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Cloning of the complete infectious cDNA of the plum pox virus strain PPV-Rec

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Summary. – Plum pox virus (PPV) is the causal agent of Sharka, considered to be the most detrimental viral disease of *Prunus* spp. worldwide. So far, several PPV strains have been recognized, three of them (PPV-D, PPV-M, and PPV-Rec) having shown serious economic impact in the European area. Infectious cDNA clones of plant RNA viruses are excellent tools for functional studies of viral genomes. Preparation and use of PPV-D and PPV-M infectious clones have been previously reported. Here we describe the construction of an infectious cDNA clone of the strain PPV-Rec (isolate BOR-3) by the strategy involving the subsequent exchanges of homologous BOR-3 genome parts in the backbone of the previously prepared PPV-D infectious construct. The infectivity of each intermediate chimeric cDNA as well as that of the final construct (pIC-PPV-Rec) was confirmed by biolistic transfection of *Nicotiana benthamiana* plants. Complete sequence of the cloned viral BOR-3 cDNA revealed 0.14% of difference at the nucleotide level compared to original BOR-3 sequence, resulting in four amino acid changes. This slight inequality was related to the population heterogeneity of the initial BOR-3 isolate; no difference in the amino acid sequence resulted from the cloning steps performed.

Keywords: inter-strain chimera; biolistics; genome sequence

Plum pox virus (PPV, the family *Potyviridae*) belongs to the most destructive viruses of stone fruit trees. PPV is a (+)ssRNA virus with the genome coding for a polyprotein which is processed to functional polypeptides (Salvador *et al*., 2006). Seven PPV strains are currently recognized (Ulubaş-Serçe *et al*., 2009). Three of them (PPV-M, PPV-D, and PPV-Rec) are epidemiologically important in middle Europe. Besides their variation in the genome sequence, few peculiar epidemiological properties can be linked with a particular strain, especially the ability to infect or its preference to natural *Prunus* host species (Crescenzi *et al*., 1997; Candresse and Cambra, 2006). Although PPV-Rec isolates are able to infect peaches under experimental conditions (Glasa *et al*., 2004), the infection of peaches under natural field conditions by PPV-Rec is very scarce (M. Glasa and Z. Šubr, unpublished data; Kamenova *et al*., 2011).

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Abbreviations: CP = capsid protein; PPV = plum pox virus

PPV-Rec originated from a homologous recombination event between PPV-D and PPV-M in the region coding for viral replicase NIb (Glasa *et al*., 2004).

Infectious clones of PPV-M and PPV-D have been prepared (Sáenz *et al*., 2000; Varrelmann *et al*., 2000; Raghupathy *et al*., 2006). The previous attempt to prepare infectious cDNA of PPV-Rec by long RT-PCR or by joining few separate PCR products in unique restriction sites didn´t succeed and none of such obtained full-length clones were able to infect plants (Nagyová *et al*., 2011). Therefore, a strategy involving the modification of the infectious clone pIC-PPV (López-Moya and García, 2000) by step-by-step exchange of the PPV genome parts by their homologues from the PPV-Rec isolate BOR-3 (Glasa *et al*., 1997) were engaged using natural or constructed restriction sites. The main advantage of this approach was that the infectivity of each intermediate construct could be verified before further cloning steps were performed.

Infectious clone pIC-PPV was kindly obtained from Prof. J. A. García (CSIC Madrid). It is a cDNA of a PPV-D genome cloned in a pGEM3-based vector (Promega). cDNA of the PPV isolate BOR-3 was prepared as described earlier (Glasa *et al*., 2004). LA

Taq DNA polymerase (TaKaRa) was used for subsequent PCR and all amplimers were verified by sequencing. FastDigest restriction endonucleases and T4 DNA ligase (all from Fermentas) were applied in the cloning procedures described below. DNA fragments were gel-purified using Wizard SV gel and PCR clean up system (Promega). Plasmids were multiplied in *Escherichia coli* JM109 and purified by Pure yield plasmid miniprep system (Promega). The experimental plants were maintained in the growth chamber at 20°C and 14h/10h light/dark period. The *Nicotiana benthamiana* plants were biolistically transfected by cDNA constructs as described previously (Predajňa *et al*., 2010). Other host plant species (*N. occidentalis* and *Pisum sativum* cv. Colmo) were infected by mechanical reinoculation from *N. benthamiana*. The infectivity was evaluated by *in situ* symptom observation and by immunoblotting of capsid protein (CP) using polyclonal anti-PPV antiserum (Šubr and Matisová, 1999).

