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A natural population of recombinant *Plum pox virus* is viable and competitive under field conditions

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Abstract

The isolate BOR-3, collected in Slovakia in 1996, was recently identified as a natural recombinant between an M and D type of *Plum pox virus* (PPV). Biological assays demonstrated its capacity to be aphid- and graft-transmitted to various *Prunus* spp. hosts. A study was carried out to determine the further presence of PPV recombinants in two epidemiologically distinct areas – Slovakia and France. Tools based on PPV-M and D subgroup typing, targeting P3–6K_1, CI and CP regions of the PPV genome were used for recombinant identification. Closely related recombinant variants were detected in different *Prunus* spp. during a survey conducted in Slovakia in 2001, but not within a set of selected PPV isolates from France collected between 1985 and 2001. Sequence analysis of the (Cter)NIb–(Nter)CP region of 10 recombinant isolates from Slovakia showed their high homology, reaching more than 98%. All the recombinant isolates shared the same recombination breakpoint situated in the C terminus of the NIb gene. Our study demonstrates that the PPV recombinants are viable and competitive with conventional PPV-M and D isolates. The present work indicates that the occurrence of recombinants within PPV isolates might be more common than previously assumed.

Introduction

Plum pox virus (PPV), a member of the genus Potyvirus, is the causal agent of sharka, the most detrimental disease of stone-fruit trees world-wide. The PPV genome consists of a single-stranded positive-sense RNA of about 10 kb and encodes a single polyprotein to be proteolytically processed by self-encoded proteases into as many as 10 functional proteins (Revers et al., 1999).

Two main subgroups of PPV isolates have been identified, i.e. PPV-M and PPV-D (Candresse et al., 1998). Isolates of these subgroups occur together in most European countries (Smith et al., 1994; Kölber et al., 2001). Additional minor subgroups include the geographically limited El Amar isolates from Egypt (PPV-EA, Wetzel et al., 1991) and the cherry-adapted isolates (PPV-C, Nemchinov et al., 1996). Currently, all the techniques used for subgroup discrimination of isolates focus exclusively on the coat protein (CP) or the CP gene (Wetzel et al., 1992; Bousalem et al. 1994; Candresse et al., 1998). Molecular variability in the P3–6K_1 region of several PPV isolates has been recently evaluated and a simple RT-PCR/RFLP method targeting this region was optimised to enable typing of PPV-M and D isolates independently of the CP (Glasa et al., 2002). Using this approach, both PPV-M and PPV-D isolates were confirmed to occur in Slovakia and France.

Recombination is considered to play an important role in RNA virus evolution and can provide additional sources of variation with unpredictable effects on virus

pathogenicity (Worobey and Holmes, 1999). Reports on natural recombinant isolates within potyviruses are still limited. Based on the 3′ terminal sequence, the PPV-o6 isolate was found to be the product of a natural recombinant event between two wild types of PPV (Cervera et al., 1993). Later, a systematic search for recombinant events of the available database potyviral sequences in the CP gene and the 3′ NCR in natural isolates was performed (Revers et al., 1996). The authors demonstrated that the frequency of occurrence of recombinants in potyvirus varies greatly with different viruses. Despite a large dataset, recombinants were not detected within PPV. On the contrary, several *Potato virus Y* (PVY) and *Bean common mosaic virus* (BCMV) isolates were found to be derived from recombination events. The high genetic diversity and intraspecies recombination events among natural populations of *Yam mosaic virus* (YMV) were reported by Bousalem et al. (2000). A natural PPV recombinant was very recently reported in Slovakia (Glasa et al., 2001).

There have been very limited descriptions of the effect of recombination event on the potential fitness of plant viruses. In this work, several assays were undertaken to evaluate the biological properties of the natural recombinant isolate. Furthermore, the finding of a naturally occurring PPV recombinant prompted us to perform a more detailed study of PPV isolates recovered from two epidemiologically distinct areas – Slovakia and France – using a tool developed for recombinant identification. We report a high occurrence of viable PPV recombinants on *Prunus* spp. in several localities of western and middle Slovakia. Our work indicates that the occurrence of recombinants within PPV isolates might be more common than previously assumed.

Material and methods

Isolates

The PPV natural recombinant isolate BOR-3 was isolated in the summer of 1996 in an orchard in western Slovakia and its preliminary characterisation was reported (Glasa et al., 1998; 2001). We looked for further possible recombinant isolates in fruit-growing localities in western and middle Slovakia in the spring and summer of 2001. Leaf samples were taken from naturally-infected plum and myrobalan trees. Overall, sampling was conducted in six localities (Bojnice, Borovce, Krajné, Lozorno, Myjava, Podkylava). Each disease focus sampled was spatially isolated from other focuses. However, the foci were in regions with high stone-fruit growing activity. A total of 23 Slovak samples were collected and PPV positivity was tested in triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) using universal monoclonal antibody (MAb) produced in the IV SAS laboratory (Šubr and Matisová, 1999). Moreover, 30 French isolates selected from a virus collection kept at INRA were analysed.

