Sour Cherry Strain of Plum Pox Potyvirus (PPV): Molecular and Serological Evidence for a New Subgroup of PPV Strains

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ABSTRACT

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Properties of the unusual sour cherry strain of plum pox potyvirus (PPV-SoC) were investigated by sequencing its 3′-terminal 1,360 nt and examining its serological reactivity with several monoclonal antibodies (MAbs) to PPV. A significantly low degree of identity of the sequenced region has been found between PPV-SoC and other isolates of PPV. This is due to the high nucleotide divergence between the 5′-terminal region of the coat protein (CP) coding region of PPV-SoC and that of other isolates. Most nucleotide substitutions in the sequenced region are not silent and result in amino acid changes, especially near the N terminus of the CP. The unique 5′ terminus of the PPV-SoC CP coding sequence has been utilized to develop a cRNA probe that hybridizes to PPV-SoC but not to other isolates/members of the D or M subgroups of PPV. The N-terminal region of the PPV-SoC CP contains the recognizable DAG motif that determines aphid transmissibility of potyviruses. Aphid transmission of PPV-SoC to herbaceous and woody hosts has been confirmed experimentally. Serological reactivity of PPV-SoC with 10 MAbs of PPV suggest that PPV-SoC represents a new serotype of PPV that does not fall into the conventional D or M serotypes/subgroups. We propose that PPV-SoC represents a new subgroup of PPV strains, termed PPV-cherry (PPV-C), and that PPV-SoC is its prototype member.

Additional keywords: detection, differentiation, insect transmission, nucleotide sequence of coat protein, phylogeny.

Plum pox virus (PPV) is a member of the genus *Potyvirus* and causes the devastating plum pox (sharka) disease of stone fruits (27). Its genome consists of a positive-strand RNA molecule, approximately 10 kb long with a 3′ poly(A) tail (20). It contains a unique open reading frame (ORF), the product of which is proteolytically processed by virus-encoded proteases into smaller functional proteins, including the coat protein (CP) located at the C terminus of the large polyprotein (7,12,20,29). The complete nucleotide sequences and the nucleotide sequences of the 3′-terminal regions of the genome of several PPV isolates have been determined (7,20,28,32,34). With the exception of the El-Amar strain from Egypt and the PS and o6 isolates from the former Yugoslavia, the 3′-terminal region of all PPV isolates previously sequenced show a high level of nucleotide identity. All of these isolates infect plum, peach, and apricot trees and represent two main serotypes/subgroups of PPV: PPV-M (Marcus strain, Greece) and PPV-D (Dideron strain, France). PPV isolates that infect these hosts have been reported not to infect cherry trees, and/or cherry trees are highly resistant to viral infection (11,27).

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Recently, we characterized a new strain of PPV that naturally infects sour cherry trees in Moldova, which we termed PPV-SoC (21–26). PPV-SoC systemically infects several cultivars of sour cherry, *Prunus cerasus* L. (25), and it also has been reported to infect other cherry species (21), plum (14), and peach (T. Candresse, L. Nemchinov, and A. Hadidi, *unpublished data*). Restriction fragment length polymorphism analysis of a 243-nt-long reverse-transcription polymerase chain reaction (RT-PCR) product amplified from the carboxy-terminal region of PPV-SoC CP suggested this virus may not belong to the conventional D or M subgroups of PPV isolates (21). However, serological assays that use polyclonal antisera against PPV did not differentiate between PPV-SoC and common plum isolates (14). To further characterize PPV-SoC at the molecular and serological levels, we sequenced the 3′-terminal 1,360 nt of the viral genome and investigated the viral serological reactivity with several monoclonal antibodies (MAbs) to PPV in indirect double antibody sandwich enzyme-linked immunosorbent assay (indirect DAS-ELISA) tests.

In this paper, we report nucleotide sequence analysis of the region of the PPV-SoC genome that contains a portion of the NIb (putative RNA replicase) coding region, the entire CP-coding sequence, and the 3′-noncoding region (3′NCR). In addition, we show molecular and serological evidence that demonstrates that PPV-SoC is a prototype of a new PPV subgroup, termed PPV-cherry (PPV-C), which significantly differs from the D or M subgroups of PPV. A preliminary account of these results has been reported (22,23,26).