Complete construction of the BOR-3 infectious cDNA clone composed of several steps (for the positions of restriction sites see the scheme in Fig. 1):

1. The extreme 3′ terminus of PPV isolate SK68 (identical to BOR-3 except one terminal nucleotide) was excized from the clone pPPV-SK68 (kindly provided by Prof. L. Palkovics, CU Budapest) by XbaI/EcoO109I digestion. Relevant part of the pIC-PPV (López-Moya and García, 2000) was replaced by this 154 bp fragment involving 63 terminal nucleotides of the PPV genome, the polyA tail and a short part of the plasmid vector.
2. A 1208 bp fragment was amplified from the BOR-3 cDNA by the primers TCTAGAGCTGATGAAAA GGAGGACGA (F) and TTTTTTTTTTTTTTTTTT TTCTCTTGTAC (R) and digested by SacI/EcoO109I to gain the fragment of 704 bp (nt positions 9021–9723). This fragment was used to replace its homologue in the previous construct (#1) digested by the same restriction enzymes.
3. A 1349 bp fragment was amplified from the BOR-3 cDNA by the primers TGGGACAAACTGCTTAGAGC (F) and CGCTTAACTCCTTCATACCAAG (R) and digested by SacI/BstXI to gain the fragment of 1276 bp (nt 7745–9020). This fragment was used to replace its homologue in the previous construct (#2) digested by the same restriction enzymes.
4. A 1315 bp fragment was amplified from the BOR-3 cDNA by the primers GCACTGGATTAGTACGAAGG (F) and GTAT CCAAGCTTCAGGAGTG (R) and digested by XhoI/BstXI to gain the fragment of 978 bp (nt 6767–7744). This fragment was used to replace its homologue in the previous construct (#3) digested by the same restriction enzymes.
5. A 3736 bp fragment was amplified from the BOR-3 cDNA by the primers TACGTACGATATCTCCG TTCGGT (F) and CCCAAGAATACTGCCGTCTC (R) and digested by XhoI/SphI to gain the fragment of 2711 bp (nt 4056–6766). This fragment was used to replace its homologue in the previous construct (#4) digested by the same restriction enzymes.
6. A 2446 bp fragment was amplified from the BOR-3 cDNA by the primers AATCTAGAAAATATAAAAAC TCAACACAACATAC (F) and CCTCCAACCAG GTATGTTTTC (R). One mismatch in the reverse primer (underlined) introduced the SexAI cleavage site in the product which was subsequently digested by RsrII/SexAI to gain the fragment of 1931 bp (nt 505–2435). This fragment was used to replace its homologue in the previous construct (#5) digested by the same restriction enzymes.
7. A 1345 bp fragment of the plasmid vector including the CaMV promoter region was amplified from the pIC-PPV by the primers ATTAATGCAGCTGGCTTATCG (F) and GAGTTTTTATATTTTCCTCTCCAAATGAAA TGAAC (R). The reverse primer included 15 nucleotides of the 5′-terminal PPV BOR-3 sequence (underlined). A 590 bp 5′-terminal fragment was amplified from the BOR-3 cDNA by the primers AATCTAGAAAATATAAAAAC TCAACACAACATAC (F) and AGGTTTCTCAATAATA TGAGGG (R). Both fragments were purified and mixed in a new amplification reaction serving each for the other as template and primer. Resulting product of 1920 bp was digested by RsrII/PvuII to gain the fragment of 1824 bp (including the promoter region and BOR-3 nt 1–504) and used to replace its homologue in the previous construct (#6) digested by the same restriction enzymes.
8. A 2538 bp fragment was amplified from the BOR-3 cDNA by the primers CGCGAACTAGCGCGATATCAG (F) and TGGAGTTGATCCAAAGGTGC (R) and digested by SexAI/DraIII to gain the fragment of 469 bp (nt 2436–2904). This fragment was used to replace its homologue in the previous construct (#7) digested by the same restriction enzymes.
9. The same 2538 bp amplimer was digested by PsyI/SphI to gain the fragment of 947 bp (nt 3109–4055). This fragment was used to replace its homologue in the previous construct (#8) digested by the same restriction enzymes.

The obtained construct preserved a 189 bp intron I from the *ST-LS-1* gene of potato (Vancanneyt *et al*., 1990) introduced into pIC-PPV by its authors (López-Moya and García, 2000) to avoid the toxicity of PPV *P3* gene for *Escherichia coli* (Maiss *et al*., 1992). Due to the absence of suitable restriction sites, 205 bp from the original pIC-PPV remained unchanged around the intron sequence. However, this short pIC-PPV-derived genome part differs from the BOR-3 sequence only by four nucleotides.

