

## Natural deletion is not unique in the coat protein (CP) of recombinant *Plum pox virus* (PPV) isolates in Hungary

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### Abstract

Three *Plum pox virus* (PPV) isolates (Soskut1, Godollo2, Szigetcsép1), collected from apricot (*Prunus armeniaca* L.) trees in Hungary in 2008, were characterized in this study by sequence analysis of the RT-PCR amplified 3' part of the viral genome spanning the 3' part of the N1b gene the complete CP gene and the 3'UTR [3'N1b-CP-3'UTR] and also by restriction analysis of the PCR products derived from the 3' part of the P3, the complete 6K1 and the 5' end of the CI genes [3'P3-6K1-5'CI]. Phylogenetic analysis of the 3'N1b-5'CP region showed that one isolate (Godollo2) could be classified as a member of the PPV-Rec group, while the other two (Soskut1 and Szigetcsép1) belonged to PPV-D isolates. In the case of the recombinant Godollo2 isolate a 33-nucleotide (nt) in frame natural deletion was detected in the 5' part of the CP gene during the sequence analysis of the cDNA fragment corresponding to the 3'N1b-CP-3'UTR region. Currently we have reported on another Hungarian PPV-Rec isolate (PPV-B1298) collected from plum that also had a shorter CP gene bearing a much larger 135-nt in frame natural deletion at a similar position to that of the Godollo2. The PPV-D type Soskut1 isolate showed an atypical restriction pattern in the 3'P3-6K1-5'CI region using *EcoRI* and *DdeI* endonucleases, respectively. Nucleotide sequence analysis of this region indicated that its unusual pattern is as a result of a point mutation affecting the *EcoRI* restriction site.

Keywords: *Plum pox virus*, PPV, natural CP deletion mutant, *EcoRI* restriction site

### Introduction

Plum pox virus (PPV, the genus *Potyvirus*, the family *Potyviridae*) is the most important viral pathogen of *Prunus* trees in Europe. It causes serious symptoms and substantial yield losses (Németh, 1986). It was first recorded in Bulgaria around 1915 (Atanasoff, 1932), and since then it has rapidly spread throughout Europe and nowadays is present almost all over the world (Wetzel et al., 1991; Roy and Smith, 1994; Thakur et al., 1994; Milius, 1999; Thompson et al., 2001; Dal Zotto et al., 2006).

PPV has a single-stranded plus-sense genomic RNA, about 10 kilobases long. The RNA genome consists of a single open reading frame encoding a large polyprotein that is subsequently processed into functional viral proteins by viral proteinases (Urcuqui-Inchima et al., 2001). Recently a new potyviral protein has been discovered which is not part of the polyprotein (Chung et al., 2008). The RNA genome carries a virus encoded protein (the viral protein genome-linked, VPg) covalently bound to the 5' terminus and a poly(A) tail at the 3' end (Urcuqui-Inchima et al., 2001).

PPV has been classified into seven groups of isolates PPV-M, PPV-D, PPV-Rec, PPV-EA, PPV-C, PPV-W and PPV-T according to their serological and molecular features (Candresse et al., 1998; Glasa et al., 2004; James et al., 2003; Serçe et al., 2009). PPV-Rec group contains viral isolates that emerged from a natural recombination between PPV-D and PPV-M isolates with a recombination breakpoint located in the nuclear inclusion b (N1b) gene. Later on another recombination point was detected in the third protein (P3) gene (Glasa et al., 2004). A more recently identified group of closely related PPV isolates was PPV-T which is characterized by a unique recombination point in the helper component protease (HC-Pro) gene around nucleotide position 1566 (Serçe et al., 2009).

The present study reported on two PPV isolates possessing unusual molecular characteristics. One of them (Godollo2) has an 11-amino acid (aa) deletion in the N-terminal (Nt) part of the coat protein (CP) and the other (Soskut1) possesses an atypical restriction typing characteristic in the genomic region corresponding to the 3' part of the P3, the complete 6K1 and the 5' part of the cylindrical inclusion (CI) genes [3'P3-6K1-5'CI].

