A SIMPLIFIED RT-PCR-BASED DETECTION OF RECOMBINANT PLUM POX VIRUS ISOLATES

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Summary. – Closely related natural Plum pox virus (PPV) isolates derived from homologous RNA recombination between PPV-D and PPV-M have been recently identified and shown naturally spread in several European countries. As their serological properties were identical with those of conventional PPV-M isolates, they could be detected only by combined analysis of at least two different genome parts. To simplify the detection of such recombinants primers specific to PPV-M and PPV-D sequences with binding sites located on both sides of the recombination crossover situated in the C-terminal part of NIb were designed. They were used for direct differentiation of PPV-M, PPV-D and their recombinants by reverse transcription–polymerase chain reaction (RT-PCR). This method is convenient for identification of a recombinant PPV in single as well as mixed infection with PPV-M or PPV-D.

Key words: Sharka; recombination; molecular diagnostic

PPV is an aphid-transmitted potyvirus causing sharka disease of stone fruit trees. Based on their serological and molecular variability, PPV isolates identified so far have been divided into four subgroups, from which the most prevalent are PPV-D and PPV-M. They are recognizable by specific monoclonal antibodies as well as by PCR/restriction fragment length plymorphism (PCR/RFLP) techniques (Candresse *et al.*, 1998). Although the isolates of both main subgroups differ in the nucleotide sequence along the whole genome, methods based exclusively on capsid protein (CP) or CP gene have been used to discriminate between them until recently. Sequence analysis of CP and P3-6K1 of several isolates evidenced the existence of isolates arisen by homologous recombination between PPV-M and PPV-D (Glasa *et al*., 2001, 2002a, b). Furthermore, recombinant PPV isolates were found to occur in several south- and middle-European countries. Invariably of their origin and time of isolation, all the recombinant isolates presented a close molecular relationship and the same position of the recombination crossover situated in the 3-terminal part of the viral replicase (NIb) gene (Fig. 1A) (Glasa *et al*., 2004). Because of high virulence and aphid transmissibility of recombinant PPV isolates (Glasa *et al*., 2002b), their early and specific detection is highly desirable. However, conventional CP gene-based methods and specific monoclonal antibodies do not discriminate between PPV-M and recombinant isolates as the recombination crossover is positioned upstream of the CP gene. Two independent RT-PCR assays targeting different genome parts upstream and downstream of the crossover, followed by RFLP analysis, were necessary to identify recombinant PPV isolates. Alternatively, two RT-PCR assays with subgroup-specific primers could be used instead of one or both RT-PCR/RFLP assays (Glasa *et al*., 2002b).

In this study we describe an RT-PCR using subgroup-specific primers spanning the region around the recombination crossover and enabling direct discrimination between distinct PPV isolates.

Thirty PPV sequences retrieved from the GenBank database (www.ncbi.nlm.nih.gov) were multiple aligned

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Abbreviations: PPV = Plum pox virus; RT-PCR = reverse transcription–polymerase chain reaction; RFLP = restriction fragment length polymorphism; CP = capsid protein

