Identification and Differentiation of *Prunus* Virus Isolates that Cross-React with Plum Pox Virus and Apple Stem Pitting Virus Antisera

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ABSTRACT

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Prunus virus isolates (PVIs) were found that cross-reacted with plum pox virus (PPV) polyclonal antisera in immunosorbent electron microscopy, immunogold labeling, and Western blot analysis. The positive reactions in Western blot analysis were not affected when bovine serum albumin, fetal calf serum, glycerol, D-glucose, or skimmed (defatted) milk were used as blocking agents. Monoclonal antibodies to PPV reacted with the PVIs in Western blot analysis. The PVIs and some PPV isolates also cross-reacted in immunosorbent electron microscopy and Western blot analysis with apple stem pitting virus polyclonal antisera. The RNAs associated with the PVIs were analyzed to evaluate their homology with PPV RNA. The double-stranded RNA (dsRNA) profile of the PVIs showed that there were 3 to 5 dsRNA species ranging in size from 2.7 to 7.4 × 10^6 Da. Oligonucleotide sequences corresponding to the 3′ noncoding region, the 5′ noncoding region, and the 3′ terminus of the coat protein coding sequence of a non-aphid-transmissible strain of PPV (PPV-NAT) were used as probes in hybridization studies and as primers in reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. The PVIs did not react. RT-PCR analysis using PPV-specific primers from the conserved 5′ terminus of the coat protein coding sequence of PPV gave negative results with all PVIs. These analyses of the viral genome indicate that the PVIs are not isolates of PPV. Further studies to determine if the PVIs were members of the potyvirus group were negative. No evidence of associated cytoplasmic cylindrical inclusions or reaction with a potyvirus-specific monoclonal antibody was observed. The PVI particles ranged in size from 740 to 791 nm in length, and 10.4 to 14.4 nm in width. The coat protein subunit molecular mass ranged from 48 to 56 kDa, and can be distinguished from PPV by Western blot analysis. The implications of the serological cross-reactions are discussed.

Serological techniques for the detection and identification of plant viruses are usually economical and reliable, and are, therefore, widely used. The inadequate specificity and sensitivity displayed by some polyclonal antibodies has been resolved to some extent by the introduction of monoclonal antibodies. However, nonspecific binding or spurious cross-reactions can be observed even when monoclonal antibodies are used (2). Hauri and Bucher (14) demonstrated that, by means of the appropriate blocking agent, nonspecific binding can be eliminated. Serological cross-reactions between certain viruses have been described (4,11) and subsequently shown to be artifacts that can be eliminated by using skimmed milk as the blocking agent (12,40). Zimmermann and Van Regenmortel (40) also demonstrated that skimmed milk did not affect genuine incidents of cross-reactions.

Plum pox virus (PPV), a member of the potyvirus group, causes plum pox disease or sharka, which is considered the most serious disease of stone fruits and may cause losses as high as 80 to 100% of a crop (32). Serological techniques such as enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM) are commonly used to detect PPV (1,18,32). Analysis of double-stranded RNA (dsRNA) has also been used to detect and identify PPV-infected tissue (28). Maiss et al. (28) reported the isolation of two species of dsRNA associated with a non-aphid-transmissible isolate of PPV (PPV-NAT) (7.8 × 10^6 and 3.2 × 10^6 Da), whereas only one species of dsRNA was associated with an aphid-transmissible isolate (PPV-AT) (7.8 × 10^6 Da).

The PPV genome consists of a positive sense single-stranded RNA of approximately 10 kilobases (kb) (29) with a virus-encoded protein (VPg) covalently linked to the 5′ terminus of the genome, and a poly(A) tail at the 3′ end. The complete nucleotide sequence of several PPV isolates has been determined (21,29,34). This information has facilitated the development of specific oligonucleotides for use as nucleic acid probes, and as primers of reverse transcriptase–polymerase chain reaction (RT-PCR) that enable accurate identification of PPV. Clones of PPV-NAT have been used successfully for the detection of PPV RNA from herbaceous and woody hosts by Northern and dot blot hybridization assays (27). Wetzel et al. (39) developed an RT-PCR assay using oligonucleotide primers based on consensus sequences from homologous regions of three published PPV sequences. This RT-PCR assay has been used to detect PPV isolates from Cyprus, Egypt, France, Germany, Greece, Italy, Spain, and Turkey; in all cases, a PPV-specific 243 base pair (bp) fragment was amplified.

Recently, viruses in certain *Prunus* species were detected by ELISA and by ISEM using polyclonal antisera to PPV (16). Since the *Prunus* species involved originated from regions with no known history of plum pox disease, the identity of these viruses needed to be confirmed. The *Prunus* virus isolates (PVIs) could be distinguished from known PPV isolates by Western blot analysis. The coat protein (CP) subunits of the PVIs ranged in size from 48 to 56 kDa, whereas known PPV isolates had CP subunits ranging in size from 32 to 37 kDa (16). Indexing on woody indicators also suggested that the PVIs were not isolates of PPV (16).

The objective of this study was to determine if the PVIs reacting with PPV-specific antisera may be regarded as PPV isolates. A number of different antisera and the effects of several blocking agents were assessed to determine if the cross-reactions observed were genuine. The genomes of the PVIs were compared with those of known PPV isolates by means of dsRNA analysis accompanied by Northern blot and RT-PCR analysis. The cytopathology of PVI-infected tissue was examined for the appearance of cytoplasmic cylindrical inclusion bodies that are characteristic of potyvirus infections (5). This paper reports the results of these studies.