### Material and methods

Leaf samples showing typical symptoms of PPV infection were collected from different apricot (*Prunus armeniaca* L.) varieties from three different locations in Hungary (Sóskút, Gödöllő and Szigetcsép) in 2008. Total nucleic acids were extracted from systemically infected leaves by the method of White and Kaper (1989). Partial molecular characterization was done by RT-PCR for amplification of (i) the 3' part of the N1b gene, the complete CP gene and the 3' untranslated region (3'UTR) [3'N1b-CP-3'UTR] using primers PolyT2 and Poty7941 (Salamon and Palkovics,

2005) and (ii) the 3'P3–6K1–5'CI region using the PCI/PP3 set of primers (Glasa et al., 2002). Purified PCR products were sequenced after cloning to pGEM-T Easy vector and the PCI/PP3 amplicons were subjected to restriction analysis using *EcoRI* and *DdeI* D-type sequence specific endonucleases (Glasa et al., 2002).

Obtained nucleotide (nt) and deduced amino acid sequences were compared to other PPV sequences available in GenBank database overlapping the genomic region examined. Sequence comparisons and phylogenetic analysis were performed using the neighbor-joining method of the MEGA 3.1 software with 1000 bootstrap replicates (Kumar et al., 2004).

## Results

Three PPV isolates were investigated in this study (Table 1). CPs of all three isolates started with alanine and the DAG aa motif associated with aphid transmission (Atreya et al., 1990) found in all sequences at the Nt end of the protein. CPs of the Soskut1 and the Szigetcsep1 isolates were identical in size (330-aa residues), while the CP of the Godollo2 isolate was shorter. In the case of the Godollo2 in frame natural deletion was detected in the CP gene during the sequence analysis of the RT-PCR amplified cDNA fragment corresponding to the 3'N1b–CP–3'UTR region. The Godollo2 isolate had a 33-nt deletion at the 5' end of the CP gene, which corresponds to an 11-aa deletion in the Nt region of the CP downstream to the DAG motif (Figure 1).

**Tab. 1** Original hosts and specific molecular groups of PPV isolates investigated in this study and their GenBank accession numbers

Isolates	Localities	Original hosts	Groups	Accession numbers
PPV-Soskut1	Sóskút	<i>P. armeniaca</i> 'Bergeron'	PPV-D	FN179152 <sup>a</sup> , FN179155 <sup>b</sup>
PPV-Godollo2	Gödöllő	<i>P. armeniaca</i>	PPV-Rec	FN179153 <sup>a</sup>
PPV-Szigetcsep1	Szigetcsép	<i>P. armeniaca</i> hybrid 10/7	PPV-D	FN179154 <sup>a</sup>