Biological assays with the recombinant BOR-3 isolate

Graft and mechanical inoculation. For mechanical transmission tests, BOR-3 infected leaves of *Nicotiana benthamiana* were ground 1/20 (w/v) in 0.03 M Na_2HPO_4 (supplemented with 0.2% sodium diethyl dithiocarbamate) and the crude sap extract was inoculated onto leaves of 3-week-old seedlings of *Prunus persica* GF305, *P. armeniaca* cv. Manicot and *P. insititia* × *P. domestica* St. Julien no. 2. Chip-bud transmissions from GF305 to Manicot, St. Julien, *P. persica* cv. Montclar and back to GF 305 were made on 3-month-old seedlings. Plants were maintained under containment at a temperature of 25 °C/18 °C (day/night), with a 16-h/day photoperiod and tested by DAS-ELISA (Clark and Adams, 1977) using polyclonal antiserum produced in the UMR BGPI laboratory.

Aphid transmission. Experimental aphid transmission was performed under controlled conditions using a technique involving a controlled acquisition access period (Labonne et al., 1995) using a clonal culture of *Myzus persicae* Sulzer. PPV-BOR-3 infected *N. benthamiana* were used as inoculum source. French isolate 91.003 was used as control. Peach GF305 seedlings, *N. benthamiana* and *Pisum sativum* cv. Colmo were used as test plants. Fifty aphids were set on each test plant after a 3-h starvation period followed by a 6-min acquisition period on the source leaves. Evaluations were carried out after a 3-week incubation period using DAS-ELISA.

In vitro cultures were established from stem segments of St. Julien no. 2 plant infected with BOR-3. *In vitro* propagation and graft experiments were carried out according to standard laboratory methods (Lansac et al., 1998).

CP-based subgroup typing

Standardised protocols based on specific MAb reaction (Candresse et al., 1998) and *Rsa*I polymorphism of the C-terminal part of the CP gene (Wetzel et al., 1992) were used for subgroup determination of isolates.

Reverse transcription polymerase chain reaction (RT-PCR) and restriction analyses

Plant extracts were prepared by grinding plum and myrobalan leaves 1/20 (w/v) in phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 and 2% (w/v) polyvinyl pyrrolidone (PVP-40). For French isolates, lyophilised GF305 or *N. benthamiana* leaves were used. One hundred microlitre of clarified sap were incubated overnight at 4 °C in tubes pre-coated with 1 μg ml^{-1} of anti-PPV IgG prepared in the laboratory. Then the tubes were washed twice with sterile PBS-T.

A two-step RT-PCR protocol was used. To synthesise first strand cDNA, 20 μl of mixture containing 0.2% TritonX100 and 2 μM of hexanucleotide random primers (Promega) were added to tube and incubated at 65 °C for 10 min, then immediately chilled on ice. The reverse transcription (RT) mixture was complemented to a final volume of 30 μl with 5 × RT buffer (Promega), 0.7 mM of each dNTP (Pharmacia Biotech) and 7.5 units of AMV reverse transcriptase (Promega). Samples were incubated for 45 min at 42 °C. RT was inactivated at 94 °C for 3 min.

One microlitre of RT reaction was added to PCR containing 49 μl of reaction mix [10 × Expand High Fidelity (HF) buffer, 15 mM $MgCl_2$]; 250 μM of each dNTP, 1 μM of each of the two primers (Table 1); 2.5 units of Expand HF PCR Systeme enzyme mix (Roche Molecular Biochemicals). For PCI/PP3 (targeting P3–6K$_1$) and Potyvirid2/P4 primer sets, targeting (Cter)NIb–(Nter)CP region, PCR was carried out in a thermal cycler (UNO Thermoblock, Biometra) programmed for one denaturation step at 94 °C for 5 min, followed by 35 cycles (94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min) and the extension temperature at 72 °C for 10 min.

For specific PCR targeting the CI gene (using CIf/CIM or CID primer sets), 0.5 μl of RT reaction was used and PCR was performed in a 25 μl volume using TaKaRa *Taq* DNA polymerase (Takara Biomedicals) for target amplification. The PCR cycling conditions were as follows: denaturation at 94 °C for 5 min followed by 35 cycles (94 °C for 45 s, 54 °C for 30 s, 72 °C for 45 s). The final extension step at 72 °C lasted 10 min. The specificity of the PCR products amplified with CIf/CIM or CID primers was verified by cleavage using *Bam*HI, *Dde*I, *Eco*RV and *Sma*I endonucleases. Aliquots of PCR products obtained with the PCI/PP3 primer pair were subjected to RFLP analysis with restriction endonucleases *Dde*I and *Eco*RI to enable subgroup typing as previously reported (Glasa et al., 2002).