MATERIAL AND METHODS

Plant source. PPV-SoC–infected sour cherry tissues were collected in Moldova (21,25) and used in the United States for RT-PCR amplification of PPV-SoC cDNA fragments, which were cloned and sequenced. In Beltsville, MD, under quarantine, the virus was maintained on two cherry rootstocks, *P. avium* L. 'Mazzard' (sweet cherry) and *P. mahaleb* L., which showed systemic infection after grafting with sour cherry budwood. PPV-SoC also was mechanically transmitted to *Nicotiana benthamiana* Domin. from infected tissue of sweet cherry.

Primers and RT-PCR. Three sets of PPV-specific primers were utilized for PCR amplification in this study. The first set (13,17) primed the amplification of the 3′NCR of PPV RNA; the second set (35) directed the amplification of the 3′-terminal region of the PPV CP-coding sequence; and the third primed the amplification of the 3′-terminal region of the NIb-coding sequence, the whole CP gene, and the 3′NCR combined: 5′-CGGGGATCCTCGAGA-AGCTTTTTTTTTTTTTTTTTT-3′ (Poly-T, downstream; *Bam*HI, *Xho*I, and *Hin*dIII internal sites); and 5′-GGAATTCCCGCGG-AAAAGCCCCGTACATTGC-3′ (upstream; *Eco*RI and *Ksp*I internal sites; position 8433 to 8452 [20]). Samples were prepared for RT-PCR by Gene Releaser (Bio Ventures, Inc., Murfreesboro, TN) polymeric matrix or immunocapture of virions with PPV antiserum, as described previously (18,21,25,35,36).

Cloning. Polyacrylamide gel-purified PCR products originating from the PPV-SoC genome (derived from PPV-infected leaves of *P. cerasus*) were cloned into the pCR II cloning vector (Invitrogen, San Diego, CA) or the pGEM 3zf(+) vector (Promega, Madison, WI) according to the manufacturers' directions.

Nucleotide sequence. Each nucleotide was sequenced at least once on each strand of the cloned cDNA. The dideoxy nucleotide chain termination reaction was carried out by the fmol sequencing system (Promega) or the nonradioactive digoxigenin (DIG) *Taq* DNA sequencing kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturers' directions.

Phylogenetic analysis. The predicted amino acid sequences of the CP from several PPV strains were aligned by the Clustal method, version 5, using DNASTAR LaserGene software (DNASTAR, Inc., Madison, WI). Phylogeny was determined by cladistic analysis (phylogenetic analysis using parsimony, PAUP, version 3.1, written by D. L. Swofford, University of Illinois, Urbana) on a Power Macintosh computer, model 8100/100, using branch and bound search, employing tobacco etch virus (TEV) (1) as the outgroup. Bootstrap analysis with 100 replicates also was performed to assess the robustness of the generated clusters.

Probe synthesis and hybridization. Nonradioactive, DIG-labeled cRNA probes were generated from the pCR II cloning vector containing the PPV inserts by T7 RNA polymerase, using the GENIUS system (Boehringer Mannheim) according to the manufacturer's directions. Dot hybridization of tissue extracts with a cRNA probe was performed under standard conditions, as previously described (21,25). Hybridization signals were visualized by the GENIUS nonradioactive detection kit (Boehringer Mannheim) with Lumi-Phos 530 substrate (Boehringer Mannheim) and Hyperfilm-MP (Amersham Corp., Arlington Heights, IL).

Western blot. PPV-SoC–infected leaves of *N. benthamiana* or *P. avium* 'Mazzard' were ground, 1:7 (wt/vol), in 50 mM sodium citrate buffer, pH 8.3, containing 20 mM DIECA (diethyldithiocarbamate) and 5 mM EDTA. Sap was clarified by centrifugation; collected supernatant was mixed with an equal volume of Laemmli buffer (15), boiled for 5 min, and placed on ice. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15) together with PPV-D and PPV-M isolates from infected *N. benthamiana* as PPV CP markers (both strains of PPV were provided by M. Barba, Istituto Sperimentale per la Patologia Vegetale, Rome). Gels were electroblotted onto nitrocellulose (0.45-μm pore size; Schleicher & Schuell, Keene, NH). Blots were reacted with PPV polyclonal antiserum from Sanofi (Libourne Cedex, France) or Bioreba (Basel, Switzerland) diluted 1:1,000 and allowed to react with PPV CP for 16 h at 4°C overnight. Goat anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate was used as the secondary antibody. Substrate consisted of buffered tablets of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Chemical Company, St. Louis) used for colorimetric reaction.