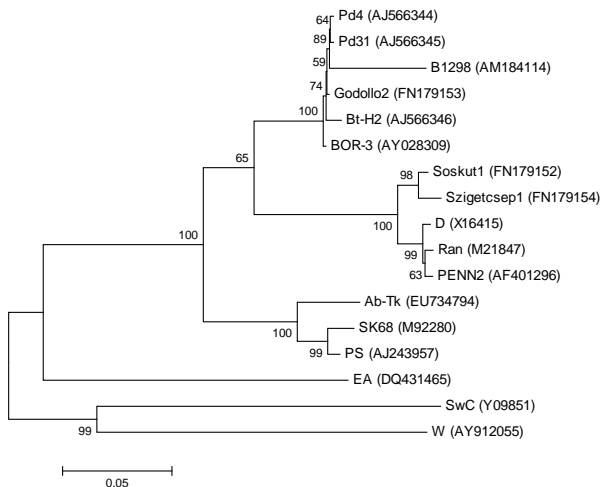
a: 3'N1b–CP–3'UTR, b: 3'P3–6K1–5'CI

	1	92
Soskut1	ADEREDEEEVD <u>AG</u> KPIVVTAPAATSPILQPPVVIQAPRATAPMFNPIFTPATTQPATTPVSRVSGPQLQTFGTGHGNE <span style="text-decoration: underline;">DAS</span> SPNSNALVTTN	
Szigetcsep1	ADEREDEEEVD <u>AG</u> KPIVVTAPAATSPILQPPVVIQAPRITAPMFNPIFTPATTQPATKPVSRVSGPQLQTFGTGYGNE <span style="text-decoration: underline;">DAS</span> SPNSNALVNTN	
Godollo2	ADEKEDDEEVD <u>AG</u> KPVTVTAATVATTQPAPVIQPAIQTTTPMFNPIFTPATTQPAT-----QSGVGYGNE <span style="text-decoration: underline;">GAS</span> SPSTNTLVNTG	
B1298	ADEKEDDEEVD <u>AG</u> KPPVVTAPAATVATTQPAPVIQPAIQTTT-----LVNTG	
NAT	ADEREDEEEVD <u>A</u> -----LQPPVVIQAPRITAPMLNPIFTPATTQPATKPVSVSGPQLQTFGTYSHE <span style="text-decoration: underline;">DAS</span> SPNSNALVNTN	
SH	ADEREDEEEVD <u>A</u> -----ILQPPVVIQAPRITAPMFNPIFTPATTQPATKPVSVSGPQLQTFGTGYGNE <span style="text-decoration: underline;">DAS</span> SPNSNALVNTN	
KAZ	ADEREDEEEVD <u>AG</u> KPIVVTAPAATSPILQ--VIQAPRITAPMFNPIFTPATTQPATKPVSVSGPQLQTFGTGHGNE <span style="text-decoration: underline;">DAS</span> SPNSNALVNTN	

**Fig. 1** Multiple amino acid alignment of the Nt CP region of PPV isolates. The DAG motif is underlined. Soskut1 (Acc. No.: FN179152), Szigetcsep1 (Acc. No.: FN179154), Godollo2 (Acc. No.: FN179153), B1298 (Acc. No.: AM184114), NAT (Acc. No.: D13751), SH (Acc. No.: X81073), KAZ (Acc. No.: AY591253).

Nucleotide and deduced amino acid sequences of the CP regions of Soskut1 and Szigetcsep1 Hungarian apricot isolates were most identical to PPV-D isolates, while Godollo2 showed the highest sequence similarities to isolates having M-type CP region (members of the PPV-M and the PPV-Rec groups), and it was the most similar to PPV-Rec isolates. It is known that recombination causes changes in the sequence of the CP, or in the CP gene, but the 3' recombination point is located upstream to the CP coding region, at the 3' end of the N1b gene (Glasa et al., 2004).

To examine the recombinant nature of these three Hungarian PPV isolates phylogenetic analysis was performed using nt sequence data corresponding to the 3' end of the N1b and the 5' end of the CP genes [3'N1b–5'CP, nt 8050–8902]. The phylogenetic tree clearly showed the clustering of the Godollo2 isolate with some previously characterized recombinant (PPV-Rec) isolates, while the Soskut1 and the Szigetcsep1 clustered with PPV-D isolates (Figure 2).



**Fig. 2** Phylogenetic tree of PPV isolates based on nucleotide sequence data corresponding to the 3'NIB-5'CP (nt 8050-8902) genomic region. Bootstrap values are presented next to tree nodes. The scale bar represents 0.05 substitutions per site. Accession number of PPV isolate is in brackets after the name of isolate.

