Occurrence and Genetic Diversity of Winona-Like *Plum pox virus* Isolates in Russia

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Abstract

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In studying the distribution and genetic diversity of *Plum pox virus* (PPV) in Russia, over a dozen new PPV isolates belonging to the strain Winona (PPV-W) were identified by immunocapture reverse-transcription polymerase chain reaction with the PPV-W-specific primers 3174-SP-F3/3174-SP-R1. Isolates were detected in two geographically distant regions of European Russia (Northern Caucasus and Moscow regions) in naturally infected plum (*Prunus domestica*), blackthorn (*P. spinosa*), Canadian plum (*P. nigra*), and downy cherry (*P. tomentosa*). The new PPV-W isolates were shown to be serologically related but not identical by triple-antibody sandwich enzyme-linked immunosorbent assay and Western blotting analysis using the monoclonal

antibody (MAb) 5B-IVIA and MAbs specific to the N-terminal epitopes of PPV-W isolate 3174. Analysis of nucleotide and deduced amino acid sequences of the (C-ter)NIB-(N-ter)CP genome region indicate great genetic diversity among isolates, with phylogenetic analysis revealing seven clades. Isolates P1 and P3 found in plum in the south of Russia clustered closely with the putative ancestral PPV-W isolate LV-145bt from Latvia, while isolate 1410-7 found in *P. nigra* in Moscow appears to be closely related to the Canadian isolate W3174. The data obtained indicate wide dissemination of PPV-W isolate in stone fruit in the European part of the former USSR.

Plum pox virus (PPV; genus *Potyvirus*; family *Potyviridae*) is the causal agent of sharka disease and is considered the most important viral pathogen of stone fruit crops (2). The virus is characterized by marked genetic diversity. Seven PPV strains (PPV-M, PPV-D, PPV-C, PPV-EA, PPV-W, PPV-Rec, and PPV-T), differing in nucleotide sequences of genomic RNA, antigenic properties, epidemiological behavior, geographic distribution, host range, and pathogenicity for various *Prunus* spp., are recognized (10,31). A number of serological and molecular methods for PPV detection and strain typing have been developed (3,29,34,39).

Three PPV strains D, M, and Rec are found commonly in Europe, while others are considered to have rare or local distribution (6). PPV-W is a recently described strain (14,15). The first isolate belonging to the W strain (W3174) was discovered in Ontario, Canada, in plum (*Prunus domestica*) and described as possibly being “introduced into Canada from Eastern Europe” (16). The results of molecular characterization and phylogenetic analysis of the complete nucleotide sequence indicated that W3174 was distantly related to other PPV isolates and represented a new strain of PPV (14,15). Another W-like isolate, 44191, was found in plum germplasm illegally imported into the United States from Ukraine (25). Because no W-like isolates were found in other regions of North America, and based on the homeowner description of the W3174 plant, it was proposed that PPV-W exists in and perhaps originated from Eastern Europe (16,25).

Subsequent findings of PPV-W isolate 1410 in Russia (32) and four isolates in Latvia (12) supported this assumption. Isolate 1410 was found in naturally infected seedborne Canadian plum (*P. nigra*) originating from plants introduced to the Moscow Botanical Garden many years ago. The results of partial molecular charac-

terization by immunocapture reverse-transcription polymerase chain reaction (IC-RT-PCR) and (Cter)NIB-CP-3'NCR nucleotide sequencing showed that 1410 was closely related to isolates W3174 and 44191 that composed the W strain. At the same time, some differences within coat proteins (CPs) of these isolates were found, indicating genetic diversity within the PPV-W strain (32).

Analysis of partial and complete genome sequences of the Latvian isolates indicate that PPV-W intrastrain diversity is greater than that of the other PPV strains (12). Remarkably, this variability was not distributed evenly along the entire genome. Two regions (P1/HcPro and VPg portion of NIa) showed >20% divergence when LV-141pl or LV-145bt and W3174 genomes were compared. This is due to the mosaic structure of the W3174 genome which contains PPV-M- and PPV-D-specific recombinant fragments in these regions, respectively. Thus, isolate W3174 is apparently chimeric and not typical of other PPV-W isolates. In contrast, LV-141pl and LV-145bt appear not to be affected by recombination and may quite possibly represent the “ancestral” or “typical” PPV-W genome. It was also suggested that PPV-W isolates identified in Latvia may have been introduced from other countries of the former USSR through uncontrolled exchange of propagation material, and that at least two independent introductions took place many years ago.