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MATERIALS AND METHODS

Virus sources. *Prunus mume* Siebold & Zucc. 'Bungo' (Q1256-01) and *P. mume* cv. Ting Ting (Q1256-03) were received from Japan in 1985. *Prunus persica* (L.) Batsch 'Ta Tao' (Q375-02), *P. persica* cv. Ku Chu'a Hung (Q375-18), and *P. persica* cv. Ta Tao (Q375-23) were obtained from the USDA Plant Introduction Station, Chico, CA, in 1974. This material had originated from China in 1933. *Prunus persica* cv. Agua (4-N-6) was brought to Ontario, Canada, from South Carolina and is believed to have come from Mexico originally. The PVIs were mechanically transferred from each of these *Prunus* sources to *Nicotiana occidentalis* Wheeler '37B' (VA Sisson, USDA, Oxford, NC). The PVIs were also transferred to *P. persica* cv. GF305 by bud inoculation.

PPV-ATCC, PPV-TAF, and PPV-Beltsville (#24830A) in the herbaceous host *N. benthamiana* Domin., and PPV-Spain in the woody host *Prunus tomentosa* Thunb., were obtained from A. Hadidi, USDA, Beltsville, MD. Samples of PPV-Marcus were obtained from F. Dosba, Bordeaux, France.

The apple stem pitting virus (ASPV) isolate B39 was obtained from W. Howell, IR2/NRSP5 Virus-tested Deciduous Fruit Tree Collection, Prosser, Washington.

Electron microscopy. Disks (1 cm in diameter) from symptomatic leaves were ground in 100 µl 0.03 M potassium phosphate, pH 7.0, containing 5 mM magnesium chloride and 0.02% sodium azide. Formvar-carbon coated copper grids were allowed to bind particles, after which the grids were washed, stained with 2% uranyl acetate, and examined by a transmission electron microscope calibrated with a grating replica (JBS #401: J. B. EM Services Inc., St. Laurent, Quebec). Normal lengths of the particles were calculated using a procedure similar to that described by Monette and James (30).

ISEM was carried out using a procedure similar to that described by Van Regenmortel (36). After trapping and decorating, the grids were stained with 2% uranyl acetate. The following antisera were tested: apple chlorotic leaf spot virus (ACLSV), apple stem grooving virus (ASGV), bean yellow mosaic potyvirus (BYMV), potato virus T (PVT), ASPV-Yanase, PPV-Dunez, and PPV-ATCC (#PVAS-709).

Immunogold labeling was carried out by trapping virions from symptomatic leaves as described above. The grids were washed with 0.01 M sodium potassium phosphate, pH 7.2, containing 0.14 M sodium chloride, 0.02% sodium azide (phosphate-buffered saline, PBS) containing 0.05% Tween 20 (PBST) (25) and blocked for 1 h with 0.1% bovine serum albumin (BSA) in water. The grids were washed with PBST and decorated by floating the grids for approximately 30 min on polyclonal antiserum diluted 1:100 in PBS. The grids were exposed for 1 h to gold-labeled (10 nm) goat anti-rabbit IgG (Sigma, St. Louis, MO) diluted 1:100 in PBS containing 0.02% polyethylene glycol (M.W. 20,000). The grids were washed in sequence with PBST, PBS, and filter-sterilized double distilled water, then stained with 2% uranyl acetate.

To determine if the same population of virions was reacting with antisera to ASPV and PPV, two techniques were used. In the first method, particles of the PVIs were trapped with ASPV antiserum, and exposed to purified anti-PPV IgG labeled (37) with colloidal gold particles (15 nm) (26). In the second method, the particles were trapped with PPV antiserum, exposed to ASPV antiserum, then exposed to gold-labeled PPV IgG for a further 30 min. The grids were then washed as described above and stained with 2% uranyl acetate.

Western blotting. Sap was extracted from fresh or freeze dried leaves of healthy or PVI-infected *Prunus, N. occidentalis,* and *N. benthamiana* plants. Proteins were resolved under denaturing conditions by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (20) and electroblotted (Bio-Rad, Mississauga, Ontario, Semi-Dry Transfer Unit) onto nitrocellulose (Schleicher & Schuell, Keene, NH, 0.45-µm pore size). Blots were screened with PPV polyclonal antisera from five sources: Casper, Plantest, Bioreba, ATCC, Dunez; with the PPV monoclonals (PPV-MAbs) 5B, 4DB12, 1EB6, and 4DG5 obtained from M. Cambra, IVIA, Valencia, Spain, and the PPV-MAbs 03(C11), 04(H11), 05(F12), 06(G11), 07(D9), and 08(G8) from M. Navratil, Palacky University, Czech Republic. Blots were also screened with ACLSV, BYMV, and ASGV polyclonal antisera and with a potyvirus-specific monoclonal antibody (Mab) (Agdia). Polyclonal antisera against ASPV from three sources (Jelkmann, Verderevskaja, and Yanase) were also used. Protocol A (see below) was used for screening all antisera. Protocols B and C were used for any antiserum that reacted in Protocol A.

Protocol A. The membranes were blocked overnight at 4°C, in 0.05 M Tris, 0.15 M sodium chloride, pH 7.4, (TBS) containing 1% BSA; washed three times (10 min each) in TBS with 0.05% Tween 20 (TTBS). The primary antisera, except PPV-MAbs, were diluted 1:2,000 in TBS containing 1% BSA; the PPV-MAbs were used at a 1:1,000 dilution. Goat anti-rabbit or anti-mouse alkaline phosphatase conjugate was used as the secondary antibody.

Protocol B. TBS containing 5% skimmed milk powder was used as the blocking agent.

Protocol C. The membranes were blocked overnight at 4°C in PBS containing 10% fetal calf serum, 10% glycerol, 1 M D-glucose, and 0.5% Tween 20 (PBS-FGGT). The antisera, except the PPV-MAbs, and the conjugates were diluted 1:2,000 in PBS-FGGT. The PPV-MAbs were used at a concentration of 1:1,000 in PBS-FGGT. All washes were in TTBS.

All membranes were washed in TBS for 5 min prior to immersion in the substrate consisting of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, used as recommended by the supplier (Bio-Rad).

