STRAIN VARIABILITY OF PLUM POX VIRUS ISOLATES FROM WESTERN SLOVAKIA

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Summary. – Leaf tissues of stone fruit trees (plum, apricot, peach and myrobalan) carrying symptoms of plum pox virus (PPV) infection and of peach GF 305 seedlings and *Nicotiana benthamiana* infected experimentally with PPV were assayed for PPV by polymerase chain reaction (PCR). The expected 243 bp PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis with restriction endonucleases *Alu*I and *Rsa*I. All of the PCR products contained the *Alu*I site. The *Rsa*I restriction profiles of the PCR products demonstrated the prevalence of PPV-M subgroup in the tested samples from western Slovakia.

Key words: plum pox virus; polymerase chain reaction; restriction fragment length polymorphism; strain variability

Introduction

On the basis of present knowledge, PPV isolates may be divided in four subgroups (groups) (Candresse *et al.*, 1994; Nemchinov *et al.*, 1996; Pasquini and Barba, 1997). The PPV-D subgroup, represented by the Dideron strain from France, was firstly differentiated from other isolates by a double diffusion antibody agar test (Kerlan and Dunez, 1979). This subgroup contains mostly isolates from western Europe. Most isolates from southern and eastern Europe belong to the PPV-M subgroup, represented by the Marcus strain from Greece. The third subgroup, PPV-EA, contains only the isolate El Amar from Egypt and differs from other PPV isolates mainly by N-terminal region of the capsid protein (Wetzel *et al.*, 1991a). Recently, Nemchinov *et al.* (1996) characterised a new strain of PPV (PPV-SoC) that naturally infects sour cherry trees in Moldova and proposed this strain as a prototype member of the new subgroup, PPV-C. Molecular analysis of genomic RNAs of various PPV isolates (Varveri *et al.*, 1988; Teycheney *et al.*, 1989; Maiss *et al.*, 1989; Lain *et al.*, 1989; Wetzel *et al.*, 1991a) enabled to design oligonucleotide primers amplifying in reverse transcription-PCR (RT-PCR) and/or immunocapture RT-PCR (ICRT-PCR) a specific fragment of the carboxy-terminus of the capsid protein gene (Wetzel *et al.*, 1991b, 1992). Based on the nucleotide sequence of the amplified fragment, the PPV isolates may be classified in the subgroups by restriction fragment length polymorphism (RFLP) analysis. With exception of PPV-SoC, all the so far characterised PPV isolates contain in the 3′-terminal region of the coat protein gene the conserved *Alu*I recognition sequence. In contrast to PPV-M strains, PPV-D strains contain in the amplified fragment the *Rsa*I restriction site. The above mentioned primers and RFLP analysis are widely used for characterisation of PPV isolates.

In this study, we have investigated PPV isolates from western Slovakia using RT-PCR, ICRT-PCR and RFLP analysis of the PCR products.

Abbreviations: DAS-ELISA = double sandwich enzyme-linked immunosorbent assay; ICRT-PCR = immunocapture RT-PCR; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; PNRSV = prunus necrotic ringspot virus; PPV = plum pox virus; RT = reverse transcription; RTase = reverse transcriptase; RT-PCR = reverse transcription-PCR; RFLP = restriction fragment length polymorphism

Materials and Methods

Plant samples and extracts. Samples from plum, apricot, peach and myrobalan trees carrying symptoms of PPV infection were collected (Table 1). For PPV detection by RT-PCR and ICRT-PCR, we used leaf tissues of trees and leaf tissues of peach GF 305 seedlings and *N. benthamiana* infected with PPV by chip-budding and mechanical inoculation, respectively (Glasa *et al.*, 1997). To obtain a plant extract, the leaves were ground in sterile distilled water (1:10, w/v) and the resulting suspension was centrifuged for 10 mins in a microcentrifuge. Triton X-100 (Fluka) was added to the supernatant to final concentration of 1% (v/v) and the virus particles were disrupted at 65°C for 10 mins (Wetzel *et al.*, 1991b). The sample was then centrifuged for 5 mins and the supernatant was saved for PCR.