These findings suggest that PPV-W isolates may be widely disseminated over the European part of the former USSR but were not recognized because both PPV distribution and, especially, strain diversity are poorly studied in this region to date. Indeed, in 2010 and 2011, a number of field surveys of stone fruit plantings in some regions of European Russia resulted in the identification of several more PPV-W isolates. Their identification and partial characterization are described.

Materials and Methods

Virus isolates. The samples P1, P2, and P3 were collected from wild symptomatic *P. domestica* trees growing at a long distance from each other in Pyatigorsk district, Northern Caucasus region, during field surveys performed by the All-Russian Plant Quarantine Center. Leaf samples of isolates 1410-1, 1410-7, and 1410-12

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were collected from asymptomatic individual Canadian plum (*P. nigra*) trees growing on a plot in the Moscow Botanical Garden, Moscow (32). The samples Pd1, Pd2, Pd3, Pd4, and Pd5 were obtained from symptomatic blackthorn (*P. spinosa*) root offshoots within the same plot. These isolates were found during the course of PPV monitoring in stone fruit collections. Some samples were supplied kindly for analysis by owners of private gardens. The samples STNB1 and STNB2 were taken from two *P. domestica* trees grown in the immediate vicinity of a private garden located in Leninsky district, Moscow region. The sample RD4 was found on a downy cherry (*P. tomentosa*) tree in a backyard in Ramensky district, Moscow region. Fresh leaves were used for the initial PPV detection. Also, infected leaves were cut into small pieces and divided randomly into portions of 0.2 to 0.5 g weight, which were lyophilized and stored at 4°C until analyzed.

Double-antibody sandwich enzyme-linked immunosorbent assay. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed using the ELISA reagent set SRA 31505 (Agdia), and according to the manufacturer's protocol. Approximately 0.2 g of the leaf tissue was triturated in extraction buffer consisting of phosphate-buffered saline (PBS), 0.02% Tween-20, 2% polyvinylpyrrolidone (molecular weight [MW] of about 24,000), and 0.5% Triton X-100 (30) using a 1/20 ratio (g/ml). The extracts were clarified by low-speed centrifugation, added to the microplate wells (MaxiSorp microtiter plates; Nunc), and incubated in a temperature-controlled ST-3 shaker (ELMI, Latvia) for 2 h at 37°C (450 rpm). The virus concentration in the plant extracts was determined by DAS-ELISA using a calibration curve obtained with a purified PPV preparation. The isolate PPV-NAT, kindly provided by E. Maiss (Institute of Plant Disease and Plant Protection, University of Hannover, Germany), was propagated in *Nicotiana benthamiana* plants and purified as described (21).

Triple-antibody sandwich ELISA. Triple-antibody sandwich (TAS)-ELISA was used for strain identification of the isolates detected, and to study the reactivity of W3174-specific monoclonal antibodies (MAbs) with the PPV-W isolates. Identification of M (Rec and T), D, and C strains was performed using K11B, K12B, and K14B kits (AgriTest S.r.l., Valenzano, Italy), respectively, following the kit instructions.

All virus samples listed above were tested with a panel of 4A11, 10G7, and 5C1 W-specific MAbs (7). Rabbit PPV-specific immunoglobulin G (IgG) from the SRA 31505 ELISA reagent set (Agdia) was used as the capture antibody. The antibodies, diluted 1/200 in 0.05 M carbonate coating buffer, pH 9.6, were added (100 µl/well) to MaxiSorp microtiter plates (Nunc) and incubated overnight at 4°C.

The same plant extracts and incubation conditions were used for both DAS- and TAS-ELISA. The culture supernatants containing MAbs and diluted 1/200 in PBS-0.5% bovine serum albumin (BSA; albumin bovine fraction V; MP Biomedicals) were added to the wells and incubated 1 h at 37°C. Horseradish peroxidase-labeled anti-mouse IgG (W4021, Promega Corp.), diluted 1/20000 in PBS-0.5% BSA, was used as the detection antibody. The plates were washed five times with PBS-0.1% Tween-20 after each incubation step. The mixture of 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid)-2NH₄-salt (0.04%) and hydrogen peroxide (0.01%) in 0.05 M sodium citrate buffer, pH 4.4, was used as a chromogenic substrate. The optical densities were measured 20 min after substrate addition at a wavelength of 405 nm using a Titertek Multiscan Plus reader (Eflab Oy, Helsinki, Finland).