Cytopathology. Pre-chilled healthy and infected tissues were fixed in cold 2.5% glutaraldehyde as described by Langenberg (22). After dehydration in an acetone series, the samples were gradually embedded in Spurr's resin containing 1% Dow 200 fluid (23). Ultrathin sections were stained by immersion in 25% uranyl acetate in methanol for 5 to 10 min, followed by treatment with 0.1% basic lead citrate.

Epidermal strips were taken from fresh leaves of *Prunus* and herbaceous indicator plants infected with the PVIs or known PPV isolates that were included as positive controls. The strips were stained in phloxine as described by Van Oosten and Van Bakel (35), then examined and photographed using a Nikon (Chiyoda, Tokyo) Optiphot light microscope.

Isolation of dsRNA. Five grams of *N. occidentalis* tissue was ground in liquid nitrogen and the dsRNA extracted using a procedure previously described by James (15) except that the eluate was treated with RNase T1 (10 U ml^{-1}) for 30 min, adjusted to 30 mM magnesium chloride, and treated with 10 µg ml^{-1} DNase for 30 min prior to ethanol precipitation. The samples were analyzed by polyacrylamide gel electrophoresis and silver stained. The dsRNA sizes were estimated by comparison with the dsRNA from *Phaseolus vulgaris* L. (Black Turtle Soup bean), molecular mass = 8.0×10^6 Da; and the dsRNAs of carnation mottle virus, molecular mass = 3.0, 1.2, and 1.0×10^6 Da (R. Jordan, personal communication). All extractions and analyses were repeated.

Slot-blot hybridization analysis of RNA. Total RNA was isolated from 7 g of healthy or virus-infected herbaceous tissue essentially according to Morris and Dodds (31), except that the ethanol concentration during binding to Whatman CC-41 cellulose was increased to 30% to retain single-stranded RNA (7). Nuclease digestions were omitted. After two successive ethanol precipitations, the total RNA was dissolved in 100 µl of DEPC-treated water. To 20 µl of RNA, 0.5 µl of 1 M methyl mercury hydroxide was added. After 15 min, each sample was diluted with 1.3 ml of 10× SSC (SSC = 0.15 M sodium chloride; 15 mM sodium citrate, pH 7.0) containing 14 mM β-mercaptoethanol. In each well of a vacuum manifold (Bio-Rad), 300 µl of each sample or a 1:10 dilution in 10× SSC was applied to a nylon membrane (GeneScreen, DuPont, Mississauga, On-

tario) previously equilibrated with 10× SSC. Each well was washed with 300 µl of 10× SSC. RNA was cross-linked to the filter by UV light, washed in 10× SSC, and air dried.

Plasmids pPPV-NAT274 and pPPV-NAT65 were digested with *Pst*I to recover the complete PPV insert. Plasmid p4PPV-NAT309 was digested with *Bam*HI/*Eco*RI to release two fragments of PPV sequence. In all cases, the inserts were resolved by gel electrophoresis and recovered by gelase (Epicentre Technologies, Madison, WI) digestion. The resulting fragments (Table 1) were labeled with [^{32}P]-dCTP (6) and used for hybridization with Northern blots and detected by autoradiography. A separate blot was used for each probe.

Table 1. Hybridization probes used to compare plum pox virus (PPV) and *Prunus* virus isolates

Description	Position[a]	Source
NAT274	12-1630	ATCC
NAT65	7030-poly(A)	E. Maiss
NAT309	8890-poly(A)	E. Maiss
NAT309-5′	8890-9273	See text
NAT309-3′	9274-poly(A)	See text

[a] Nucleotide numbers are relative to the corresponding non-aphid-transmissible (NAT) strain of PPV sequence (29).

RT-PCR analysis using primers corresponding to PPV-NAT sequences. RT was performed, using 5 µl of total RNA extracted from herbaceous hosts as described above, in a total volume of 20 µl containing 50 mM Tris pH 8.3, 75 mM potassium chloride; 3 mM magnesium chloride; 10 mM dithiothreitol, 500 µM dNTPs, 40 pmole 3′ primer (Table 2) and 75 U cloned Moloney murine leukemia virus RT (Superscript, Gibco/BRL, Burlington, Ontario) for 45 min at 42°C.

Table 2. Reverse transcriptase–polymerase chain reaction primers used to compare plum pox virus (PPV) and *Prunus* virus isolates

Description	Position[a]	Sequence	Reference
CP-5′	8592	5′-GACGAGGAGGAAGTTGATGC-3′	29
CP-3′	8721	5′-TGCAGTTGAGGTCCTGACAC-3′	29
NCR-5′	9522	5′-TAGTGGTCTCGGTATCTATC-3′	29
NCR-3′	9652	5′-CGACAATAACAGACTAGAAC-3′	29
PPV-2	9292	5′-CAGACTACAGCCTCGCCAGA-3′	39
PPV-3	9515	5′-CCCTCACATCACCAGAGCCA-3′	39

[a] Nucleotide numbers are relative to the corresponding non-aphid-transmissible strain of PPV sequence (29).

