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DIFFERENTIATION OF PLUM POX VIRUS ISOLATES BY SINGLE-STRAND CONFORMATION POLYMORPHISM AND LOW-STRINGENCY SINGLE SPECIFIC PRIMER PCR ANALYSIS OF HC-Pro GENOME REGION

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Summary – Single-strand conformation polymorphism (SSCP) and low-stringency single specific primer (LSSP)-PCR were assessed for suitability and reliability in genotyping of Plum pox virus (PPV) isolates. Examined PPV isolates included 16 PPV-D, 12 PPV-M, and 14 PPV-Rec isolates collected in Czech Republic. The analysis was performed on the helper component protease (HC-Pro) region of the PPV genome. SSCP and LSSP-PCR allowed the differentiation of PPV strain, but SSCP was not able to distinguish isolates within the same strain. The individual genotyping of each PPV isolate was obtained by LSSP-PCR. Nevertheless, both SSCP and LSSP-PCR techniques are suitable for preliminary screening of genetic variability of plant RNA viruses.

Keywords: Plum pox virus; strains; isolates; single-strand conformation polymorphism; low-stringency single primer PCR; HC-Pro region

Introduction

PPV is a member of the genus *Potyvirus* and is the causal agent of sharka disease. PPV contains single stranded positive-sense RNA genome of about 10 kb with a single ORF (Riechmann *et al*., 1992). Recently, six strains of PPV (PPV-C, PPV-D, PPV-EA, PPV-M, PPV-Rec, and PPV-W) have been identified (James and Varga, 2005; Glasa *et al*., 2004; Candresse *et al*., 1998; Nemchinov and Hadidi, 1996; Wetzel *et al*., 1991; Kerlan and Dunez, 1979). The occurrence of the virus isolates belonging to the three major strains PPV-D, PPV-M, and PPV-Rec was confirmed in Czech Republic (Gadiou *et al*., 2008; Navrátil *et al*., 1998). Potyviral HC-Pro protease is a multifunctional protein involved in a wide spectrum of processes, such as vector transmission, replication, virus movement, and suppression of plant defense (Salvador *et al*., 2006). Currently only limited data are available about the molecular variability of this genome segment (Škopek *et al*., 2003). Most sequence studies are focused upon the opposite 3' terminal part of the PPV genome (Glasa *et al*., 2004).

SSCP and LSSP-PCR techniques represent an interesting tool for genotyping of viruses. They were successfully applied for a variability study of Hepatitis C virus, Grapevine leaf roll associated virus 3, Cucurbit yellow stunting disorder virus, and also for the study of genetic polymorphism in *Trypanosoma cruzi* (Brito *et al*., 2008; Turturo *et al*., 2005; Rubio *et al*., 2001; Stamenkovic *et al*., 2001). The SSCP technique requires a denaturation of cDNA followed by a 3-dimensional folding of the single strand, what results in a different conformation according to the DNA sequence. This allows differentiation of the sequences by gel electrophoresis that separates different conformations of DNA strands (Orita *et al*., 1989). The mechanism by which LSSP-PCR works is not completely understood. It is assumed that the production of multiple fragments is related

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Abbreviations: HC-Pro = helper component protease; IC-RT = immuno-capture RT; LSSP-PCR = low-stringency single specific primer PCR; PPV = Plum pox virus; SSCP = single-strand conformation polymorphism

to the specificity of primer annealing to its complementary extremity and with lower specificity to various sites present in the interior of fragment, what produces the fragments with variable sizes (Pena *et al*., 1994).

In this paper, we have extended the knowledge about a variability of HC-Pro gene and compared application of SSCP and LSSP-PCR techniques for genetic characterization of 42 PPV isolates collected in Czech Republic.

Materials and Methods

Virus isolates. A total of 42 PPV isolates from different localities of the Czech Republic were analyzed. The isolates belonged to PPV-D (16), PPV-M (12), and PPV-Rec (14) strains.