Sequencing and phylogenetic analyses

Potyvirid2/P4 amplified RT-PCR products were purified from an agarose gel and directly cloned into a pGEM-T Easy® plasmid vector (Promega) according to the manufacturer's instructions. At least two independent clones of each isolate were sequenced in both directions using standard forward and reverse primers of pUC plasmids. Alternatively, the PCR products were directly sequenced by means of a dideoxy sequencing method using specific primers for PCR (Table 1). The obtained sequences were compared

Table 1. Characteristics of the primers used in this study

Primer[1]	Target region	Nucleotide position[2] and length	Reference
PP3 5′TTATCTCCAGGARTTGGAGC3′ sense PCI 5′TTGAGTCAAATGGRACAGTTGG3′ antisense	(Cter)P3–6K$_1$–(Nter)CI	2915–3750, 836 bp	3
CIf 5′GCAAAGTAAYGAAGTTGCAC3′ sense CID 5′TCGTGTATCGAACGAGACGA3′ antisense CIM 5′TCGTATAACGAACAAGTCGG3′ antisense	CI	3710–4671, 962 bp	
Potyvirid2 5′GGBAAYAAYAGYGGDCARCC3′ sense P4 5′TGCCTTCAAACGTGGCACTG3′ antisense	(Cter)NIb–(Nter)CP	7940–8911, 972 bp	4 5
P2 5′CAGACTACAGCCTCGCCAGA3′ sense P1 5′ACCGAGACCACTACACTCCC3′ antisense	(Cter) CP	9337–9579, 243 bp	6

[1]Redundancy code: R = A/G; Y = C/T; B = C/G/T; D = A/G/T; [2]numbered according to PPV-PS GenBank AJ243957; [3]Glasa et al. (2002); [4]Gibbs and Mackenzie (1997); [5]Candresse, unpublished; [6]Wetzel et al. (1992).

to previously published sequences overlapping at least NIb-CP-3′NCR (M92280, D13751, S57404, AY028309, AJ243957, M21847, Y09851, X16415, X56258, X81803, X97398) or CP-3′NCR (X57975, X57976, X81073-X781082, X81084, U27652, AF332871, AJ306420, AJ000340). Sequence analyses and comparisons were made using the ClustalX (Thompson et al., 1997) and GAP program of the Genetic Computer Group (GCG) Sequence Analysis Software Package v.10.0 (Devereux et al., 1984). The alignments were used as input data to construct phylogenetic trees with the neighbour-joining distance method implemented in ClustalX; each time the bootstrap option was run with 1000 resampling. The trees were visualised with the Treeview v.1.6.1 program. The recombinant sequences and the location of recombination breakpoints were detected by PhylPro v. beta 0.8 (Weiller, 1998). RNA folding of sequences was done by www service mfold (Mathews et al., 1999).

Results

Biological assays demonstrated the capacity of natural recombinant BOR-3 isolate to be aphid- and graft-transmitted to various Prunus *hosts*

The experimental host range of BOR-3 was assayed by chip-budding and/or sap-inoculation of young seedlings of several *Prunus* species. Infection of St. Julien no. 2 seedlings was expressed by intensive leaf chlorotic spots and rings and infected *P. armeniaca* cv. Manicot seedlings developed leaf necrosis. Interestingly, sap- or chip-inoculated *P. persica* GF305 and Montclar seedlings remained symptomless, or infection induced only weak, time-limited symptoms localised on a few leaves, despite positive serological reactions.

Based on *in vitro* assays, the PPV-BOR-3 isolate was easily maintained *in vitro* on susceptible St. Julien no. 2. After 10 subcultures, the isolate was transmitted by *in vitro* grafting to *P. mariana* cv. GF8.1, *P. armeniaca* cv. Screara and back to St. Julien no. 2 with a high rate of efficiency (data not shown). The true-to-typeness of the isolate was verified after intensive *in vitro* multiplication and grafting by direct sequencing of (C-ter)NIb–(N-ter)CP region. We conclude that the viability of BOR-3 was not affected by long-term *in vitro* maintenance.

Notwithstanding the fact that the amino acid conserved Asp-Ala-Gly (DAG) block, which is essential for potyvirus aphid transmission (Atreya et al., 1995), was present in the BOR-3 N-terminal region of CP, we used green aphids (*M. persicae*) to verify the ability of recombinant isolate to be naturally transmitted by aphids. The transmission experiments confirmed that BOR-3 was aphid-transmitted from infected *N. benthamiana* to peach GF305, plum St. Julien, *N. benthamiana* and *P. sativum* cv. Colmo (Table 2). Comparing woody indicators, the transmission rate of BOR-3 from *N. benthamiana* was significantly higher to St. Julien than to GF305 seedlings. As the transmission rates were similar when comparing BOR-3 and 91.003 isolates on St. Julien, *N. benthamiana* and *P. sativum*, but distinctive on GF305 ($\chi^2 = 7.89$; df $= 1$), it seems that the BOR-3 isolate tested in these trials was less adapted to peach than to plum.

Recombinant isolates were detected in Slovakia, but not in France

Having recovered the recombinant BOR-3 isolate in a specific apricot tree in 1996, a survey was carried out in 2001 to determine whether further recombinant isolates were established in Slovakia. Overall, 23 isolates from six distinct localities (orchards) were analysed (Table 3).

Subgroup typing focusing three regions of PPV genome (CP, CI and P3–6K$_1$) showed that 16 of 23

Table 2. PPV transmission by *Myzus persicae* from infected *N. benthamiana*

Isolate	Test plants			
	P. persica GF305	*P. insititia*× *P. domestica* St. Julien no. 2	*N. benthamiana*	*P. sativum* Colmo
BOR-3	3/16*	8/10	27/28	3/16
91.006	12/19	6/10	26/30	6/20

*Positive transmission/total plant used.