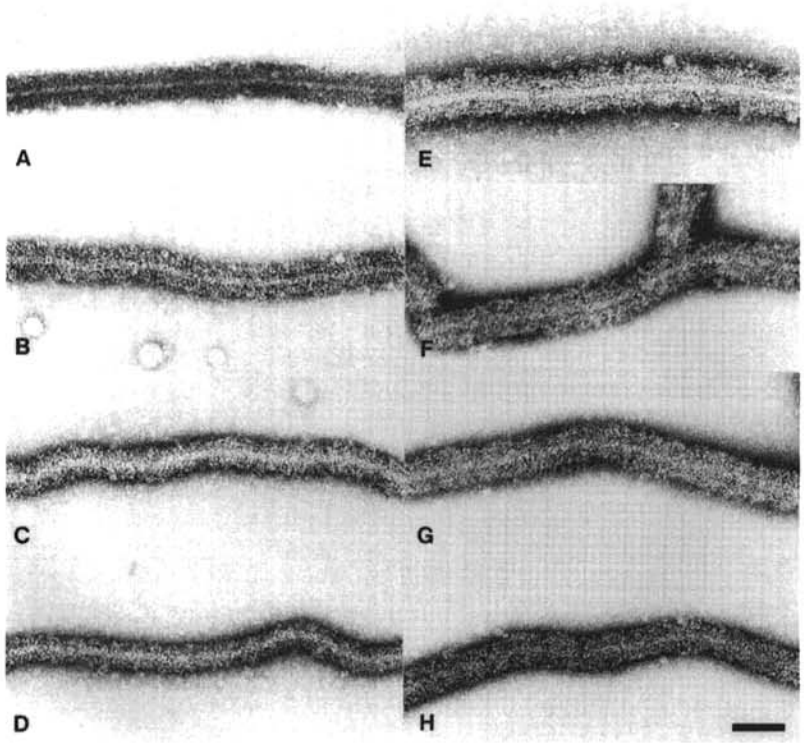

Fig. 1. Electron micrographs showing serological reactions of *Prunus* virus isolates (PVIs), plum pox virus (PPV) isolate PPV-Beltsville, and apple stem pitting virus (ASPV) to PPV and ASPV antisera. Virus particles were trapped by PPV-Dunez **(A–D)** or ASPV-Yanase **(E–H)** antisera from leaf extracts of *Nicotiana occidentalis* infected with PVI 4-N-6 **(A, E),** PVI Q1256-01 **(B, F),** PVI Q375-02 **(C, G),** and ASPV **(H)** or from extracts of *N. benthamiana* infected with PPV-Beltsville **(D).** Bar = 100 nm.

For amplification, 1 µl of RT product was added to 100 µl of reaction cocktail containing 10 mM Tris pH 8.8; 50 mM potassium chloride; 1.5 mM magnesium chloride; 0.01% gelatin; 200 µM each dNTP; 1 µM each primer (Table 2), and 1.25 U AmpliTaq DNA polymerase (Perkins-Elmer Cetus, Norwalk, CT). The samples were denatured for 1 min at 96°C, then given 30 cycles consisting of annealing at 45°C for 45 s, extension at 72°C for 30 s, and denaturation at 92°C for 30 s with a thermocycler (model PHC-2, Techne, Princeton, NJ). A 10-µl sample of the reaction mixture was analyzed by gel electrophoresis in a 2% Nu-Sieve agarose gel (FMC BioProducts, Rockland, ME) followed by ethidium bromide staining.

For RT-PCR of samples from leaves from woody hosts, extraction and RT were performed as described by Wetzel et al. (39). After amplification and gel analysis as described above, the identity of the products as PPV sequences was confirmed and the sensitivity of the assay enhanced by Southern blotting of the resulting gel and hybridization with ^{32}P-labeled probes. To prepare the probes, the 140-bp fragment of pNAT65 was amplified by PCR using the 3′ noncoding region primer pair. The product was gel purified and labeled with [^{32}P]-dCTP (6).

RT-PCR analysis using PPV-2 and PPV-3 primers. Nucleic acids were isolated from woody and herbaceous tissues using the method of Langeveld et al. (24) followed by RT as described above, except that primer PPV-3 was used. For PCR, 2.5 µl of RT reaction was added to a 22.5-µl master mix consisting of 50 mM Tris, pH 8.3; 1.5 mM magnesium chloride; 200 µM dNTPs; 500 µg BSA per ml; 1% Ficoll-400; 1 mM tartrazine dye; 50 pmoles each primer; and 0.05 U/µl *Taq* DNA polymerase (Promega, Madison, WI). Samples were subjected to 45 cycles of amplification (denaturation at 94°C for 5 s, annealing at 62°C for 15 s, and extension at 74°C for 30 s), using an air thermocycler (model 1605, Idaho Technologies Inc., Idaho Falls, ID). Fifteen-microliter aliquots were subsequently analyzed by gel electrophoresis on 1.5% agarose in the presence of ethidium bromide.

RESULTS

Electron microscopy. PVIs are flexuous rods with a normal distribution of the particle lengths; isolates Q1256-01, Q375-02, and 4-N-6 had modal lengths of 791 (n = 84), 783 (n = 121), and 740 nm (n = 123), and average widths of 13.8 (n = 23), 14.4 (n = 25) and 10.4 nm (n = 21), respectively. PPV (ATCC), included as a positive control, has a modal length of 813 nm and an average width of 11.4 nm (n = 16).

ISEMs using antisera to ACLSV, ASGV, BYMV, or PVT were all negative, that is, no trapping or decoration of the PVIs was

observed. However, all three PVIs reacted positively in ISEM analysis with PPV-Dunez antiserum and ASPV-Yanase antiserum (Fig. 1, A–C and E–G, respectively). PPV-Dunez antiserum reacted well with all PPV isolates included as positive controls, with the possible exception of PPV-Beltsville. Very few particles of PPV-Beltsville were trapped on the grids but the particles that were trapped were well decorated (Fig. 1D). ISEM analysis also revealed a similar degree of reaction between ASPV and PPV-Dunez antiserum (Fig. 2A). PPV-Beltsville also reacted positively in ISEM with ASPV-Yanase antiserum (Fig. 2B). PPV-Spain was trapped and decorated equally well by either ASPV-Yanase antiserum or PPV- Dunez antiserum. PPV-TAF and PPV-ATCC did not react with the ASPV-Yanase antiserum.