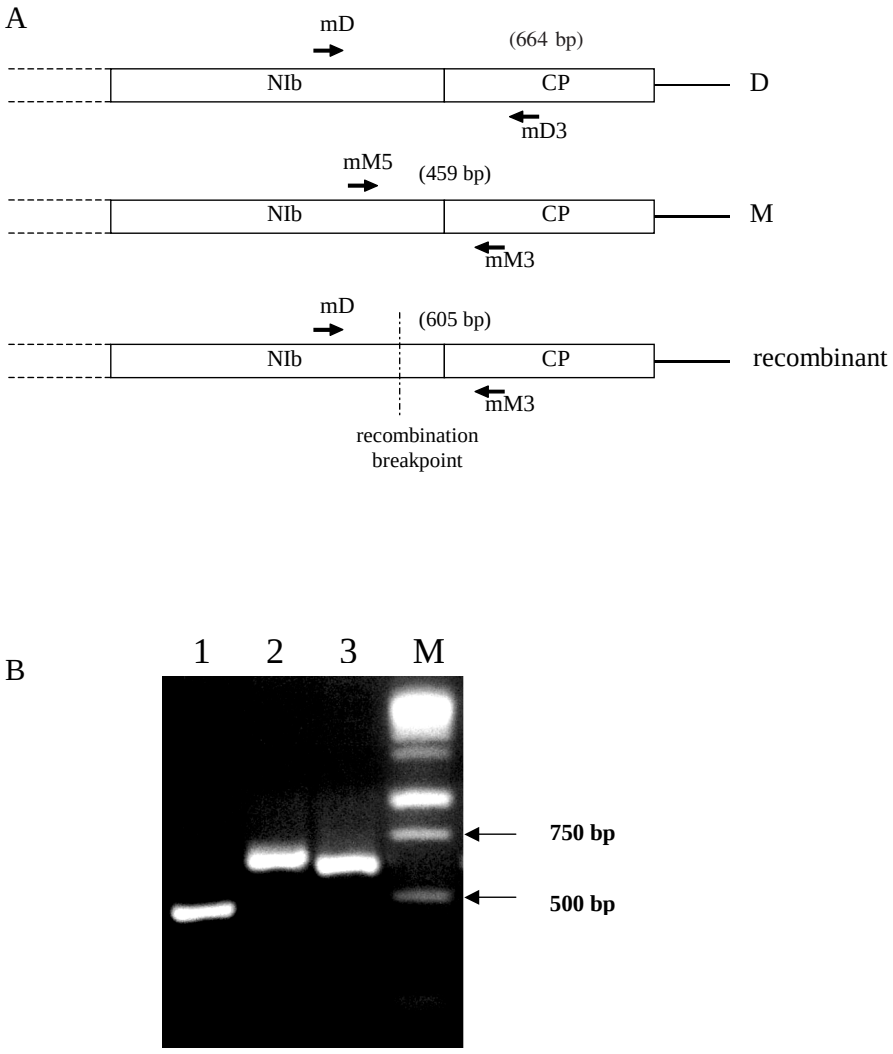

Fig. 1

RT-PCR of PPV of different subgroups

Agarose gel (2%) electrophoresis. Staining with ethidium bromide.

A. Scheme of the 3'-terminal parts of genomes of PPV-D, PPV- M and recombinant PPV showing the location of primer binding sites and respective amplimer lengths. Positions of the primers mD5 (nt 8207–8228), mD3 (nt 8851–8870), mM5 (nt 8350–8370), and mM3 (nt 8786–8808) are based on the sequence of PPV-PS (GenBank Acc. No. AJ243957).

B. Specific detection of PPV-M, PPV-D and a recombinant PPV with relevant primer pairs. Isolates and primers used: CAH-2, mM5/mM3 (lane 1), BOJ-3, mD5/mD3 (lane 2), BOR-3, mD5/mM3 (lane 3). The BenchTop 1 kbp ladder (Promega, lane M).

using ClustalX (Thompson *et al*., 1997) in order to find the best primer binding sites in the loci with high intra- and low intersubgroup sequence variability (PPV-D vs. PPV-M) both up- and downstream of the recombination point (Glasa *et al*., 2001). The location of binding sites of the primers mM5 (5'-GCTACAAAGAACTGCTGAGAG-3'), mM3 (5'-CAT TTCCATAAACTCCAAAAGAC-3'), mD5 (5'-TATGTC ACATAAAGGCGTTCTC-3'), and mD3 (5'-GACGTCCC TGTCTCTGTTTG-3') is shown in Fig. 1A.

The detection method was optimized using the well characterized PPV isolates CAH-2 (PPV- M), BOJ-3 (PPV-D) and BOR-3 (natural PPV recombinant) (Glasa *et al*., 1997) and subsequently applied to test more than 20 PPV isolates kept at this Institute. Total RNA was isolated from original *Prunus* host or mechanically inoculated *Nicotiana benthamiana* leaf tissues using the RNeasy Plant Mini Spin Kit (Qiagen) according the manufacturer's instructions. RT was performed with Avian myeloblastis virus reverse