Table 3. Results of subgroup typing based on different target regions of PPV isolates collected during the 2001 survey in Slovakia

Isolate	Focus	Host, year of plantation	Target region			Reference
			CP	CI	P3–6K$_1$	
KRN-1	Krajné	Plum, cv. Cacak Beauty, 1984	M	D	D+[3]	AF421121[1], AF450314[2]
KRN-2	Krajné	Plum, cv. Cacak Beauty, 1984	D	D	D	
KRN-3	Krajné	Plum, cv. Cacak Beauty, 1984	M	D	D+	
KRN-4	Krajné	Plum, cv. Cacak Beauty, 1984	M	D	D+	AF421122[1]
KRN-5	Krajné	Plum, cv. Cacak Best, 1984	D	D	D	
KRN-6	Krajné	Plum, cv. Cacak Best, 1984	M	D	D+	
KRN-7	Krajné	Plum, cv. Cacak Best, 1984	M	D	D	AF421123[1]
KRN-8	Krajné	Plum, cv. Cacak Best, 1984	M	D	D+	
BRC-2	Borovce	Myrobalan, unknown	D	D	D	
BRC-3	Borovce	Myrobalan, unknown	M	D	D	AF421118[1]
BRC-4	Borovce	Myrobalan, unknown	M	D	D	AF421119[1]
BRC-8	Borovce	Plum, unknown	M	D	D	AF421120[1]
BRC-9	Borovce	Plum, unknown	D	D	D	
LOZ-3	Lozorno	Plum, cv. Domestic plum, 1985	M	D	D	AF450312[1]
LOZ-4	Lozorno	Plum, cv. Domestic plum, 1985	M	D	D+	
MYV-1	Myjava	Plum, cv. Cacak Best, 1991	M	D	D+	AF 450315[2]
MYV-2	Myjava	Plum, cv. Cacak Beauty, 1991	D	D	D	
MYV-3	Myjava	Plum, cv. Cacak Beauty, 1991	M	D	D+	AF450313[1]
BNE-5	Bojnice	Plum, cv. Cacak Beauty, 1981	D	D	D	
BNE-6	Bojnice	Plum, cv. Cacak Best, 1981	M	D	D	
BNE-10	Bojnice	Plum, cv. Stanley, 1994	M	D	D	AF420311[1]
POD-1	Podkylava	Plum, cv. Cacak Fruitful, 1988	D	D	D	
POD-2	Podkylava	Plum, cv. Cacak Beauty, 1988	M	D	D	

[1](Cter) NIb–(Nter) CP; [2](Cter)P3–6K$_1$–(Nter)CI; [3]atypical *Dde*I restriction pattern (see text).

isolates collected in 2001 in Slovakia presented discordance in typing, depending on targeted region (Table 3). Each of these 16 isolates was typed as PPV-M based on *Rsa*I polymorphism of the C-terminal part of the CP gene and reaction with specific MAbs. However, typing of the CI and P3–6K$_1$ region assigned them to the PPV-D subgroup, indicating their recombinant character. Seven further isolates could be clearly typed to the PPV-D subgroup. We did not detect mixed infections by several isolates in any samples, as confirmed by specific RT-PCR targeting CI gene (Figure 1). The suitability of this method to detect mixed infection of a PPV-D and an M isolate was confirmed by testing of artificially-prepared mixed samples from infected *N. benthamiana* plants. Different ratios of PPV-M and PPV-D in mixed sample ranging from 1 : 10 to 10 : 1 did not affect the detection specificity.

A set of 30 PPV isolates originating from nine focuses from southern France (recovered between 1985 and 2001) was tested as described above. PPV-M and PPV-D isolates occurred simultaneously in only one of these foci. All isolates tested could be classed

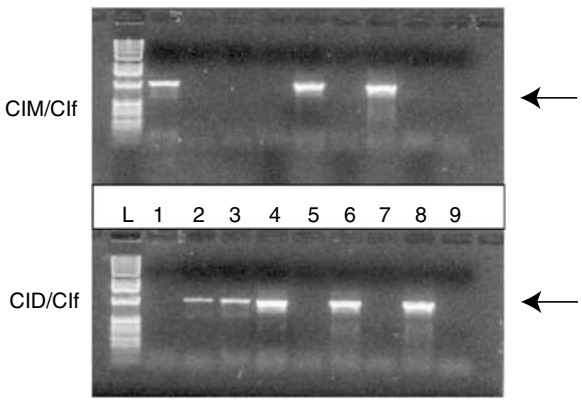

Figure 1. Specific detection of PPV-M and PPV-D sequences in the CI genomic region. No detectable cross-reaction was observed. RT-PCR was carried out with CIf/CIM (PPV-M specific) and CIf/CID primers (PPV-D specific). Arrows indicate the expected size of PCR products. L = 1 kb DNA ladder (Gibco BRL); lanes 1, 5 and 7 = PPV-M isolates (91.003, 91.006, 0.030); lanes 2–4 = recombinant isolates (KRN-1, MYV-3, BRC-3); lanes 6 and 8 = PPV-D isolates (85.001, 92.011), lane 9 = uninfected control.

into PPV-M or PPV-D subgroups, respectively, and not even in a single recombinant isolate was detected (Table 4).