In heterologous decoration tests of PVI 4-N-6 using PPV-Dunez antiserum and ASPV-Yanase antiserum, in which one antiserum was used for trapping and the other for decoration, efficient trapping of 4-N-6 virions was always observed. No undecorated or partially decorated particles were observed. Similarly, gold-labeled PPV-Dunez IgG uniformly labeled particles trapped by either PPV-Dunez or ASPV-Yanase antiserum (Fig. 3A and B, respectively). Gold-labeled PPV-Dunez IgG was used to decorate the PVIs after trapping with ASPV-Yanase antiserum. The particles were also trapped with PPV-Dunez antiserum, partially decorated with ASPV-Yanase antiserum, then decorated with gold-labeled PPV-Dunez IgG (Fig. 3C). Particles of PPV-ATCC and PPV-Spain were well decorated by gold-labeled PPV IgG, or by gold-labeled goat anti-rabbit IgG following decoration with PPV-Dunez antiserum.

Western blotting. ACLSV, ASGV, potyvirus-specific MAb (Agdia) and PPV-Plantest polyclonal antisera did not react with PPV isolates or PVIs from woody or herbaceous hosts in Western blots. Nonspecific binding was observed when either PPV-Casper or BYMV antisera were used; no virus-specific bands associated with the PVIs could be identified. The PPV-Bioreba polyclonal antiserum reacted with PPV isolates but did not react with any of the PVIs.

PPV-Dunez and PPV-ATCC antisera reacted with all of the PPV isolates and PVIs included in this study (Fig. 4A). A faint reaction with the CP subunits of ASPV was also observed (Fig. 4A, lane 8). The PPV isolates had CP subunit sizes ranging from 32 to 37 kDa (Table 3). PVIs Q1256-01, Q375-02, and 4-N-6 had CP subunit sizes estimated at 56, 52, and 48 kDa, respectively (Fig. 4; Table 3).

PPV-MAb 4DB12 reacted with PVI Q375-02, revealing a band with an approximate size of 52 kDa (Fig. 5, lane 3). Reaction with the positive control PPV-Marcus revealed a band of approximately 37 kDa (Fig. 5, lane 1) plus higher molecular mass bands possibly representing subunit dimers and trimers. The healthy control was negative (Fig. 5, lane 5). PPV-MAb 05(F12) reacted with all PVIs and PPV isolates tested (Fig. 6). No reactions were detected with the other PPV MAbs.

All ASPV antisera reacted with the ASPV control. ASPV-Jelkmann antiserum did not react with any of the PVIs. However, ASPV-Yanase and ASPV-Verderevskaja antisera reacted with all PVIs, and with PPV-Beltsville and PPV-Spain (Fig. 4B; Table 3). These antisera did not react with PPV-Marcus, PPV-ATCC, or PPV-

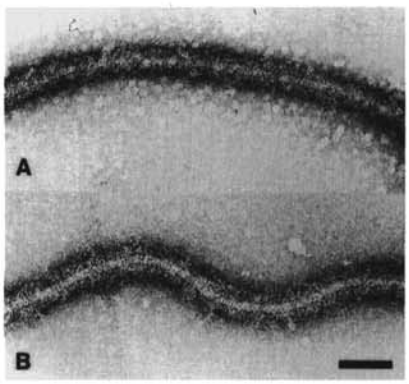

Fig. 2. Electron micrographs showing cross-reactions of plum pox virus (PPV)–Dunez antiserum and apple stem pitting virus (ASPV)–Yanase antiserum. **(A)** ASPV particle from *Nicotiana occidentalis* trapped and decorated with PPV-Dunez antiserum; **(B)** PPV-Beltsville particle from *N. occidentalis* trapped and decorated with ASPV-Yanase antiserum. Bar = 100 nm.

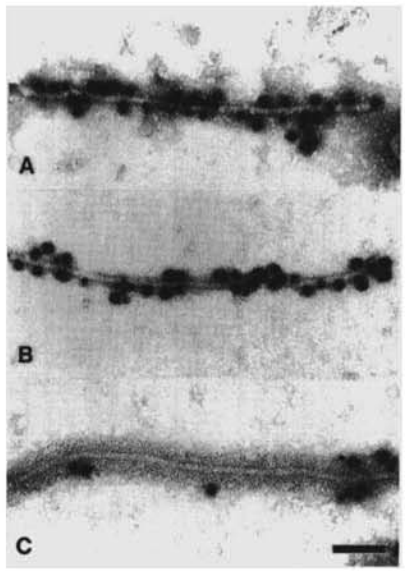

Fig. 3. Immunogold labeling of *Prunus* virus isolate PVI 4-N-6 from infected *Nicotiana occidentalis*. The particles were labeled with gold-labeled plum pox virus (PPV)–Dunez IgG following **(A)** trapping by PPV-Dunez antiserum, **(B)** trapping by apple stem pitting virus (ASPV)–Yanase antiserum, and **(C)** trapping by PPV-Dunez antiserum and decoration with ASPV-Yanase antiserum. Bar = 100 nm.

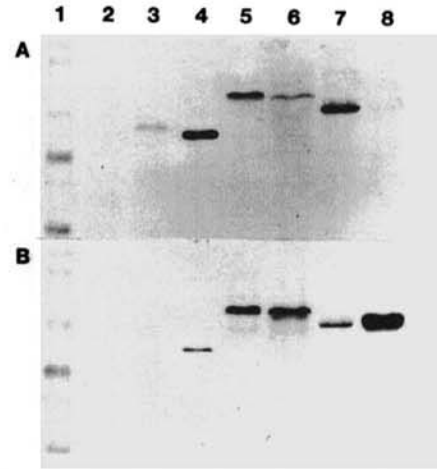

Fig. 4. Western blot analysis of *Prunus* virus isolates (PVIs), plum pox virus (PPV), and apple stem pitting virus (ASPV) coat proteins. Blots were incubated with either **(A)** PPV-Dunez antiserum or **(B)** ASPV-Yanase antiserum. Lane 1: Bio-Rad prestained molecular mass standards; lane 2: Healthy cv. GF305; lane 3: PPV-Marcus in GF305; lane 4: PPV-Beltsville in *Nicotiana benthamiana*; lane 5: Q1256-01 in GF305; lane 6: Q375-02 in GF305; lane 7: 4-N-6 in GF305; and lane 8, ASPV in *N. occidentalis*. Western blot analysis with PPV-ATCC is indistinguishable from **(A)**.