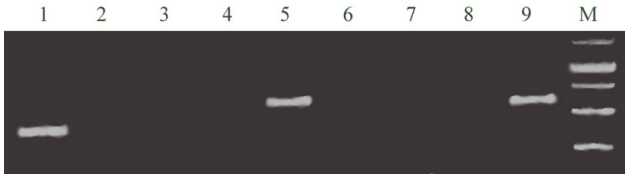

Fig. 2

Demonstration of RT-PCR specificity

Agarose gel (2%) electrophoresis. Staining with ethidium bromide. cDNA obtained from the isolates CAH-2 (lanes 1–3), BOJ-3 (lanes 4–6), and BOR-3 (lanes 7–9) were amplified using the primer pairs mM5/mM3 (lanes 1, 4, and 7), mD5/mD3 (lanes 2, 5, and 8), and mD5/mM3 (lanes 3, 6, and 9). The BenchTop 1 kbp ladder (Promega, lane M).

transcriptase and random hexanucleotide primers (both from Promega) as described previously (Glasa *et al*., 2002b). PCR was carried out with 2.5 U of *Taq* DNA polymerase (Promega) in a reaction buffer containing 2 mmol/l $MgCl_2$, 10 mmol/l dNTPs, 100 nmoles of each primer in a thermal cycler (Biometra) as follows: initial denaturation at 94°C for 3 mins, 35 cycles of 94°C/45 secs (denaturation), 60°C/30 secs (annealing), and 72°C/60 secs (elongation), and final extension at 72°C for 7 mins.

When applied to the CAH-2, BOJ-3 and BOR-3 isolates, the RT-PCR with respective primer pairs resulted in products of expected size (Fig. 1B). No cross-reactivity with subgroup non-specific primers was observed (Fig. 2). Furthermore, the described RT-PCR protocol has been successfully applied to the testing of more than 20 isolates of PPV-M, D and PPV subgroup recombinants (data not shown). Its suitability for detection of a mixed infection with PPV-D and PPV-M, PPV-D or PPV-M with recombinant PPV, and their triple mix was confirmed by separate testing of artificially prepared mixed samples from total RNAs with the three primer pairs. The results of PCR amplification demonstrated that this method is suitable for detecting possible mixed infections with PPV of different subgroups including recombinant PPV (Fig. 3).

Specific and fast detection of harmful viruses is essential to ensure efficient phytosanitary measures. In case of PPV, the typing method of the first choice is routinely based on immunochemical properties of the virus (subgroup-specific antibodies) or restriction analysis of the 3'-terminal part of the CP gene (Candresse *et al*., 1998). However, in the light of recent knowledge of the PPV variability and existence of recombinant PPV, a combination of these techniques with the elaborated RT-PCR using relevant primer pairs may be regarded as satisfactory and reliable PPV typing protocol for confirmation of a PPV-D or for differentiation between a PPV-M and a recombinant isolate.

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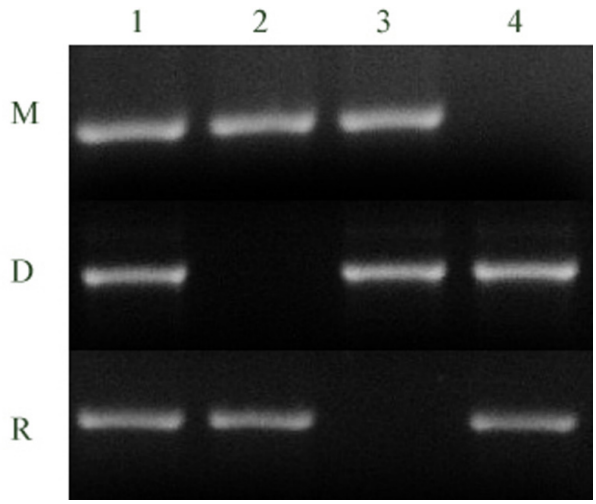

Fig. 3

Detection of RNAs of individual PPVs of different subgroups in their artificial mixtures by RT-PCR

RNAs of PPV isolates: CAH-2, BOJ-3, and BOR-3 (lane 1), CAH-2 and BOR-3 (lane 2), CAH-2 and BOJ-3 (lane 3), and BOJ-3 and BOR-3 (lane 4). Primer pairs: mM5/mM3 (row M), mD5/mD3 (row D), and mD5/mM3 (row R).

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