Slovak recombinant isolates are closely related and share the same recombination breakpoint position

The region encompassing the C-terminal part of NIb and the N-terminal part of the CP gene of 10 isolates from Slovakia presenting discordance in subgroup typing was then amplified and sequenced in view of determining a recombination break-point position suspected to occur in this portion as indicated by previous work with BOR-3 (Glasa et al., 2001). Indeed, visual inspection of the sequence alignment suggested a recombination event in the C-terminal part of the NIb gene at the nt portion 8433–8450. Based on multiple alignment comparison, the region of recombination had a high level of sequence homology among recombinant PPV isolates, whose sequences were available (Figure 2). The exact position of recombination break-point could be determined by PhylPro analysis. This computer-graphic method, suited for detecting recombinant sequences, is based on the principle that phylogenetic relationships derived from different regions of a multiple sequence alignment of particular and reference sequences will be discordant when recombination has occurred. Recombination signals appeared on the graph as areas of low phylogenetic correlation, shown by a single sharp-pointed downward peak in the graph. For all recombinant sequences,

Table 4. Results of subgroup typing based on different target regions of selected PPV isolates from France

Isolate*	Original host	Target region		
		CP	CI	P3–6K$_1$
92.011, 93.080, 97.078, 98.042, 98.063, 98.065, 98.066, 98.067, 98.071, 98.074, 98.075, 99.036, 99.059, 99.062, 99.064, 01.020, 01.024, 01.042	Peach	D	D	D
99.035	Nectarine	D	D	D
85.001, 94.055, 98.069	Apricot	D	D	D
98.043	Plum	D	D	D
98.058, 99.044, 01.030	Peach	M	M	M
91.003, 91.006	Nectarine	M	M	M
98.055	Apricot	M	M	M
94.061	Plum	M	M	M

*Code: year of isolation.specific number.

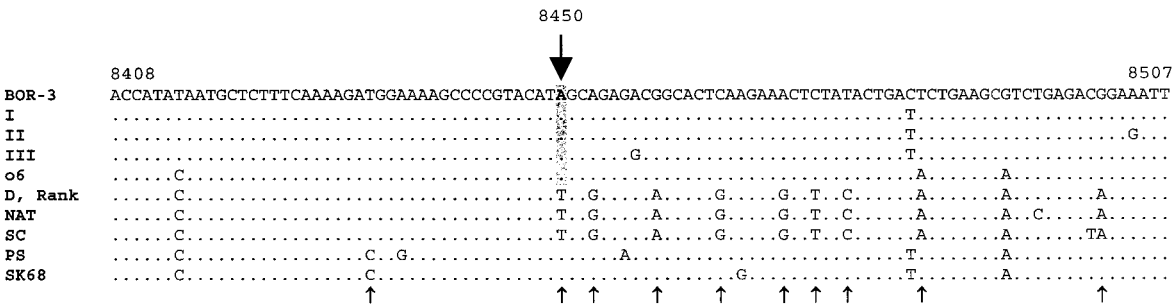

Figure 2. Multiple alignment of recombinant sequences of Slovak isolates and o6 with the sequences of PPV-M (PS, SK68) and PPV-D isolates (Dideron, Rank, NAT, SC). I-BRC-3, BRC-4, KRN-7, LOZ-3; II- BRC-8, KRN-1, KRN-4, MYV-3; III-BNE-10. Only the region around the recombination crossover is shown. The position of the crossover indicated by PhylPro analysis is marked by an arrow. Subgroup-specific nucleotide positions are indicated by small arrows below the alignment. Dots indicate nucleotides identical to the BOR-3 sequence.