Extracts from a healthy plum and an apricot infected with prunus necrotic ringspot virus (PNRSV) were used as negative controls. As a positive control, an extract from tobacco leaves infected with PPV-D, kindly provided by Dr. J.B. Quiot, INRA, Montpellier, France, was used.

Double sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed as described previously (Glasa *et al.*, 1997).

RT-PCR. The following primers were used for PCR: P1 (5'-ACCGAGACCACTACACTCCC-3', sense) and P2 (5'-CAGAC TACAGCCTCGCCAGA-3', antisense) (Wetzel *et al.*, 1991b).

Ten µl of a sample, 4 ml of 5 x RT buffer (Promega), 1 µl (0.1 – 1 µg) of RT primer and 2 µl of sterile bidistilled water were added to a PCR tube (0.5 ml). The mixture was heated at 90°C for 3 mins, cooled and left at room temperature for 30 mins. After primer annealing, 1 µl of 10 mmol/l dNTPs (Boehringer), 1 µl (200 U) of Moloney murine leukemia virus reverse transcriptase (RTase) and 1 µl (40 U) of RNasin (both from Promega) were added. The mixture was incubated at 42 °C for 45 mins, and then at 90 °C for 3 mins. Following this step, 80 µl of PCR buffer (Promega) supplemented with 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 1 µmole of each of PCR primers and 2.5 U of Taq DNA polymerase (Promega) were added, and the mixture was covered with 60 µl of mineral oil (Sigma). The amplification was carried in 30 – 40 cycles (94°C for 45 secs, 62°C for 30 secs, and 72°C for 45 secs) in a Gene ATAQ Controller (Pharmacia). The final extension step at 72°C lasted 7 mins.

ICRT-PCR. The assay was performed according to the procedure of Jansen *et al* (1990) modified for PPV detection by Wetzel *et al.* (1992). Plant extracts were prepared by grinding leaves in PBS containing 2% (w/v) polyvinylpyrrolidone (PVP, Sigma) and 0.05% (v/v) Tween 20. Briefly, a PCR tube (0.5 ml) was coated with antibodies diluted in a coating buffer (Koenig, 1981) at 37°C for 4 hrs. The coating solution was then removed, a plant sample was added to the coated PCR tube and incubated overnight at 4°C. After washing the tube with a phosphate-buffered saline (PBS)-Tween 20 buffer (Koenig, 1981), the RT-PCR was carried out as described above.

Gel electrophoresis. Aliquots (5 – 10 µl) of PCR mixtures were analysed by electrophoresis in 1.8 % agarose (SeaPlaque GTG, FMC) gel. The bands were stained with ethidium bromide and visualized under UV light.

RFLP analysis was performed by cleaving the amplified fragments with *Rsa*I and *Alu*I restriction endonucleases (Promega) (Wetzel *et al.*, 1991b). The digests were loaded on 3% agarose (MetaPhor, FMC) gel, electrophoresed, and the obtained bands were visualised as above. The DNA Molecular Weight Marker VIII (Boehringer) was used in each run.

Results

We analysed 20 samples of leaves (13 plums, 4 apricots, 2 myrobalans and 1 peach) carrying PPV symptoms collected from commercial orchards and private gardens on the territory of western Slovakia (Table 1) by DAS-ELISA and PCR. All the samples were positive in DAS-ELISA and produced the expected 243 bp fragment in PCR. In accordance with published reports (Wetzel *et al.*, 1991b, 1992; Krczal and Avenarius, 1994; Candresse *et al.*, 1994), PCR detection of PPV in tested samples was of greater sensitivity in comparison with DAS-ELISA (data not shown). The specificity of PPV detection by RT-PCR and/or ICRT-PCR was demonstrated on negative (healthy plum and apricot infected with PNRSV) and positive controls (Figs. 1 and 2). The amplified fragments were further analysed by cleavage with *Alu*I and *Rsa*I restriction endonucleases. All the amplified fragments contained the *Alu*I site (Fig. 3).