The full-length sequence of the BOR-3 isolate (referred as pIC-PPV-Rec) was deposited in the GenBank under Acc. No. JQ794501. The complete intron-subtracted sequence of pIC-PPV-Rec differs from the originally published BOR-3 (AY028309, Glasa and Šubr, 2005) by 14 of 9787 nucle-

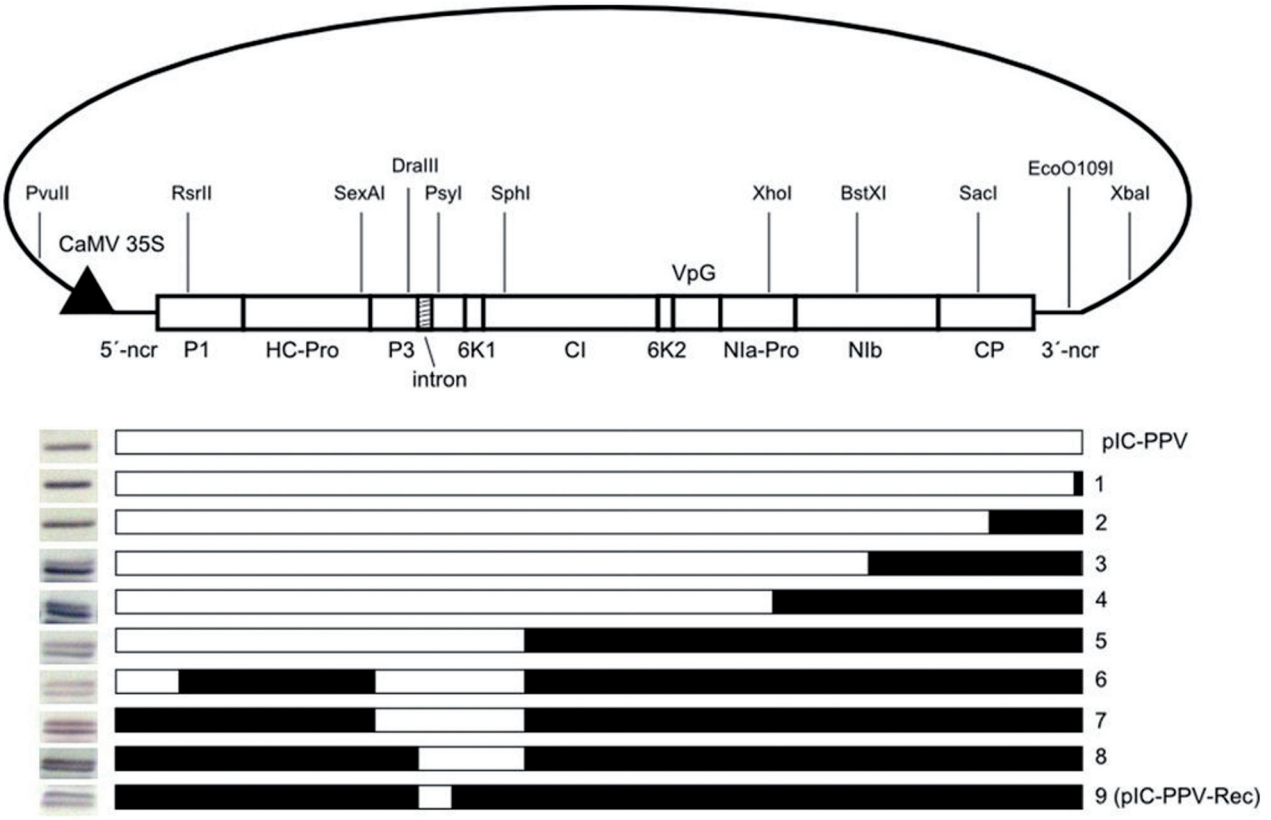

Fig. 1

Scheme of the infectious clone pIC-PPV-Rec

PPV genome with inserted intron (shaded) and restriction sites used for cloning is shown in the top. The boxes representing PPV-D (white) and PPV-Rec (black) sequences demonstrate the cloning steps 1–9 described in the text. Immunoblots of sap from *N. benthamiana* infected by particular construct are shown in the left margin.

otides (0.14%) resulting in four amino acids substitutions (0.13%). Six of the nucleotide differences resulted from the cloning procedures as they originated from the donor clones (pIC-PPV, SK68) or from the primer (see #6 of the detailed protocol). All of these six exchanges were silent. On the other hand, four nucleotide differences along the genome reflected also changes at the amino acid level. In each of these cases, several PCR products were sequence-verified before cloning, so the differences were not artifacts of inaccurate amplification. They rather originated from viral genomic RNA and reflected the heterogeneity of RNA population.

All intermediate chimeras as well as the final construct pIC-PPV-Rec were infectious in *N. benthamiana*, *N. occidentalis,* and *Pisum sativum* cv. Colmo. Immunoblotting analyses confirmed the changed electrophoretic profile of the viral CP to a double-band typical for PPV-Rec (Šubr *et al*., 2010) after replacing the genome part coding the N-terminal CP region (Fig. 1).

Presented protocol took in advance the highly efficient biolistic transfection (Predajňa *et al*., 2010) which enabled clear infectivity verification of the constructs in experimental herbaceous plants in a short time (7–10 days). Prepared cDNA clone of the BOR-3 isolate will be implemented in further research. It should help to identify the genetic determinants involved in the pathogeny of the PPV-Rec in different *Prunus* host plants, map specific PPV-Rec biological properties and, in larger extend, to clear the role of particular PPV genes in virus-host interactions.

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