Table 3. Coat protein subunit sizes of the *Prunus* virus isolates (PVIs), plum pox virus (PPV), and apple stem pitting virus (ASPV), as determined by Western blot analysis

	Coat protein subunit size (kDa)	
	PPV antisera	ASPV antisera
Isolate	(PPV-Dunez and PPV-ATCC)	(ASPV-Yanase and ASPV-Verderev.)
PVI (Q1256-01)	56	56
PVI (Q375-02)	52	52
PVI (4-N-6)	48	48
PPV (ATCC)	32	NR[a]
PPV (Belt.)	34	34
PPV (Marcus)	37	NR[a]
PPV (Spain)	37	37
PPV (TAF)	32	NR[a]
ASPV	48	48

[a] No reaction observed.

TAF (Fig. 4B; Table 3). Where there was joint reaction, ASPV antisera and the PPV antisera reacted with proteins of identical migration rates during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fig. 4A,B). The observed antibody binding was not affected by the different blocking conditions (Protocols A, B, and C); all experiments were done at least twice using both woody and herbaceous hosts.

Cytopathology. No cytoplasmic cylindrical inclusions were observed in either *Prunus* or *Nicotiana* tissue infected with PVIs Q1256-01, Q375-02, or 4-N-6, but were evident in *N. benthamiana* infected with PPV-ATCC.

RNA analysis. Gel electrophoresis revealed five dsRNA species with estimated molecular masses of 7.1, 6.8, 6.3, 5.4, and 2.7×10^6 Da associated with PVI Q1256-01; three dsRNA species with molecular masses of 6.9, 6.2, and 5.3×10^6 Da associated with PVI Q375-02; and three dsRNA species with molecular masses of 7.4, 7.0, and 6.3×10^6 Da associated with PVI 4-N-6 (Fig. 7). PPV-ATCC and PPV-TAF each had two dsRNA species, a major band of 7.9×10^6 Da and a minor band of 7.4×10^6.

The 482-bp fragment NAT309-3′ spans 230 bases of the PPV 3′ noncoding region. Hybridization of this fragment to the genome of PVIs would provide strong evidence that the PVIs are isolates of PPV. There was no hybridization of the NAT309-3′ fragment with the dsRNA of any of the PVIs (Table 4); this probe hybridized strongly with reference strains of PPV under identical conditions. The 5′ noncoding region was represented by probe NAT274, and the 3′ terminus of the CP coding sequence was represented by probes NAT65 and NAT309-5′. None of these fragments hybridized with the PVI-associated RNAs under conditions that resulted in strong binding to the RNA of the PPV reference isolates (Table 4).

Total RNA extracted from *Nicotiana* spp. infected with PPV reference strains Beltsville and ATCC yielded positive results when analyzed by RT-PCR using primers based on the PPV-NAT sequences (Table 5). Amplification products derived from the N terminus of the CP coding region, flanked by primers CP-5′ and CP-3′, have an estimated size of 210 bp and 190 bp for the ATCC and Beltsville isolates of PPV, respectively. This primer pair also yielded an amplification product of 140 bp when the control plasmid pNAT65 was used as template. The size difference is, in part, the consequence of a 45-nucleotide deletion detected in the sequence of the 5′ terminus of the PPV-NAT genome relative to other PPV isolates (27). Amplification of the 3′ noncoding region yielded 140-bp fragments from both PPV isolates and from the plasmid pNAT65. None of the PVIs in *N. occidentalis* yielded RT-PCR products from total RNA when either set of primers was used under identical reaction conditions.

To determine if PPV was present in the original source material but had not been successfully transferred to herbaceous hosts, RT-PCR with the 3′ noncoding region primers was used to test extracts directly from leaves of woody hosts. Extracts of *P. tomentosa* infected with PPV-Spain yielded the predicted product (Table 5) while extracts of PVIs in *P. persica* cv. GF305 or *P. mume* did not yield similar products. This observation was confirmed by Southern blot analysis with ^{32}P-labeled probes; no hybridization of the probe occurred in lanes corresponding to the PVI reaction products (data not shown).

When RT-PCR analysis was carried out using primers PPV-2 and PPV-3, electrophoretic analysis of the RT-PCR products of the known PPV isolates, PPV-ATCC, PPV-TAF, and PPV-Beltsville, yielded a single DNA band (Table 5) that corresponded in size to the predicted fragment of 243 bp. A similar 243-bp fragment was not detected in association with any of the PVIs in either woody or herbaceous hosts. Occasionally, a band of less than 243 bp was detected in association with Q1256-01.

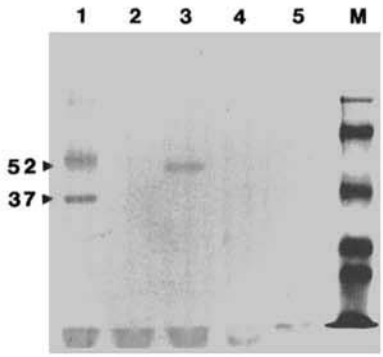

Fig. 5. Western blot analysis of the *Prunus* virus isolates (PVIs), plum pox virus (PPV)–Marcus using PPV-MAb 4DB12 with skimmed milk powder as blocking agent. Lane 1: PPV(Marcus); lane 2: Q1256-01 in cv. GF305; lane 3: Q375-02 in GF305; lane 4: 4-N-6 in GF305; lane 5: healthy GF305; and lane M: Bio-Rad prestained molecular mass standards.