IC-RT-PCR. IC-RT-PCR, designed originally for PPV detection by Wetzel et al. (41), was performed according to the diagnostic protocol for regulated pests (4,8). PPV-specific IgG was purified from the antiserum prepared by immunization of rabbits with PPV-NAT. Universal 3'-noncoding region (3'-NCR)-specific primers (22) were used for PPV detection. For strain typing, P1/PD, P1/PM (28), SoC-2/dn, SoC-2/up (27), mD5/mM3 (36), and 3174-SP-F3/3174-SP-R1 (14) primers were used for D, M, C, Rec, and W strain identification, respectively. Experimental conditions for the RT-PCR reactions were as described originally for each primer set.

PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Western blot analysis. Western blot analysis was carried out as described by James et al. (16). Leaf samples of equal weights (0.3 g) were homogenized in 0.03 M HEPES buffer, pH 7.5 (1/5, g/ml). Crude extracts were mixed immediately with an equal volume of the 2× loading buffer, clarified by low-speed centrifugation, and heated for 7 min at 96°C. Proteins were resolved under denaturing conditions by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) (20), and transferred to polyvinylidene difluoride membranes (Hybond-P; Amersham) using a Semi-Dry Transfer Cell (Bio-Rad). Membranes were blocked with 5% skim milk powder (Fluka), diluted in PBS, and probed with either the W3174-specific MAbs (see above) or universal 5B-IVIA MAbs from a K10B kit (AgriTest S.r.l.). The W3174-specific MAbs were used at the 1/200 to 1/600 dilution while the universal MAb 5B was used at a 1/1,000 dilution. Horseradish peroxidase-labeled anti-mouse IgG (W4021; Promega Corp.) was used at the 1/10,000 dilution as the detecting antibody. All the blocking and detection steps were performed for 1 h at room temperature. PBS-0.1% Tween-20 was used for membrane washing and dilution of immunospecific reagents. Bound antibodies were detected using ECL Western blotting detection reagents (Amersham).

Positive and negative controls. Lyophilized positive controls (universal, D, M, and C strain-specific) and negative controls purchased from AgriTest S.r.l. were used in ELISA, IC-RT-PCR, and Western blot analyses. The PPV-W positive controls were leaf extracts of *P. nigra* and *N. benthamiana* plants infected with isolate 1410 (32). Also, isolates SoC (PPV-C) and 44191 (PPV-W), kindly provided by V. Mavrodieva (National Plant Germplasm and Biotechnology Laboratory, United States Department of Agriculture, Beltsville, MD), were used as positive controls for the strain-specific RT-PCR. Leaves of a PPV-free plum were used as negative controls.

5'-Terminal CP gene sequencing and analysis. The (C-ter)N1b-(N-ter)CP nucleotide sequences of PPV-W isolates, which encode the entire N-terminus of CPs and flanking regions, were amplified with the primer pair antisense 5'-AGCTTTCAAC-CGTGGTACTG-3' and sense 5'-ACATAGCAGAGACAGCATTG-3' primers. The primers, designed originally by Candresse et al. (5), were adapted for W-specific sequence amplification using the corresponding genome region of the isolate W3174 (AY912055) as a template. IC-RT-PCR was performed as described above using M-MuLV reverse transcriptase (Fermentas) for the first-strand cDNA synthesis and the proofreading Encyclo DNA-polymerase (Evrogen, Russia) for amplification. The specific 472-bp PCR product was excised from 2% agarose gel, purified using a Mini Spin column kit (Cytokine, St. Petersburg, Russia), and sequenced in both directions with coincident results using Evrogen facilities (Moscow). The nucleotide and deduced amino acid sequences were aligned using the ClustalW v.1.83 program. The percent homology was calculated by Martinez-NW method (24) using the MegAlign v.7.1.0 program. The phylogenetic tree was reconstructed from (C-ter)N1b-(N-ter)CP nucleotide sequences using PHYLML version 3.0 of the maximum likelihood algorithm (13) and graphically presented using MEGA (v. 4.0.1) software (37). The MWs of the deduced proteins of PPV-W isolates W3174 and LV-145bt were determined using the program Compute pI/Mw tool at http://au.expasy.org/tools/pi_tool.html (11).

