal epitope located within the N-terminal domain of the CP. As a result, they either fail to be detected with the monoclonal antibody 5B (Chirkov et al., 2013a) or can be still recognized by this antibody after lowering the pH of the extract (Glasa et al., 2013). This mutation has not yet been discovered in other PPV strains.

The identification of the strains C and CR is based on reverse transcription-polymerase chain reaction (RT-PCR) using primers targeting the (Cter)NIb - (Nter)CP genomic region of PPV-C (Nemchinov et al., 1998; Nemchinov and Hadidi, 1998; Szemes et al., 2001) or PPV-CR (Glasa et al., 2013). Triple antibody sandwich (TAS) ELISA with the monoclonal antibody AC was also developed for the specific detection of strain C (Myrta et al., 2000). Employing these methods, a large number of PPV-C isolates was found on sour and sweet cherries in the countries of the former USSR: Moldova (Kalashyan et al., 1994; Nemchinov and Hadidi, 1996), Belarus (Malinowski et al., 2012), and Russia (Chirkov et al., 2013b; Glasa et al., 2013, 2014). In addition, isolates belonging to the strain C were sporadically observed in Italy (Crescenzi et al., 1997; Fanigliulo et al., 2003), Romania (Isak and Zagrai, 2006), Hungary (Nemchinov et al., 2008) and Croatia (Kajić et al., 2012). PPV-CR was found only in Russia (Chirkov et al., 2013a; Glasa et al., 2013).

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Fig. 1. Symptoms on the sour cherry leaf infected with the Plum pox virus isolate Tat-2.

Sour cherry is the most common stone fruit crop that is widespread throughout the European Russia. Surveys of numerous local cultivars in orchards and private gardens as well as wild trees in many geographically distant regions revealed the high incidence of PPV on sour cherry. The Russian cherry-adapted isolates have been shown to belong to the strains C or CR (Chirkov et al., 2013b, 2015; Glasa et al., 2013, 2014).

In 2015, some unusual PPV isolates were discovered under survey of sour cherry plantings in the middle Volga river region of Russia. Typical symptoms of PPV infection were observed on the leaves (Fig. 1). Despite the fact that these isolates were found on sour cherry, they were not recognized by RT-PCR with commonly employed PPV-C or PPV-CR-specific primers. At the same time, some of them could be successfully detected by RT-PCR assay using the PPV-D-specific primers P1/PD. This work was aimed at serological and molecular characterization of these isolates. The study on the 3'-terminal genomic regions showed that they represent a new group of cherry-adapted PPV isolates which does not seem to belong to any known strain of the virus.

2. Materials and methods

2.1. Plant material

The PPV isolates were sampled in cultivar and hybrid sour cherry collections situated in the middle Volga river region, the Republic of Tatarstan, Russia. Due to their location, new cherry-adapted isolates were named here as “Tat isolates”. The isolates Tat-2, Tat-3 and Tat-4 were found in some abandoned collection plots on cherry root offshoots of unknown origin. The isolate Tat-26 was detected on a hybrid of local sour cherry cultivars. Samples of the leaves displaying typical symptoms of sharka disease were subjected to serological and molecular analyses. Fresh leaves were used for the PPV detection, initial characterization of the isolates and extraction of total RNA. Additionally, symptomatic leaves were cut into small pieces, divided randomly into portions of approximately 0.5 g weight, which were lyophilized and stored at 4 °C until use.

2.2. Detection and identification of the virus by ELISA and RT-PCR

The suspected samples were analyzed by double antibody sandwich (DAS) ELISA using a reagent set SRA 31505 (Agdia, USA) and by TAS ELISA with the universal monoclonal antibody 5B, PPV-D-specific monoclonal antibody 4DG5 (Cambra et al., 1994) and PPV-C-specific monoclonal antibody AC (Myrta et al., 2000) supplied with the K-10B, K-12B and K-14B kits (Agritest, Italy), respectively. Approximately 0.1*g* of the leaf tissue was triturated in extraction buffer (2 ml) consisting of phosphate-buffered saline (PBS), 0.02% (v/v) Tween 20, 2% (w/v) polyvinylpyrrolidone (molecular weight of about 40,000), 0.5% (v/v) Triton X-100% and 0.02% (w/v) sodium azide, as was described previously (Sheveleva et al., 2012). The extracts were clarified by low speed centrifugation, added into the microplate wells (MaxiSorp microtiter plates, Nunc) and incubated in a temperature-controlled shaker for 2 h at 37 °C (450 rpm). The subsequent steps were performed following the kit supplier's instructions. The optical densities were measured 30 min after substrate addition at the wavelength of 405 nm using a Titerteck Multiscan microplate reader (Eflab Oy, Finland).