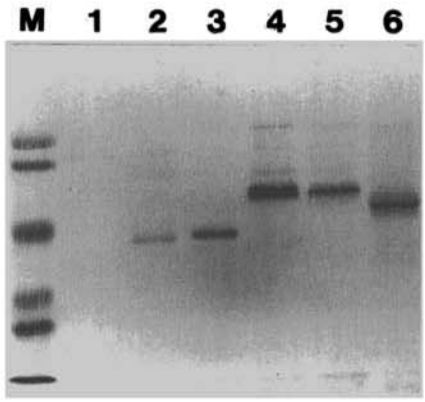

Fig. 6. Western blot analysis of the *Prunus* virus isolates (PVIs) and two plum pox virus (PPV) isolates using PPV-MAb 05(F12) with skimmed milk powder as blocking agent. Lane M: Bio-Rad prestained molecular mass standards; lane 1: healthy cv. GF305; lane 2: PPV (Marcus) in GF305; lane 3: PPV (Spain) in *Nicotiana occidentalis*; lane 4: Q1256-01 in GF305; lane 5: Q375-02 in GF305; and lane 6: 4-N-6 in GF305.

DISCUSSION

Plum pox (sharka) is a very important disease of *Prunus,* and reliable and accurate identification is essential. In this study, the PVIs reacted positively with the polyclonal PPV-Dunez antiserum when evaluated by several methods including ISEM, immunogold labeling, and Western blot analysis. Western blot analysis also revealed reactivity of the PVIs with PPV-ATCC polyclonal antiserum and PPV-MAb 05(F12), and of PVI Q375-02 with PPV-MAb 4DB12. Positive reactions between the PVIs and PPV antisera in ELISA were previously reported (16). Western blot analysis and indexing on woody and herbaceous indicators were used to differentiate these viruses.

There is a possibility that the observed cross-reactivity with the polyclonal antisera results from a mixed virus preparation used in the production of the antisera. This is unlikely since the viruses used as immunogen for each of the antisera originated from distant geographical areas. Furthermore, reaction of the PVIs with PPV-MAb 05(F12) and the recognition of PVI Q375-02 with PPV-MAb 4DB12 indicate that these viruses share some common epitopes with PPV.

Zimmermann and Van Regenmortel (40) showed that spurious serological cross-reactions between unrelated viruses can be abolished by using defatted or skimmed milk as a blocking agent. Birk and Koepsell (2) indicated that glycerol and D-glucose can reduce nonspecific binding of antibodies to proteins in immunoblot assays. James et al. (16) used skimmed milk as a blocking agent in ELISA without effect on the cross-reactions of the PVIs to PPV-Dunez antiserum. In this study, several blocking agents were assessed including BSA, fetal calf serum, glycerol, D-glucose, and skimmed milk powder. None of the blocking agents affected the observed cross-reactions between the PVIs and PPV antisera. In addition, cross-reaction of the PVIs with ASPV antisera in ISEM and Western blot analysis was also observed. Two known isolates of PPV cross-reacted with ASPV antisera, suggesting that the PVIs and some isolates of ASPV and PPV share common epitopes. The uniform heterologous decoration observed in the ISEM studies indicates that the epitopes are abundant and uniformly distributed along the entire length of the

virions. Sequential decoration of a single particle with both ASPV and gold-labeled PPV antibodies suggests that the antibodies recognize different epitopes on the virion or that the time used for decoration by the first antiserum was insufficient to saturate all available epitopes. The fact that the PPV-MAb 4DB12 recognized PVI Q375-02 but not ASPV or any of the other PVIs supports the hypothesis of different epitopes being involved in recognition by the different antibodies.

The PVIs described in this study can be distinguished from PPV by Western blot analysis (16). They have CP subunits ranging in molecular mass from 48 to 56 kDa. The PPV isolates included in this study had CP subunits ranging in molecular mass from 32 to 37 kDa, which is consistent with previous reports (32,33). The heterogeneity of the CP of PPV may result either from differences in the number of amino acid residues (38), or differences in the conformation of the proteins. ASPV is an unclassified virus with a CP subunit molecular mass of 48 kDa, a single species of RNA of molecular mass 3.1×10^6 Da, and multiple peaks when particle length distribution is analyzed (17,19). PVI 4-N-6 has a CP molecular mass estimated at 48 kDa, but none of the PVIs appear to be isolates of ASPV. The dsRNA profiles of the PVIs differ significantly from that of ASPV (D. James, unpublished); the symptomatology, and the woody and herbaceous host ranges, also differ (16). The particle length distributions of the PVIs are normal rather than multi-modal as is that of ASPV.

After rigorous examination, no cylindrical inclusions could be detected in plants infected with any of the PVIs. This indicates that these viruses are not potyviruses. This hypothesis is further supported by the negative reactions of the potyvirus-specific MAb, and the inability of *Aphis spiraecola* and *Myzus persicae* to transmit the PVIs (D. James, unpublished). These vectors were selected because they are efficient vectors of most PPV isolates (32).

The PVIs could also be distinguished from PPV by their dsRNA profiles. The PVIs have 3 to 5 dsRNA bands, compared with the 2 bands associated with PPV. The size of the major PPV-associated band is consistent with the high molecular mass dsRNA reported by Maiss et al. (28). The minor band associated with the PPV isolates was not always observed and may be spurious or subgenomic, or it may represent infection by another virus.

Genomic analysis can also be used in the identification of potyviruses (8,38). The 3′ noncoding regions of different potyviruses have a relatively low degree of sequence homology, approximately 45%, while strains of the same potyvirus have a much higher degree of homology, exceeding 97% in the case of PPV (8). The CP coding region and the 5′ noncoding region tend to be more conserved between different potyviruses than the 3′ noncoding region (8); thus, sequence homology in these regions would be indicative of a potyvirus but not necessarily of a PPV isolate. Oligonucleotides based on sequences from these regions were used successfully to amplify fragments of the genome of the PPV controls, and for detection by Southern blot analysis. No amplification or hybridization was achieved with the PVIs.