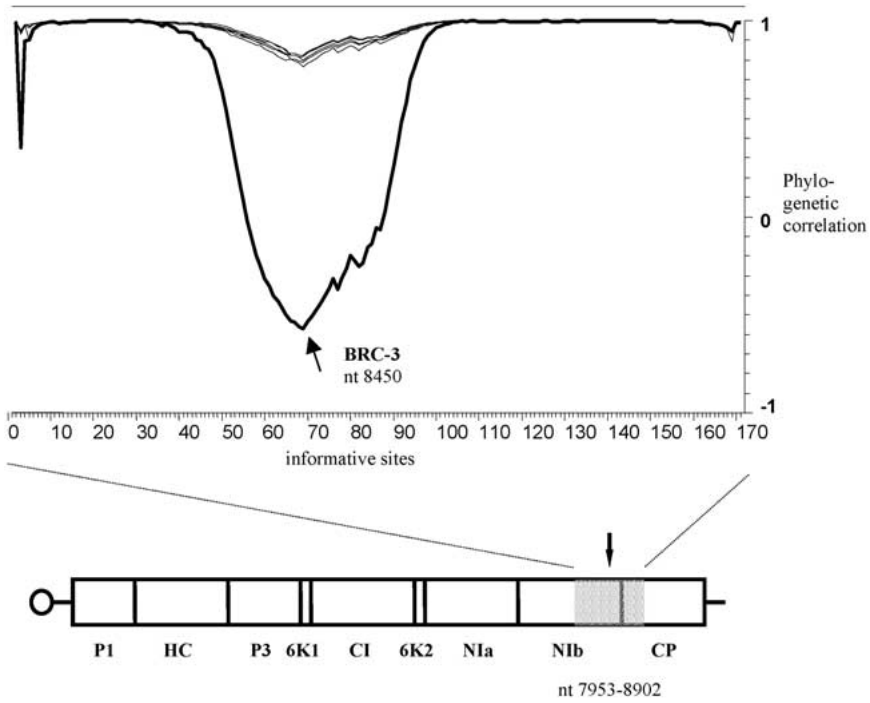

Figure 3. PhylPro analysis was performed on all sequenced recombinant isolates (BOR-3, BRC-3, BRC-4, BRC-8, KRN-1, KRN-4, KRN-7, BNE-10, LOZ-3, MYV-3), PPV-M-type isolates (PS, SK68) and PPV-D-type isolates (Dideron, SC, Rank). Only phylogenetic profiles of BRC-3, PPV-M and D-type isolates are shown. The *X*-axis of PhylPro plot represents parsimonious informative sites. The *Y*-axis gives the phylogenetic correlation on a default axis of −1 to 1. The parsimonium option (only phylogenetically informative sites) was used to calculate pairwise distances. A schematic diagram of the PPV genome shows the location of the sequenced and analysed region.

we observed a single peak, indicating a recombination breakpoint at the nucleotide position 8450 (Figure 3).

Predicted amino acid sequences of recombinant isolates preserved amino acid motifs connected with functions of some virus encoded proteins and biological properties. The NIb portion of all sequenced isolates contained conserved RdRp GQPSTVVDNT and GDD motifs (Quadt and Jaspars, 1989). The C-terminal part of NIb was terminated by the NIVIHQ↓A motif possessing the potential of the NIa protease cleavage site (Palkovics et al., 1993; Riechmann et al., 1995). The DAG motif that participates in aphid transmission occurred downstream of this sequence.

Computer analysis of (Cter)NIb–Cter(CP) sequences showed strong similarities with previously sequenced BOR-3 (AY028309) and newly sequenced Slovak recombinant isolates, reaching 98.42–100%. Phylogenetic analysis showed that the recombinant PPV isolates are closely related (Figure 4).

A variation within Slovak recombinant isolates could be determined in the P3–$6K_1$ region using *Dde*I restriction analysis of RT-PCR products (836 bp) amplified with the PP3/PCI primer set. Based on previous work (Glasa et al., 2002), the *Dde*I site was strictly conserved among PPV-D isolate sequences (*Dde*I cleavage yielded two fragments of 185 and 651 bp). Restriction analysis of Slovak and French PPV-D isolates always resulted in the expected restriction patterns. However, 8 of 16 Slovak recombinant isolates (Table 3) presented new pattern. The RFLP results were validated by direct sequencing of PP3/PCI amplified products of the two isolates KRN-1 and MYV-1 and the exact positions of *Dde*I sites were mapped. The sequence of these isolates included two *Dde*I sites and cleavage yielded three fragments of 185, 185 and 466 bp (observed as two bands on the gel, Figure 5).

Multiple sequence alignment of sequences from the N-terminal CP region of recombinant isolates (including Yugoslavian o6) and sequences available in GenBank (26 isolates) designated five amino acid positions in this region (K^{2814}, I^{2848}, T^{2852}, I^{2868}, T^{2878}) specifically conserved only within recombinant sequences. It is noticeable, that three other isolates