As an additional step for molecular characterization of the three Hungarian PPV isolates the 3'P3-6K1-5'CI (nt 2976-3696) region were also investigated. The RT-PCR amplified products derived from this genomic region were subjected to restriction analysis using *EcoRI* and *DdeI* endonucleases, respectively, in order to characterize this region. These restriction enzymes cleave cDNA fragments amplified from isolates having a D-type genome in the analysed region (members of PPV-D and PPV-Rec groups), while do not recognize cDNAs derived from isolates having an M-type genome (PPV-M isolates) in the 3'P3-6K1-5'CI region (Glasa et al., 2002). The *DdeI* enzyme could cleave cDNA fragments amplified from all three Hungarian PPV isolates, while *EcoRI* could only cut PCR products derived from the PPV-Rec type Godollo2 and the PPV-D type Szigetcssep1, but could not cleave cDNA obtained from the PPV-D type Soskut1 isolate similar to the control PPV-M type sequence. Thus, the RFLP analyses using D-type sequence-specific enzymes resulted in an atypical restriction pattern in the case of the Soskut1 isolate. The PCR fragment could be recognized only by *DdeI*, but not by *EcoRI*, although this isolate was determined as PPV-D type according to the 3'NIB-5'CP region. Such unusual typing behaviour could arise from point mutations affecting the recognition site but may also indicate a possible recombination event between PPV-D and PPV-M isolates. Sequence analysis revealed that the sixth base of the *EcoRI* site (3410 nt position, GAATTC>GAATTT) has been changed from C to T. Phylogenetic analysis performed using nucleotide sequences of PPV isolates corresponding to the 3'P3-6K1-5'CI region showed that despite the lack of the *EcoRI* restriction site the Soskut1 isolate has a D-type genome in the 3'P3-6K1-5'CI region (data not shown).

## Discussion

On the basis of the sequence similarities of the CP region and phylogenetic analyses generated using nucleotide sequences corresponding to the 3'NIB-5'CP (nt 8050-8902) genomic region, one isolate (Godollo2) out of the three belonged to the PPV-Rec group, while the remaining two (Soskut1, Szigetcssep1) could be classified as members of the PPV-D group.

The atypical typing property of the Soskut1 isolate in the 3'P3-6K1-5'CI region resulted from a point mutation in the *EcoRI* cleavage site as compared to D-type sequences, the unusual restriction pattern was not as a consequence of a recombination event in this region. Previously we have reported on a Bulgarian isolate (PPV-Troy6) collected from plum that showed also an abnormal typing property in the 3'P3-6K1-5'CI region (Szathmáry et al., 2009b). In that case the PCR product could only be cleaved by *EcoRI*, but not by *DdeI*, although this isolate was determined as a member of PPV-Rec group according to the 3'NIB-5'CP region. In the case of the PPV-Troy6 a point mutation (A3102 to C3102, CTNAG>CTNCG) was also responsible for the lack of the *DdeI* cleavage site. Our earlier and present study indicates

some limitation of restriction enzyme mapping for proper classification of PPV isolates. The accurate identification of specific PPV isolate groups can only be achieved using different methods (restriction analysis, sequence analysis) targeting the same genomic region or by parallel investigation of different genomic regions.

In the case of the Godollo2 Hungarian PPV-Rec isolate in frame deletion was detected in the CP gene during the sequence analysis of the RT-PCR amplified cDNA fragment corresponding to this region. Currently we have reported on another Hungarian PPV-Rec isolate (PPV-B1298) collected from plum bearing a much larger, 135-nt (45-aa) in frame natural deletion at a similar position to that of Godollo2 (Szathmáry et al., 2009a). There are only three other known examples for natural CP deletion mutant PPV isolates (PPV-NAT, PPV-SH, PPV-KAZ) (Maiss et al., 1989; Deborré et al., 1995; Spiegel et al., 2004). In all cases, deletions were located in the N-terminal (Nt), hypervariable region of the CP similar to Godollo2. It is known that deletions in the CPs of PPV-NAT and PPV-SH isolates affect the DAG motif, while this motif in the CPs of PPV-B1298 and PPV-Godollo2 isolates are not affected by the deletions.

Different forms of ELISA technique are widely used in almost all quarantine laboratories worldwide for the detection of plant viruses for decades: ELISA based on the detection of the viral CP using antibodies produced usually against the Nt region. Thus, a deletion affecting the Nt domain should have a significant role in virus detection as we currently showed in the case of PPV-B1298 isolate (Szathmáry et al., 2009a). Occurrence of deletions in the highly immunogenic regions could result in a failure of detection in spite of using well-characterized and widely used serological diagnostic reagents for plant virus identification, which could be dangerous with regard to the safe detection of PPV infection.

Our present study and previous data suggest that the presence of a natural deletion in the N-terminal part of the CP in the Hungarian recombinant PPV population is not unique.

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