Probe NAT309-5′ is nested within the CP-2 region described by Cervera et al. (3); this is a very highly conserved area of the PPV genome encoding the N terminus of the CP. As with the other PPV probes, NAT309-5′ did not hybridize with any of the PVIs, while strong signals were generated with slot-blots of PPV reference strains. The hybridization assays target relatively large domains of the PPV genome,

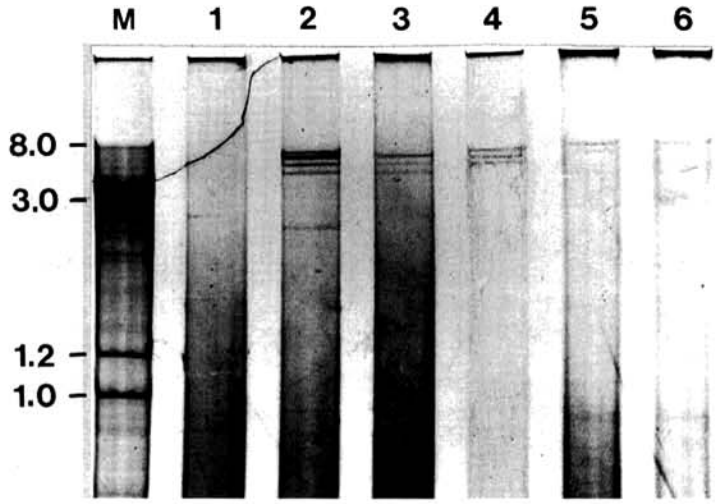

Fig. 7. Electrophoretic analysis of double-stranded RNA (dsRNA) extracted from leaves of healthy and infected *Nicotiana occidentalis* plants. Lane 1: Healthy tissue; lane 2: *Prunus* virus isolate (PVI) Q1256-01; lane 3: PVI Q375-02; lane 4: PVI 4-N-6; lane 5: plum pox virus (PPV)–TAF, and lane 6: PPV-ATCC. Lane M: dsRNA of *Phaseolus vulgaris* cv. Black turtle soup bean, molecular mass = 8.0 $\times 10^6$ Da; and the dsRNAs of carnation mottle virus, molecular mass = 3.0, 1.2, and 1.0 $\times 10^6$ Da.

Table 4. Analysis of *Prunus* virus isolates (PVIs) and plum pox virus (PPV) by slot blot hybridization

		Hybridization with indicated probe			
Virus isolate	**Source (host)**	**NAT65**	**NAT274**	**NAT309-3′**	**NAT309-5′**
PPV (ATCC)	*Nicotiana occidentalis*	+	+	+	+
PPV (Beltsville)	*N. benthamiana*	+	+	+	+
PVI (4-N-6)	*N. occidentalis*	–	–	–	–
PVI (Q375-02)	*N. occidentalis*	–	–	–	–
PVI (Q1256-01)	*N. occidentalis*	–	–	–	–
Uninoculated	*N. occidentalis*	–	–	–	–

Table 5. Analysis of plum pox virus (PPV) and *Prunus* virus isolates (PVIs) by reverse transcriptase–polymerase chain reaction (RT-PCR)

		PCR products		
Sample	**Host**	**CP-5′/CP-3′**	**NCR-5′/NCR-3′**	**PPV-2/PPV-3**
PPV (ATCC)	*Nicotiana occidentalis*	210 bp	140 bp	243 bp
PPV (TAF)	*N. occidentalis*	ND[a]	140 bp	243 bp
PPV (Beltsville)	*N. occidentalis*	ND	ND	243 bp
	N. benthamiana	190 bp	140 bp	ND
PPV (Spain)	*P. tomentosa*	ND	140 bp	ND
PVI (4-N-6)	*N. occidentalis*	–	–	ND
	GF305	ND	–	–
PVI (Q1256-01)	*N. occidentalis*	–	–	(<150 bp[b])
	Prunus mume	ND	–	–
	GF305	ND	–	–
PVI (Q375-02)	*N. occidentalis*	–	–	ND
	GF305	ND	–	–
pNAT65	NA	140 bp	140 bp	ND
Uninoculated	*N. occidentalis*	–	–	ND
	GF305	ND	–	–

[a] ND = not determined; NA = not applicable

[b] A minor product that appears only sporadically.

and since the PVIs fail to hybridize with PPV-specific probes, these data provide strong evidence of a heterologous origin of the PVIs and PPV genomes.

Primers for RT-PCR target a much smaller unit of the viral genome. However, a review of the GenBank and EMBL databases suggests that all PPV isolates with published sequence information (3,21,29, 34,38) would yield RT-PCR products of similar size with primers NCR-3′/NCR-5′, although primer NCR-3′ contains 2 nucleotides that are not strictly conserved (underlined in Table 2).

A sensitive RT-PCR assay for PPV was developed by Wetzel et al. (39) utilizing primers PPV-2 and PPV-3 to amplify a region of the viral genome encoding the C terminus region of the CP. The anticipated product was observed in association with the PPV controls but not with the PVIs. The RT-PCR data clearly distinguished PPV from the PVIs. The lower molecular mass product occasionally observed with PVI Q1256-01 RNA as target may represent a false priming site, or a fortuitous region of homology; attempts are being made to clone and sequence the fragment.

This study provides evidence of serological cross-reaction between the plum pox potyvirus and the as yet unclassified PVIs and ASPV. Cytopathological data and preliminary genomic analysis indicate that the PVIs are not members of the potyvirus group, contrary to published reports (10,13). The significance of this cross-reactivity is not known but it may indicate a close evolutionary relationship. Goldbach (9) describes the potyvirus group as the product of successive recombination events. Studies to further characterize the PVIs are being conducted; nucleotide sequence analysis may be necessary to assist in classification of the PVIs.

The discovery of these cross-reactions is significant because of the importance of plum pox disease to the stone fruit industry. Serology is often used as a tool for the detection of PPV (1,18,32). The results of this study indicate that RT-PCR analysis and nucleic acid hybridization may discriminate better than serological assays, and that if serology alone is to be applied, a combination of testing procedures should be used.

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