SSCP analysis. cDNA was obtained after immuno-capture RT (IC-RT) as previously described by Glasa *et al*. (2002). In brief, leaf samples were ground in PBS-Tween with 0.05% PVP-40. After centrifugation, 100 µl of clarified sap was incubated overnight at 4°C in tubes pre-coated with 1 µg/ml of polyclonal anti-PPV IgG raised against necrotic PPV-Wageningen isolate. After immuno-capture treatment, the viral RNA was extracted with a 20 µl of mixture containing 0.2% Triton x 100 and 2 µmol/l of hexanucleotide random primers (Promega). The RNA mixture was then made into a final volume of 30 µl with 5x RT buffer, 0.7 mmol/l of each dNTPs, and 7.5 units of AMV reverse transcriptase (all Promega). The steps for the reverse transcription were 45 mins at 37°C and 5 mins at 94°C for enzyme inactivation.

PCR primers were designed according to multiple alignments of HC-Pro sequences available at GenBank and previously published (Jridi *et al*., 2006). The newly designed primers, HC-Pro forward: 5'-ATGCAGTGCAARCTRCGCGA-3' (according to PPV-PS, nt 1116–1135) and HC-Pro reverse: 5'-CGCATYART TCACGATAY-3' (PPV-PS, nt 1372-1390) were obtained. PCR was performed in 25 µl and the conditions were 94°C for 3 mins, 40 cycles of 94°C for 45 secs, 54°C for 60 secs, and 72°C for 60 secs followed by a final extension at 72°C for 7 mins. Afterwards, the specificity of the amplicons was verified by sequencing.

LSSP-PCR analysis. The PCR products (described above) in the expected size were purified from a gel using Qiagen gel extraction kit according to the protocol given by the manufacturer. LSSP-PCR was carried in 10 µl volume containing 2 µl of DNA template, 1x of Taq polymerase buffer, 1.5 mmol/l of MgCl2, 200 µmol/l of each dNTPs, 1.6 U of GoTaq polymerase (all Promega), and 4.8 µmol/l of one of the primers (forward or reverse). After the initial denaturation step at 95°C for 6 mins the LSSP-PCR consisted of 35 cycles of denaturation at 95°C for 1 min, annealing temperature at 30°C for 1 min, and extension at 72°C for 1 min.

PAGE. SSCP-PCR products were separated by 10% non-denaturing polyacrylamide gel in TBE 1x (89 mmol/l Tris base, 89 mmol/l boric acid, 2 mmol/l EDTA). For LSSP-PCR, 5 µl of LSSP-PCR products were separated on 8% polyacrylamide gel. SSCP and LSSP-PCR bands were revealed by silver staining (Brant *et al*., 1991). The similarities among the SSCP and LSSP-PCR profiles were compared by Gene Tools software (Syngene, Ltd).

Results and Discussion

In this work, the SSCP was performed on a set of 14 PPV-Rec, 16 PPV-D, and 12 PPV-M isolates according to the procedure by Orita *et al*. (1989). After amplification using the primers HC-Pro forward and HC-Pro reverse encompassing the N-terminus of HC-Pro, the length of the PCR products was 275 bp as expected. In total, 21 different SSCP profiles were detected. We obtained 5 different patterns for PPV-Rec isolates, 10 different patterns for PPV-D isolates, and 6 different patterns for PPV-M isolates (Fig. 1). The PPV patterns were