Results

PPV detection and strain identification. The IC-RT-PCR analysis using 3'-NCR-specific primers resulted in amplification of an expected 220-bp fragment in all the samples tested and in the positive controls as well (Fig. 1A). No amplification was observed in healthy controls (NC1 and NC2). PPV also was detected readily by DAS-ELISA (*data not shown*), confirming that these samples were infected with PPV.

The strain of the isolates was determined by TAS-ELISA and IC-RT-PCR. The primers 3174-SP-F3/3174-SP-R1, specific for

PPV-W detection, were described earlier (14). Using these primers, the expected 327-bp fragment was amplified in all the samples and the corresponding positive controls (Fig. 1B). No amplification was observed in the healthy controls. Also, no amplification was observed with the M-, C-, or Rec-specific primers, except in the case of the corresponding positive controls (*data not shown*). A weak 198-bp band was observed in the samples P2 and P3 with the D-specific primers (*results not shown*). No positive reaction (except with the appropriate positive controls) was observed in strain-specific TAS-ELISA analyses, including samples P2 and P3, possibly due to low PPV-D content. All samples appeared to be infected with isolates belonging to the Winona group, PPV strain W. It should be noted that the 3174-SP-F3/3174-SP-R1 primer set allowed reliable detection of all PPV-W isolates identified in this study.

TAS-ELISA. The linear epitopes recognized by W3174-specific MAbs 10G7, 4A11, and 5C1 were mapped in the N-terminal domain of the isolate W3174 CP (7). Based on their established specificity, the reactivity of these MAbs with a broad panel of the new PPV-W isolates was studied by TAS-ELISA. The concentration of PPV-W was determined by DAS-ELISA using a calibration curve obtained with purified preparations of PPV-NAT, which belongs to strain D. According to the manufacturer's description, ELISA Reagent set SRA 31505 detects all the known PPV strains. The same extracts were used for TAS-ELISA. The average (from triplicate) optical density means (absorbance at 405 nm [A_{405}]) are shown in Table 1. The results show that PPV concentration in the extracts varied significantly. Nevertheless, the differences of MAb reactivity with each sample could be estimated reliably. No reaction of MAb 10G7 with any sample was observed. In contrast, MAb 4A11 reacted with all isolates. The lower A_{405} in samples P2, RD4, and STNB2 was likely due to low virus concentration in these extracts. No reaction of MAb 5C1 was observed with the samples P1 and P3, while weak reactions were observed with isolates STNB2 and RD4. This could be due to low virus concentrations in the latter extracts or different binding affinities. These results indicate that the W-like isolates identified are serologically related but not identical.

Western blotting analysis. Reactivity of W3174-specific MAbs with these isolates was also studied by Western blot analysis (Fig.

2). All isolates were detected by the universal MAb 5B (Agritest S.r.l.) and 4A11; however, only isolates Pd3, 1410-1, and P2 were detected by the MAb 5C1 (Fig. 2A, B, and C, respectively). MAb 10G7 failed to react with any isolate (*data not shown*). Where detection occurred, two main bands are associated with some virus isolates (Pd3 and 1410-1), and this pattern appears to be consistent regardless of the antibody used (Fig. 2). Because all samples were treated in the same way, the additional bands observed appear to be a feature of Pd and 1410 isolates. The universal MAb 5B seems to detect a number of lower-MW bands which may represent CP degradation products (Fig. 2A). These bands are usually considered as products of cleavage of the surface-exposed N-terminal domain of the CP by cell proteases (6,21,23,35). Their binding by MAb 5B specific to the universal PPV epitope, localized in the protease-resistant CP core, is an expected result. Interestingly, no degradation products are associated with isolate RD4, the only isolate detected in *P. tomentosa*, and there is variability in the profile of all isolates, except Pd3 and 1410-1, which appear similar to each other. The bands associated with the PPV-W isolates displayed unusual electrophoretic mobility and the uppermost band, associated with the full-length CP, was estimated to have a MW of approximately 43 kDa.

All isolates appear to contain the epitope recognized by MAb 4A11 (Fig. 2B). The epitope recognized by MAb 5C1 appears to be present only in isolates Pd3, 1410-1, and P2, with P2 showing great affinity for MAb 5C1. Where reactions were observed, MAbs 4A11 and 5C1 appeared to be more specific, recognizing only the main bands, which are presumably associated with the full-size CP of the virus (Fig. 2B and C). The CP double-band pattern observed in Pd3 and 1410-1 isolates was confirmed further and seems to be consistent in all the Pd and 1410 isolates, detected in *P. domestica* and *P. nigra* species, respectively (Fig. 3).