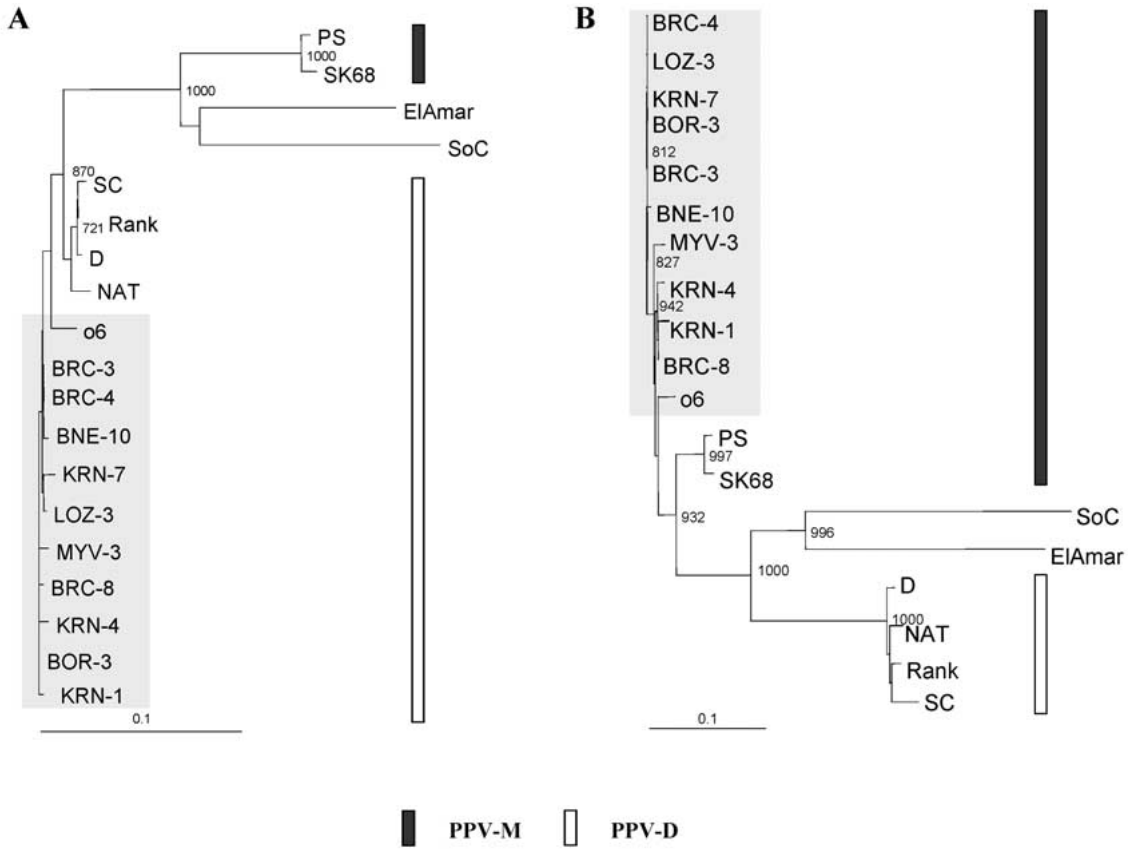

Figure 4. Phylogenetic position of recombinant isolates according to the genomic region under focus. Phylograms were reconstructed from A: the C-terminal region of the NIb gene (nt 8019–8449); B: the N-terminal region of the CP gene (nt 8577–8902). Recombinant isolates are shaded. The scale bar represents a distance of 0.1 substitutions per site.

referred as PPV-M – CG, Bulgarian (Deborré et al., 1995) and Bt-H (Salomon and Palkovics, unpublished), the sequences of which are available in the GenBank, share the same amino acids and phylogenetic analyses grouped them with recombinant isolates. Only CP sequences are available for these isolates, but it cannot be excluded that these isolates have recombinant character with a crossover situated out of the CP sequence.

Discussion

RNA recombination is thought to play an important role in the evolution, adaptation, repair and diversification of viral genomes (Lai, 1992; Cascone et al., 1993; Simon and Bujarski, 1994; Roosinck, 1997). Both homologous and nonhomologous recombination has been documented for natural isolates of several plant virus genera (e.g. Allison et al., 1989; Le Gall et al., 1995; Swanson and MacFarlane, 1999; Bousalem et al., 2000). Although homologous RNA recombination has been described for several viruses, the sequence and structural requirements of homologous crossovers are not well understood. Several models have been proposed and almost all studies have supported a copy-choice mechanism functioning during RNA synthesis (Nagy and Simon, 1997; Aaziz and Tepfer, 1999). Template switching may be relatively common in virus RNA synthesis but becomes detectable only when two different, although related, viral RNA strands occupy the same cell at the same time. This can be highly improbable because of the 'cross-protection' effect. However, once the recombination is established, the viability of a new RNA type depends on its fitness in comparison with parental molecules.

Limited data are available on the effect of recombination events on the potential fitness of plant viruses,

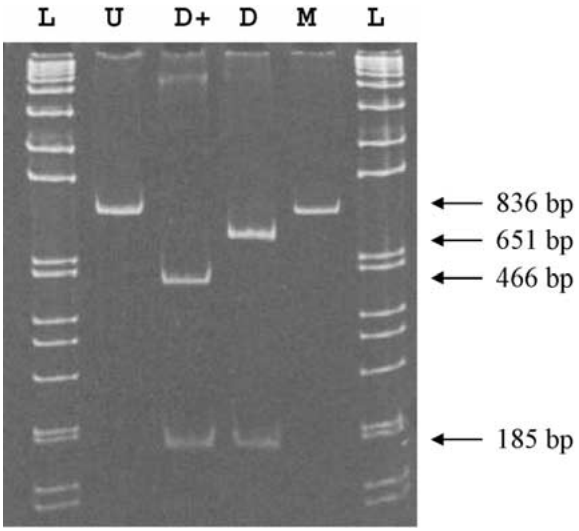

Figure 5. *Dde*I restriction profiles of RT-PCR products amplified with primer pair PP3/PCI. L = 1kb DNA ladder (Gibco BRL); U = uncleaved product; D = classical PPV-D restriction pattern (85.001 isolate); D+ = atypical restriction pattern observed in some recombinant isolates (KRN-1 isolate, see also Table 3); M = 91.003 isolate (PPV-M).

although it is generally thought to provide some selective advantage over parent variants in order to become established in nature. For instance, nonhomologous recombination of PPV-like virus and another unknown viral or host nucleic acid is considered as theoretically possible pathway of PPV–SoC evolution. This might explain the expanded host range of this type of isolate naturally infecting cherry trees (Nemchinov et al., 1998).