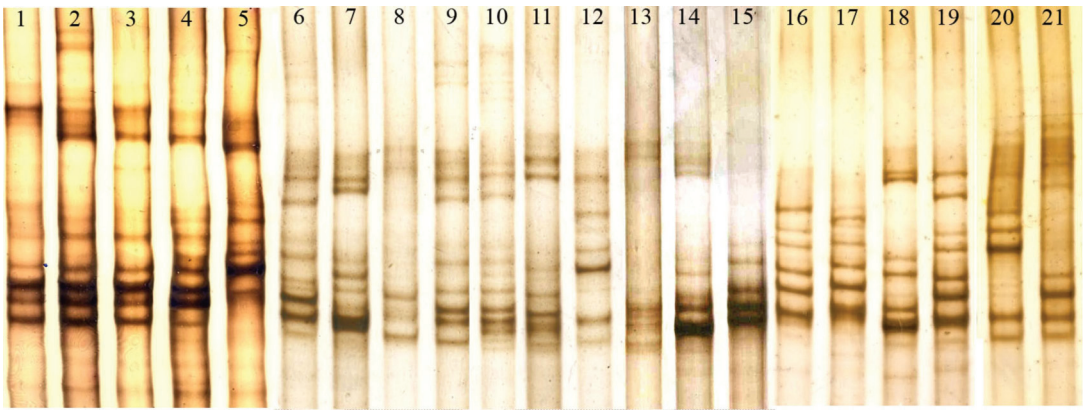

Fig. 1

SSCP profiles of HC-Pro region of PPV isolates

PPV-Rec isolates, patterns Nos. 1–5 (lanes 1–5), PPV-D isolates, patterns Nos. 6–15 (lanes 6–15), PPV-M isolates, patterns Nos. 16–21 (lanes 16–21).

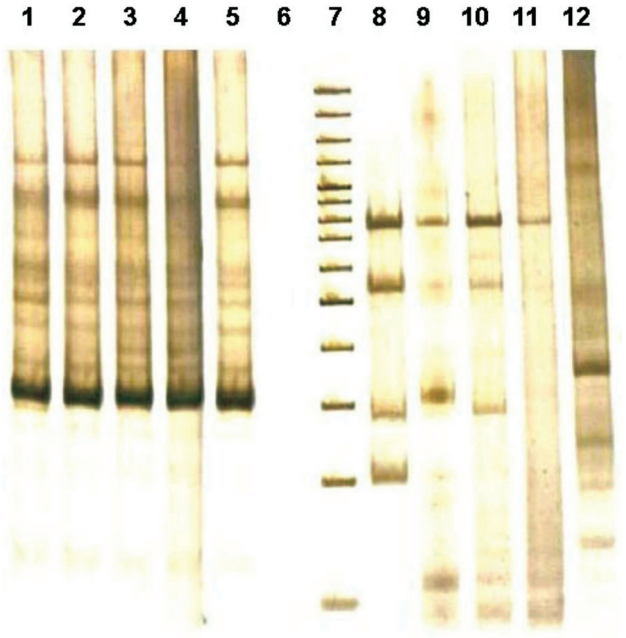

Fig. 2

LSSP-PCR profiles of HC-Pro region of PPV-M isolates

HC-Pro forward primer (lanes 1–5), HC-Pro reverse primer (lanes 8–12), negative control (lane 6), size marker (lane 7).

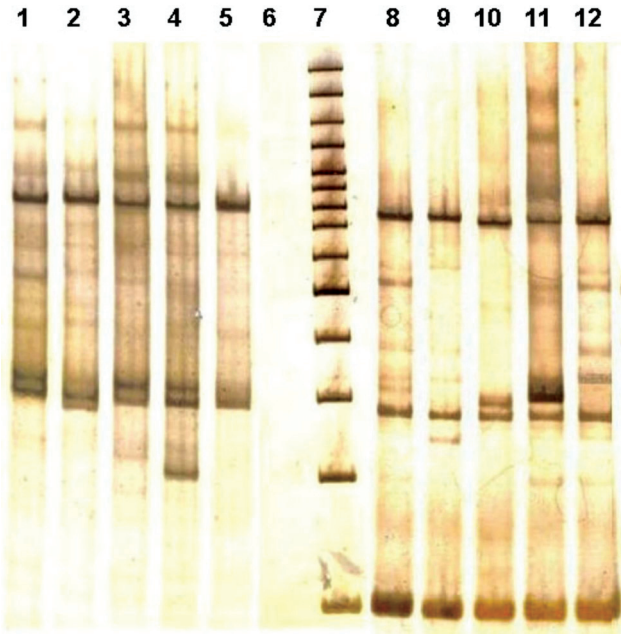

Fig. 3

LSSP-PCR profiles of HC-Pro region of PPV-D isolates

HC-Pro forward primer (lanes 1–5), HC-Pro reverse primer (lanes 8–12), negative control (lane 6), size marker (lane 7).