Nucleotide sequence and phylogenetic analyses. The (C-ter)Nlb-(N-ter)CP nucleotide sequences of PPV-W isolates were determined directly from PCR products (GenBank accession numbers JN226148, JN251107 to JN251117, JN900467, and JN900468). When compared with the isolates W3174, LV-141pl, and LV-145bt, the PCR fragments of these new isolates corresponded to the nucleotide positions 8,511 to 8,895 of W3174

Fig. 1. A and B, Analysis of *Plum pox virus* strain Winona (PPV W)-like isolates by immunocapture reverse-transcription polymerase chain reaction with A, 3'-noncoding region-specific primers and B, W strain-specific primers. PC = positive controls. A, Agritest and B, isolate 1410 on *Nicotiana benthamiana* (left) and *Prunus nigra* (right). NC1 and NC2 = negative controls (healthy plum and Agritest, respectively) and MR = GeneRuler Plus 100 bp DNA ladder (Fermentas).

(AY912055). They showed nucleotide identity levels of 88.6 to 94.5, 89.4 to 91.2, and 89.4 to 97.1%, respectively. Taken together with the data of IC-RT-PCR analysis using PPV-W-specific primers, these results indicated that all isolates belonged to the PPV-W group.

The phylogenetic tree presented in was created using the nucleotide sequences of the (C-ter)Nlb-(N-ter)CP region of these new PPV-W isolates. The corresponding nucleotide sequences of all other PPV-W isolates described to date—namely, W3174 (AY912055) (15); LV-140pl (HQ670745), LV-141pl (HQ670746), LV-143pl (HQ670747), and LV-145bt (HQ670748) (12); and 1410 (HQ326086) (32)—were included for comparison. The tree demonstrates clearly at least six distinct clades of the Russian isolates: Pd/1410, P1/P3, 1410-7, P2, RD4, and STNB1/STNB2. A high degree of divergence of these isolates is observed. Isolate 1410-7, which was found in a *P. nigra* collection in Moscow, clustered with W3174, and isolates P1 and P3, collected in the south of Russia (Pyatigorsk district), clustered with the putative typical PPV-W isolate LV-145bt. All other Russian isolates form distinct clades. Overall, this phylogenetic analysis seems to reflect a divergence that includes a total of seven clades, with Latvian isolates 140pl, 143pl, and 141pl forming a seventh and distinct clade.

N-terminal CP amino acid and epitope sequence analysis. An alignment of the deduced amino acid sequences encompassing the N-terminal region of the CPs, from the Nlb-CP junction to the conserved DRDVDAG sequence, is presented in Figure 5. The W3174-specific MABs evaluated in this study recognize epitopes in the N-terminus region of isolate W3174 (7); hence, this sequence was used as a reference. In addition, the corresponding deduced amino acid sequences of the other known PPV-W isolates were also included.

In most cases, the NIa protease site at the Nlb/CP junction of the W strain isolates consisted of the sequence NIVMHQ/A (Fig. 5, bold residues). Methionine (M) at the –3 position, as described by James and Varga (15), seems to be conserved in most PPV-W isolates. Interestingly, isolates STNB1 and -2, RD4, and P2 contain valine (V) at this position. This is similar to isolates of strains D, EA, and SoC (15). All of the strain W isolates studied contain the DAG motif associated with aphid transmission as well as the conserved DRDVDAG sequence representing the universal PPV-specific epitope (6).

The amino acid residues at positions 2 to 6, representing the N-terminal W-specific epitope recognized by MAb 10G7 (7), exist as NEEDD in all strain W isolates, except isolate W3174 (Fig. 5). The substitution of aspartic acid in position 2 with the inversely

charged asparagine shortened the continuous sequence of six negatively charged amino acids and, apparently, disrupts the structure of the epitope recognized by MAb 10G7. In addition, all PPV-W isolates studied to date, except W3174, lack the amino acid sequence ⁸PTIV¹¹ that was shown also to be essential for binding of 10G7. These differences explain the failure of 10G7 to bind with the CP of all isolates tested by TAS-ELISA and Western blotting analysis in this study. The only PPV isolate that reacted with 10G7 was W3174 because the epitope ²DEEDD⁶ was found only in this isolate to date.