Until now, no biological study of a recombined PPV isolate has been carried out. Under natural conditions, the PPV recombinants sampled in Slovakia did not develop particular symptoms on their hosts. Experimentally, the BOR-3 isolate showed a capacity to be transmitted by graft or aphids to several *Prunus* spp. hosts (plum, apricot, peach), although the reaction on peach GF 305 and Montclar was symptomless. The existence of isolate(s) causing asymptomatic reactions on GF 305 is noticeable as it is widely used as an effective woody indicator plant.

Fifty-three isolates originating from Slovakia and France were analysed. To detect their potential recombinant character, we used a typing strategy targeting several parts of the PPV genome. The use of a common reverse primer (hexanucleotide random primers) in RT eliminated the need for individual reverse primers. All PCRs were performed from the same RT preparation, which reduced the cost and limited the possibility of manipulation risk due to multiple RT preparations. Inclusion of an immunocapture step overcame the inhibitors present in *Prunus* leaf extracts and eliminated the need for complicated RNA extraction.

A typing method based on an RT-PCR of the P3–$6K_1$ genomic region followed by restriction analysis has been developed (Glasa et al., 2002). However, a problem could arise when a mixed infection by isolates from different subgroups occurs in the same sample. In these cases, the analysis of restriction profiles would be difficult. Therefore, this method was complemented by an additional tool based on amplification of the same portion of the CI gene with two subgroup-specific primer sets (Figure 1). The results of PCR amplification demonstrated that this method could be used for PPV-M and D subgroup discrimination and it is also reliable for detecting possible cross-infection by PPV-M and D isolates.

In the present work, closely related recombinant variants were detected in various Slovak localities. This demonstrates that recombinants are viable in competition with conventional PPV-M and D isolates and circulate in respective localities through aphid transmission. The incidence of recombinants at each site was not determined, but detection of recombinants with only a small number of samples (16/23) indicates that the incidence is very high, at least in plums. The exact initial source of recombinant spread in Slovakia is difficult to identify, but some hypothesis could be put forward. Close molecular relationships of Slovak recombinant isolates support the hypothesis that these could have a common (recombinant) ancestor and unique dissemination focus. Only minor nucleotide changes were scattered along the sequenced regions of recombinants. The nucleotide substitution patterns for (Cter)NIb–Nter(CP) and P3–$6K_1$ revealed that variability could be due to a random genetic drift during post-recombination amplification steps.

The linkage of recombinants to plum orchards of Bojnice and Krajné in western and central Slovakia planted in the early 1980s, as first noted with biological material originating from Yugoslavia, may indicate a possible way of introduction. This hypothesis is also supported by the fact that the first reported PPV recombinant originated from former Yugoslavia (Cervera et al., 1993). An alternative scenario might involve several independent parallel PPV recombination events, which suggest that an extremely active recombination site (‘hot spot’) in the PPV genome is situated in the C terminus of the NIb gene.

The 'template switching' model of RNA recombination includes specific stem–loop structures close to the recombination point, which enable the viral replicase to pause polymerisation on the donor strand and vice versa, attract the enzyme to bind to another (acceptor) RNA strand and continue elongation of the nascent strand serving as primer (Nagy and Simon, 1997). A slightly different secondary structures predicted in C terminus of NIb gene (crossover-bearing portion in recombinant sequences) for available sequences of PPV-M and D isolates could explain why only the PPV recombinant type 5′D/3′M and no 5′M/3′D was detected (data not shown). However, it cannot be excluded that other recombinant isolates with further recombination point(s) along the PPV genomic RNA will be found in the future. Highly conserved regions including helicase and polymerase genes (CI, NIb) are especially promising candidates for bearing crossovers. Further data about PPV recombinants from more geographically distant places could clarify this point.

The finding of recombinant PPV populations in Slovakia was unexpected and contrasts with a previous investigation (Revers et al., 1996). Mixed infections of PPV-M and D isolates have been detected in nature (Candresse et al., 1998; Myrta et al., 1998; Szemes et al., 2001) and provide the opportunity for recombination to occur. In addition, invisible (and very hardly detectable) recombination could occur between RNA molecules of phylogenetically similar isolates. It is possible that further PPV recombinants occur in other European regions. However, this has to be confirmed by detailed assessments because currently used typing tests focus exclusively on the CP and do not allow identification of possible recombination along the 10-kb long PPV genome.

On the other hand, no recombinants were detected from France, a country with traditionally accurate phytosanitary measures (Table 4). Although the possible presence of PPV recombinants in French orchards cannot be excluded, the possibility of a shared occurrence of PPV-M and D isolates in one locality is minimised because sharka focuses are localised and infected trees immediately eradicated. Moreover, new imports of foreign isolates with infected material are prevented by strict phytosanitary control.

Relatively easy molecular identification of recombinant isolates will enable us to study the dynamic of recombinant populations in Slovakia, where PPV has an endemic status.

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