Table 1. List of PPV isolates identified by PCR and RFLP analysis in original woody hosts and/or after transmission in test plants

Isolate	Original host	Plant tested	PPV subgroup
BIII/1	plum	plum	M
BIII/2	plum	plum, *N. benthamiana*	D
BOJ-3	plum	GF 305, *N. benthamiana*	D
BOR-3	apricot	*N. benthamiana*	M
BOR-11	plum	plum	M
CAH-2	apricot	apricot, GF 305, *N. benthamiana*	M
CFR-3	apricot	apricot	M
CFR-4	myrobalan	myrobalan	M
KRA-70	plum	plum	D
MT	plum	plum, *N. benthamiana*	M
RAK 2/9	apricot	apricot	D
SEN-1	plum	plum, *N. benthamiana*	M
SEN-3	myrobalan	myrobalan	D
TG	plum	plum, GF 305	D
TG II	plum	plum	D
TTE-1	plum	plum	M
TTE-2	plum	plum	D
TTE-3	plum	plum	M
VAR	peach	peach, GF 305, *N. benthamiana*	M
X	plum	*N. benthamiana*	M

On the basis of *Rsa*I polymorphism, the PPV-M and PPV-D subgroups could be identified in the isolates (Table 1, Fig. 4). There were following PPV-M/PPV-D proportions in the tested samples: plums – 7/6, apricots – 3/1, myro-

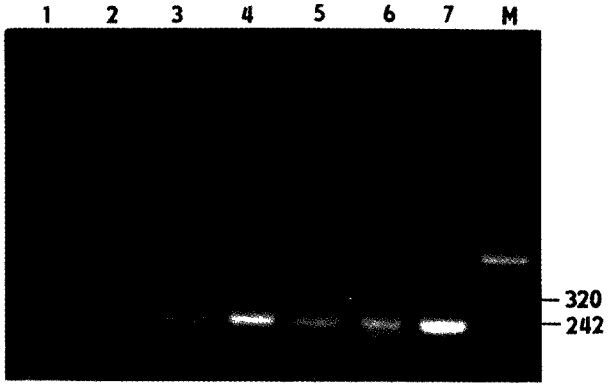

Fig. 1
Agarose gel electrophoresis of ICRT-PCR products
Healthy plum (lane 1), PNRSV-infected apricot (lane 2), tested samples from Slovakia (lanes 3–6), PPV-D infected tobacco (lane 7), DNA size markers (bp, lane M).

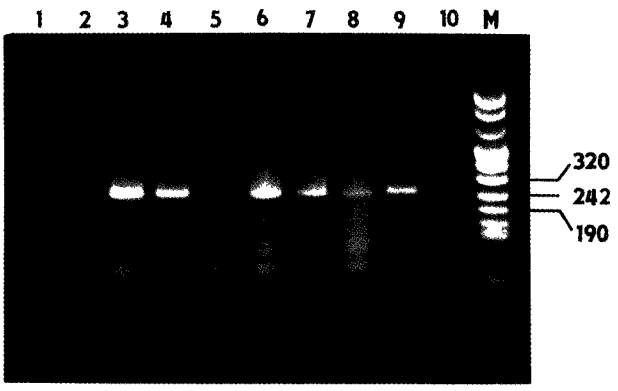

Fig. 2
Agarose gel electrophoresis of RT-PCR products
Healthy plum (lane 1), PNRSV-infected apricot (lane 2), tested samples from Slovakia (lanes 3–9), PPV-D infected tobacco (lane 10), DNA size markers (bp, lane M).

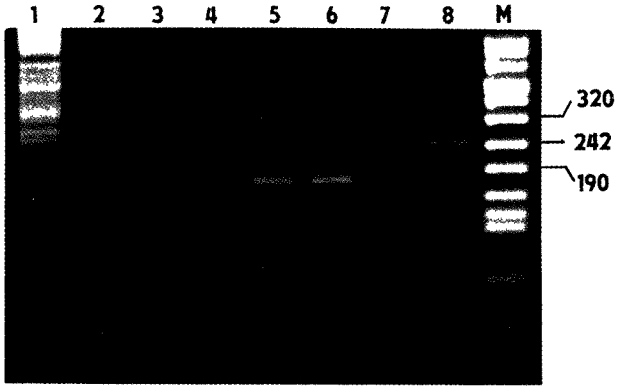

Fig. 3
Agarose gel electrophoresis of PCR products cleaved with *AluI*
Lambda DNA/*AluI* (lane 1), *AluI*-cleaved PCR products of tested samples from Slovakia (lanes 2–4, 6 and 7), *AluI*-cleaved positive control (lane 5), uncleaved positive control (lane 8), DNA size markers (bp, lane M).