Fig. 2. A–C, Western blot analysis to assess monoclonal antibody (MAb) reactivity with *Plum pox virus* strain Winona isolates. MABs: **A**, universal (Agritest); **B**, 4A11; **C**, 5C1. NC1 is a negative control (healthy plum). Arrows to the left indicate the positions of the marker proteins.

Fig. 3. Western blotting analysis of the *Plum pox virus* strain Winona-like isolates Pd and 1410 groups using monoclonal antibody 4A11.

Table 1. Reactivity of monoclonal antibodies (MABs) with *Plum pox virus* (PPV) strain Winona isolates in triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA)^a

Number	Sample	Virus concentration (µg/ml) ^b	MAB preparations		
			10G7	4A11	5C1
1	Pd1	0.14	0.11	2.55	2.39
2	Pd2	0.25	0.08	2.75	2.59
3	Pd3	0.29	0.08	2.66	2.57
4	Pd4	0.26	0.08	2.62	2.52
5	Pd5	0.32	0.08	2.67	2.61
6	1410-1	0.04	0.09	2.11	1.35
7	1410-7	0.05	0.09	2.47	1.91
8	1410-12	0.03	0.09	2.07	1.50
9	P1	0.06	0.08	2.59	0.11
10	P2	0.03	0.09	1.35	2.81
11	P3	0.12	0.09	2.74	0.13
12	RD4	<0.03	0.08	1.39	0.28
13	STNB1	0.07	0.09	2.37	0.78
14	STNB2	<0.03	0.10	1.31	0.36
15	Negative control (healthy plum)	0.0	0.10	0.09	0.09
16	Negative control (Agritest)	0.0	0.09	0.10	0.08

^a Average (from triplicate) optical density means (absorbance at 405 nm) are shown.

^b Determined by double-antibody sandwich ELISA using the purified PPV-NAT preparation as a standard.

The amino acid residues at positions 46 to 50 (⁴⁶MFNPV⁵⁰) represent the epitope recognized by MAb 4A11 (Fig. 5). There were slight differences between the isolates, in that valine or isoleucine was found at position 50; also, in the case of isolate P2, the amino acid residue at position 48, asparagine (N), is replaced by aspartic acid (D). Nevertheless, the MAb 4A11 specific to the epitope ⁴⁶MFNPV⁵⁰ recognized all PPV-W isolates, indicating that the substitution of valine to closely related isoleucine was apparently not crucial for binding. However, these differences may contribute to the variability in binding seen, for example, for isolate P2 in Figure 2A.

The sequence ⁵⁸PSVKP⁶² represents the epitope recognized by MAb 5C1 (Fig. 5). The amino acid residues at this site vary between the homologous isolate W3174 and the isolates P1, P2, P3,

STNB, and RD4. The substitution of hydrophobic valine by polar threonine at position 60 in isolates P1 and P3 appears to prevent the binding of MAb 5C1 in TAS-ELISA and Western blot analysis. The substitution of lysine by arginine at position 61 seems to prevent binding of MAb 5C1 with isolates STNB1, STNB2, and RD4 but surprisingly strong binding was observed with the isolate P2 (Fig. 2C). It may be that some surrounding substitutions influence this epitope accessibility.

In general, the amino-acid-deduced residues in the N-terminal region of the CP of the PPV-W isolates included in this study display great diversity (Fig. 5). The most variable part of the sequences appears to be the 50-amino-acid N-terminus region encompassing the epitope ²NEEDD⁶ and the DAG motif. Also, it seems that the block of amino acid residues 8 to 11 (PTIV for

Fig. 4. Phylogenetic analysis using the (C-ter)Nlb-(N-ter)CP nucleotide sequences of the *Plum pox virus* strain Winona isolates. The tree was constructed using the maximum likelihood method. Bootstrap values out of 1,000 replicates are shown. The scale bar indicates the number of substitutions per residue.

W3174) is prone to substitutions and indel events. Amino acid deletions are indicated for several isolates (Fig. 5).