Immunocapture RT-PCR assay was performed according to Wetzel et al. (1992) using polyclonal PPV-specific antibodies (Agdia, USA), an oligo-dT primer for the first strand cDNA synthesis and the primers P1/P2 (Wetzel et al., 1991) or primers specific to the 3′-NCR (Levy and Hadidi, 1994) for the PCR. Strain typing was done by conventional PCR using the same RT preparations and primers specific for the strains C, CR, W, D and M, according to the original protocols (Olmos et al., 1997; Nemchinov and Hadidi, 1998; Szemes et al., 2001; James and Varga, 2004; Glasa et al., 2013). Experimental conditions for the RT-PCR assays were as described originally for each primer set. PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

The same extracts obtained from fresh leaves were used for DAS and TAS ELISA as well as for immunocapture RT-PCR assays. Lyophilized leaves containing the isolates 1410 (Sheveleva et al., 2012), Fl-3 (Chirkov et al., 2013a), Bg66 (Chirkov et al., 2013b) and Pav-17 (Chirkov et al., 2016) from the collection of our laboratory were used as positive controls for the strains W, CR, C and D, respectively. The PPV-M positive control was provided with the K-11B kit (Agritest, Italy). The negative control was obtained from the PPV-free sour cherry leaves.

2.3. Sequencing and analysis of the sequences

Total RNA was purified from infected leaves using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instruction, with the exception that another lysis buffer was used for RNA extraction (MacKenzie et al., 1997). The first strand cDNA was synthesized using the random hexamer and oligo-dT primers. The 3′-terminal genomic region of the Tat isolates spanning the entire CP gene and flanking sequences of the NIb gene and 3′-NCR ((Cter)NIb-CP-3′NCR) was amplified using three different primer sets. The PCR products of 1340 base pairs (bp) encompassing the entire CP gene and the most part of the 3′-NCR were amplified using the forward primer PD3W (5′-ACATAGCAGAGACAGGATTG-3′) (Sheveleva et al., 2011) and the reverse primer that targets the 3′-NCR (Levy and Hadidi, 1994). Amplification of the cDNA was performed using the proof-reading Encyclo DNA polymerase (Evrogen, Russia) and included the following cycles: denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 1 min 40 s for 35 cycles with a final extension at 72 °C for 10 min. Another 3′-terminal segment of the Tat-3 and Tat-26 genomes of 1846 bp, including a part of the NIb gene and the entire CP, was amplified using the forward Potyvirid primer 2 (Gibbs and Mackenzie, 1997) and the reverse primer 4CPR1 (Matic et al., 2011), under the conditions described by Gibbs and Mackenzie (1997). The 3′-proximal third of the NIb gene of the isolates Tat-2 and Tat-4 of 987 bp was amplified using the forward potyvirid primer NIb2F (Zheng et al., 2010) and a specially designed internal reverse primer tat2cpR (5′-ACCAAAGTTACGTTATCATCA-3′) targeting positions 15–35 in the CP gene. Amplification conditions consisted of: 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 45 °C for 30 s, 72 °C for 1 min 10 s, and 72 °C for 10 min. The PCR products were purified from the agarose gel using a Cleanup Standard kit (Evrogen, Russia) and sequenced in both directions using Evrogen facilities (Moscow, Russia).

The sequences of (Cter)NIb-CP-3′NCR genomic regions were assembled from the corresponding sequences of overlapping PCR fragments and trimmed by length to 1747 nucleotides (nt). They comprised 576 nt of the NIb gene, 996 nt of the CP together with 175 nt of the 3′-NCR and were deposited in the GenBank database under accession numbers KX685593 - KX685596. Multiple alignments were obtained using the ClustalW v.2.1 program available online at the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/) or an older version of the program implemented in the BioEdit sequence alignment editor v. 7.2.4 (Hall, 1999). Nucleotide and encoded amino acid (aa) sequence identities and genetic distances between isolates were calculated using the ClustalW algorithm implemented in

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