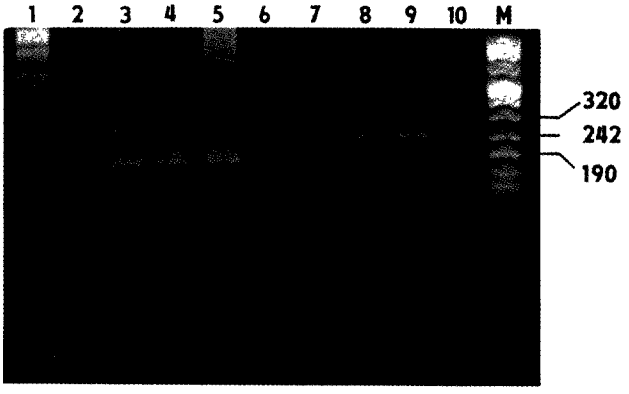

Fig. 4
Agarose gel electrophoresis of PCR products cleaved with *RsaI*
Lambda DNA/*RsaI* (lane 1), uncleaved positive control (lane 2), *RsaI*-cleaved positive control (lane 3), *RsaI*-cleaved PCR products of tested samples from Slovakia (lanes 4–10), DNA size markers (bp, lane M).

balans – 1/1 and peach – 1/0. In total, there were 12 samples with PPV-M and 8 samples with PPV-D positive in tests.

The subgroup character of several isolates was analysed in original hosts and after transmission on test plants as well (Table 1). The obtained data confirmed the same PPV subgroup in both the original host and test plant.

Discussion

PPV is the most important viral pathogen of stone fruit trees in Slovakia. The occurrence (by symptomatology) of sharka (plum pox) disease in this territory was first time evidenced around 1950 (Králiková, 1962). Later, several PPV isolates have been characterised by symptomatology on selected herbaceous plants and peach GF 305 seedlings (Paulechová, 1981; Glasa *et al.*, 1997). The results shown in this paper represent the first report on PCR detection and subgrouping of PPV isolates from Slovakia.

PCR procedures developed by Wetzel *et al.* (1991b, 1992) have demonstrated high degree of sensitivity and specificity also in our experiments. All the tested samples produced in PCR the expected fragment. Although all the assays were performed under the same conditions, we could detect in some

samples by electrophoresis various yields of the expected PCR product and also non-specific submolar fragments. The yield variability could be due to uneven distribution (concentration) of the virus in leaf tissues, and the non-specific fragments could result from mispriming and degradation, probably caused by host component in RT-PCR (Wetzel *et al.*, 1991b; Levy and Hadidi, 1994; Corvo *et al.*, 1995).

The *Rsa*I polymorphism of the PCR products revealed a mild prevalence of PPV isolates belonging to the M subgroup (12 isolates of PPV-M and 8 isolates of PPV-D). This result corresponds with the concept of geographical spread of PPV (Candresse *et al.*, 1994; Pasquini and Barba, 1997). Since the epidemic spread of PPV-M strains is more rapid in nature as compared to other strains (Krczal and Avenarius, 1994; Pasquini and Barba, 1997), the obtained result is important from the point of view of the phytosanitary measures.

The PPV-M and PPV-D character of the detected isolates was confirmed by digestion with *Alu*I restriction endonuclease. All the amplified fragments contained the *Alu*I recognition site.

To obtain a more complete information about the PPV strains spread on the whole territory of Slovakia, samples from orchards and gardens in another regions of Slovakia should be subjected by PCR and RFLP analysis.

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