Discussion

Isolates of the various strains of PPV differ in their host range (27) and in their detectability or identification using existing diagnostic tools (26). Therefore, it is important to determine the genetic diversity of isolates of PPV and their distribution to better enable accurate detection and more effective control of this very important stone virus. During PPV monitoring in European Russia, a number of new PPV isolates were detected and determined to be isolates of the newly described PPV-W strain. Their close relationship with members of the W strain was confirmed by the results of IC-RT-PCR with PPV-W-specific primers, (C-ter)Nlb-(N-ter)CP genome region sequencing and analysis, and serological analysis. No positive reactions for D, M, Rec, T, and C strain detection was observed with the corresponding strain-specific TAS-ELISA and IC-RT-PCR of these samples, except P2 and P3. This seems to indicate mixed infection of these two samples with isolates of both

strain D and W. The new PPV-W isolates were found in two geographically distant regions; namely, in the northern Caucasus (Pyatigorsk district) and Central Russia (Moscow and Moscow region) in trees growing hundreds of kilometers from each other. Taken together with previous results (12,16,25,32), our data indicate wide dissemination of PPV-W in stone fruit plantings over the European part of the former USSR.

Phylogenetic analysis resulted in the Russian isolates grouping into six distinct clades, with isolates 1410-7 and P1/P3 grouping with the previously described isolates W3174 and LV-145bt, respectively, while the other Russian isolates separated into the five other clades observed. LV-145bt, because of an absence of any recombination events, has been described as possibly representing the ancestral type (12). A high degree of divergence of these isolates is observed. Isolate 1410-7 was found in a *P. nigra* collection in Moscow while isolates P1 and P3 were collected in the south of Russia (Pyatigorsk district).

PPV is a member of the genus *Potyvirus*, and the main antigenic differences of species such as PPV are attributed to the immu-

Fig. 5. Multiple alignment of the N-terminal deduced amino acid sequences of the *Plum pox virus* strain Winona coat protein (CP). The proteinase cleavage site at the Nlb/CP junction is bolded and four mapped epitopes are shaded. Hyphens indicate sites of amino acid deletions.

nodominant N-terminus of the CP, where the majority of virus- and strain-specific epitopes are exposed (33). MABs are a convenient tool for CP analysis (17,23). The reactivity of MABs 10G7, 4A11, and 5C1 specific to the N-terminus of W3174 CP (7), as well as use of the universal PPV MAB 5B-IVIA, which is able to detect all PPV isolates to date (1), confirmed that these isolates were all PPV, with varying serological profiles.

The first Russian W-like isolate, 1410, was found in a symptomless *P. nigra* tree in Moscow (32). That planting consisted of 15 seedborne *P. nigra* trees more than 10 years old and growing in close vicinity to each other on an isolated plot. Some were asymptotically infected with PPV, while others were not. The isolate 1410 was taken from a single tree. In this work, the samples from three other infected *P. nigra* plants were investigated. In addition, five samples (designated as Pd) were collected from blackthorn root offshoots growing between *P. nigra* trees and displaying typical PPV symptoms. The results of serological tests showed that isolates collected from *P. nigra* and *P. spinosa* trees reacted similarly. In addition, all isolates of series Pd and 1410 were found by Western blotting analysis to differ from the others by the presence of a double band associated with the CP.

This double-band phenomenon is a feature of the Pd and 1410 isolates rather than some host species peculiarity because similar Western blot patterns were observed with infected extracts from *P. nigra* and *P. spinosa* and also from 1410-infected *N. benthamiana* plants (32). Mixed infection is likely not the cause. No strain other than PPV-W was found in these samples by TAS-ELISA and IC-RT-PCR analyses, and there were no ambiguities when the genomes of these isolates were partially sequenced. The PPV CP-associated double-band “phenotype” has been described previously (19,35). Site-directed mutagenesis was used to demonstrate that a single amino acid residue at position 66 of the CP may determine this phenotype, and that substitution of glycine at this position with arginine eliminated the double-band phenotype. The double-band phenotype associated with the CP of PPV-W isolates seems unrelated to the glycine-related mechanism described for PPV-Rec CP (35). Glycine is not present at position 66 of the PPV-W isolates. Alanine occupies position 66 in the vast majority of PPV-W isolates, while some isolates possess serine. Both alanine and serine are associated with the double-band phenotype in PPV-W. Glycine and serine are uncharged polar amino acid residues, whereas alanine is non-polar. Although glycine and alanine are similar in that they possess aliphatic R-groups, serine has a hydroxyl R-group. Therefore, it seems that there may be other mechanisms that contribute to this phenotype.