characteristic for each strain. Pattern No.1 was typical for PPV-Rec isolates and predominant among the 10 PPV-Rec isolates out of 14 PPV-Rec isolates examined. Pattern No. 9 was typical for PPV-D isolates and detected in 7 isolates out of 16 tested. Pattern No. 19 was characteristic for PPV-M isolates and detected in 7 isolates out of 12 tested. Thus, SSCP technique was confirmed as a useful tool for estimating the genetic diversity of RNA viruses. However, the method usefulness is limited by underestimation imposed by the primers used in RT-PCR and by the genetic diversity of the compared sequences. Due to its robustness it can be used for rapid and/or preliminary differentiation of isolates (Turturo *et al*., 2005; Rubio *et al*., 2001).

LSSP-PCR and SSCP are tools used for genotyping and never before they were used for characterizing of RNA viruses. These methods were chosen for verifying their usefulness for detection of molecular variability among randomly selected PPV isolates. Out of 15 PPV isolates, five PPV-Rec isolates showing SSCP pattern No. 1, five PPV-M isolates showing SSCP pattern No. 19, and five PPV-D isolates showing different patterns were examined by LSSP-PCR using HC-Pro forward (F) and HC-Pro reverse (R) primers. Lower resolving power had the combination FR/F that generates 10 profiles, two for PPV-M (Fig. 2; lanes 1–5), four for PPV-D isolates (Fig. 3; lanes 1–5) and four for PPV-Rec isolates (Fig. 4; lanes 1–5). Combination FR/R provided 15 clearly distinct profiles what

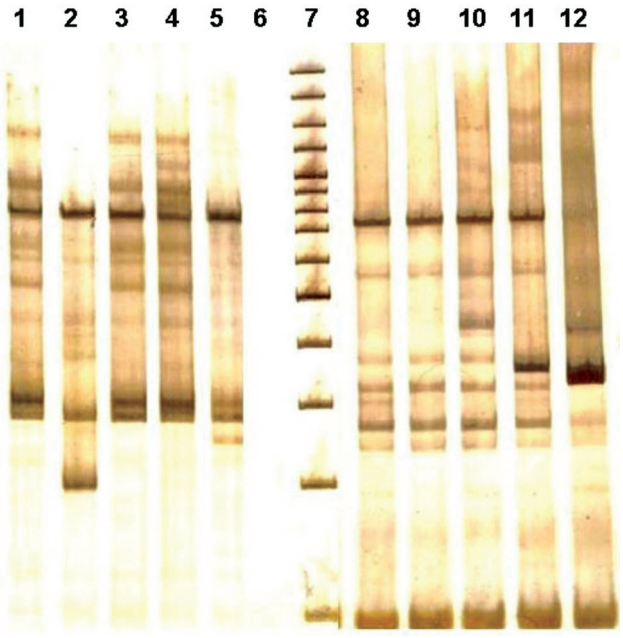

Fig. 4

LSSP-PCR profiles of HC-Pro region of PPV-Rec isolates

HC-Pro forward primer (lanes 1–5), HC-Pro reverse primer (lanes 8–12), negative control (lane 6), size marker (lane 7).

allow to discriminate each isolate (Figs 2, 3, 4; lanes 9–14). LSSP-PCR was able to detect an additional molecular variability of HC-Pro segment, what facilitate the differentiation of PPV-M and PPV-Rec isolates that was not possible using SSCP.

LSSP-PCR originally described by Pena *et al*. (1994) has been successfully used for the study of DNA variability of human papillomaviruses, *Trypanosoma cruzi*, and for the identification of *Leptospira* spp. (Oliveira *et al*., 2003; Vago *et al*., 1996; Villa *et al*., 1995). This study represents the first report of the LSSP-PCR technique being performed on plant RNA viruses with respect to their cDNA. We have demonstrated the application of SSCP and LSSP-PCR for the differentiation of PPV isolates from Czech Republic. Both of these techniques seem to be suitable for preliminary detection of the genetic variability of PPV viruses.

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