The samples STNB1 and STNB2 were collected from two adjacent *P. domestica* trees originated from the same scion-rooted plant. Unsurprisingly, these two samples were indistinguishable (except the virus titer) and may be preliminary considered to be a single isolate, STNB. The isolate RD4 appears to be closely related to STNB. Furthermore, both serological and sequencing data demonstrated close relationships between isolates P1 and P3 while the isolate P2 was at a long distance from them. Thus, in addition to isolate 1410, at least six more new PPV-W isolates (1410/Pd, 1410-7, STNB, P2, P1/P3, and RD4) have been found in Russia. They differ from each other and also from the Ukrainian and Latvian isolates when the sequencing data are compared. The phylogenetic tree based on the analysis of the (C-ter)N1b-(N-ter) CP nucleotide sequences may be considered as preliminary; however, the high bootstrap values indicate its reliability. In addition, it should be noted that the results are consistent with previous studies, and the arrangement of the four Latvian isolates and isolate W3174 in this tree is similar to that constructed by Glasa et al. (12) using complete or much more lengthy partial sequences and a different (neighbor-joining) algorithm. Isolates LV-140pl, LV-141pl, and LV-143pl; isolate LV-145bt; and isolate W3174 formed three distinct clades in both trees. Therefore, it appears that the tree (Fig. 4) reflects real phylogenetic relations between known and new isolates. This confirmed further the high levels of genetic diversity among PPV-W isolates described by Glasa et al. (12).

Unusual electrophoretic mobility was observed for all the isolates of PPV-W analyzed by Western blot analysis in this study, regardless of the detecting antibody. The MW of the PPV-W full-size CP determined by Western blotting analysis was about 43 kDa and found to be the highest among all PPV isolates investigated to date. At the same time, the CP of isolate 1410 was shown to consist of 330 amino acids and its calculated MW was about 36.4 kDa (32). Also, the MW of PPV isolates W3174 and LV-145bt were estimated at 36.6 and 36.4 kDa, respectively. It is expected that, when determined, the sizes based on the CP amino acid sequences of the new Russian isolates will likely be closer to these values rather than 43 kDa. Differences between the MW of the CP of PPV determined by sequence data analysis and by PAGE analysis have been described previously by Kollerova et al. (19). They showed that, for isolates of PPV strains D, M, and Rec, PAGE analysis gave an overestimation of the MW. It seems that this pattern is also similar for PPV-W because the MWs of PPV isolates W3174 and LV-145bt were estimated at 36.6 and 36.4 kDa, respectively. It is accepted currently that abnormally slow electrophoretic PPV CP mobility is due to posttranslational glycosylation or phosphorylation of serine and threonine residues in the N-terminal part of the CP molecule (9,19,35). The treatment of plant extracts with bacterial and calf intestine alkaline phosphatases performed as described elsewhere (38) did not alter the CP mobility (*data not shown*), suggesting that the phosphorylation, if it occurred, was of minor importance to modulate the electrophoretic CP mobility. On the other hand, it should be noted that almost one-third of the amino acids within the N-terminus of the CP of the investigated PPV-W isolates are serine and threonine residues which could be modified with *O*-linked B-N-acetylglucosamine by plant *O*-GlcNAc transferases (18). The analysis of glycoproteins by SDS-PAGE was shown to result in overestimation of MW due to lack of SDS binding by carbohydrate moieties (40). It is quite possible that the high glycosylation could be responsible for abnormally slow electrophoretic PPV-W CP mobility.

The profile seen in Western blot analysis may be an indicator of relationship in some cases, because all of the Pd and 1410 isolates, except 1410-7, group closely together in phylogenetic analysis of the (C-ter)N1b-(N-ter)CP nucleotide sequences.

Until now, the PPV-W isolates were found in European (*P. domestica*) and Canadian (*P. nigra*) plum and blackthorn (*P. spinosa*) (12,16,32). In this work, downy cherry (*P. tomentosa*) has been also identified as a natural host for PPV-W, indicating that the Winona strain is able to infect a wide range of *Prunus* spp.

In conclusion, it should be stressed that all Russian PPV-W isolates were found on scion-rooted, seedborne, or wild trees. This suggests that most, if not all, of these trees were apparently naturally infected with PPV-W and strengthens the hypothesis of an East European origin of the Winona strain (12,16). Attempts are now being made to obtain more sequence data, including complete genome sequences of some isolates, to more fully characterize these new Russian isolates of PPV-W and to better understand the genetic diversity of PPV strain W.

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