Serological characterization. Ten PPV-specific MAbs were used as intermediate antibodies in indirect DAS-ELISA (6) by the methods and antibody concentrations of Cambra et al. (5) for MAbs 5B, 4DB12, 1EB6, 3C6, 4F4, 4DB7, 4CB1, 4DG5, and 4DG11. The serotype M-specific MAb (4) was used at 0.1 μg/ml in PBS (phosphate-buffered saline). The immunoplates (Maxisorp; Nunc, Roskilde, Denmark) were coated with polyclonal antibodies (2 μg/ml in 50 mM carbonate buffer, pH 9.6) from rabbit antiserum obtained by immunization with purified PPV CP from the Spanish PPV-RB isolate. Alkaline phosphatase-linked goat anti-mouse Igs (Boehringer Mannheim) were used as detecting antibody. Four wells per plate were filled with extracts, 1:10 (wt/vol), from the different PPV sources prepared in PBS + 0.2% DIECA + 0.2% polyvinylpyrrolidone-10, pH 7.2 to 7.4. The PPV sources were PPV-NAT on *N. benthamiana* (representing serotype D), PPV-Marcus/Mp on GF 305 peach (representing serotype M), and PPV-SoC on sour cherry. Noninoculated leaves of *N. benthamiana*, GF 305 peach, and sour cherry were used as negative controls. The plates were read at 405 nm after 1 h of substrate incubation in a Titertek Multiscan Plus MKII (ICN Biomdicals, Costa Mesa, CA) automatic reader calibrated to 0.0 absorbance with an empty ELISA plate.

Insect transmission. A colony of the green peach aphid, *Myzus persicae* Sulzer, was obtained from L. Fox, USDA/ARS, Yakima, WA, and maintained in cages on leaves of healthy turnip, *Brassica rapa*, and *Datura stramonium* plants. PPV-SoC–infected sweet cherry, *P. avium* 'Mazzard,' and *N. benthamiana* plants were used as the virus source. Test plants included seedlings of *N. benthamiana*, *N. clevelandii*, and plum, *P. domestica* 'Stanley.'

In each experiment, late instars and adults of *M. persicae* were removed with a brush from colony plants onto moist filter papers in 15-cm petri dishes. Aphids were starved for 1 to 4 h. Sixty to one hundred starved aphids were placed on several PPV-SoC–infected *N. benthamiana* or sweet cherry leaves in a petri dish for acquisition access periods (AAP) ranging from 1 min to 16 h. Leaf pieces bearing 10 or more aphids were then placed on test *N. benthamiana*, *N. clevelandii*, or plum seedlings for a 24-h inoculation access period (IAP). After 24 h, the seedlings were sprayed with a pesticide to kill the aphids.

RESULTS

Amplification, cloning, and sequencing of the 3′-terminal region of the PPV-SoC genome. Amplified cDNA fragments of the expected size were obtained from PPV-SoC–infected sour cherry extracts with primer pairs for PPV 3′NCR (220 bp), for the 3′-terminal region of the CP-coding region (243 bp), or for a 1,360-bp cDNA fragment that included the coding regions of the C-terminal portion of the NIb, the CP, and the 3′NCR (data not shown). Five to ten cDNA clones that contained the amplified cDNA derived from each set of primers were obtained; three to five clones of each insert were sequenced and compared. The sequenced region included 1,360 nt of the single PPV ORF terminated by a UAG codon located 220 nt upstream from the poly(A) tail (Fig. 1). The predicted amino acid sequence included 48 C-terminal amino acids of the NIb protein and 332 amino acids of the CP-coding region, with a putative Q-A cleavage site between the NIb and the CP at nucleotide position 144 to 145.

Heterogeneity was observed among the clones sequenced in the 3′-terminal end of the CP-coding region at four positions and in

the 3′NCR at two positions (Fig. 1). Only one nucleotide substitution (A→G), at position 1,018, results in an amino acid change (N→D). Comparison of the nucleotide sequence and predicted amino acid sequence of the PPV-SoC CP-coding region with the corresponding sequences of PPV-D (32), PPV-NAT (20), PPV-AT (20), PPV-EL Amar (34), PPV-RAN (16), PPV-PS, and PPV-o6 (7) revealed an average percent identity between 77 and 82% for both nucleotide and amino acid sequences. Thus, most of the nucleotide

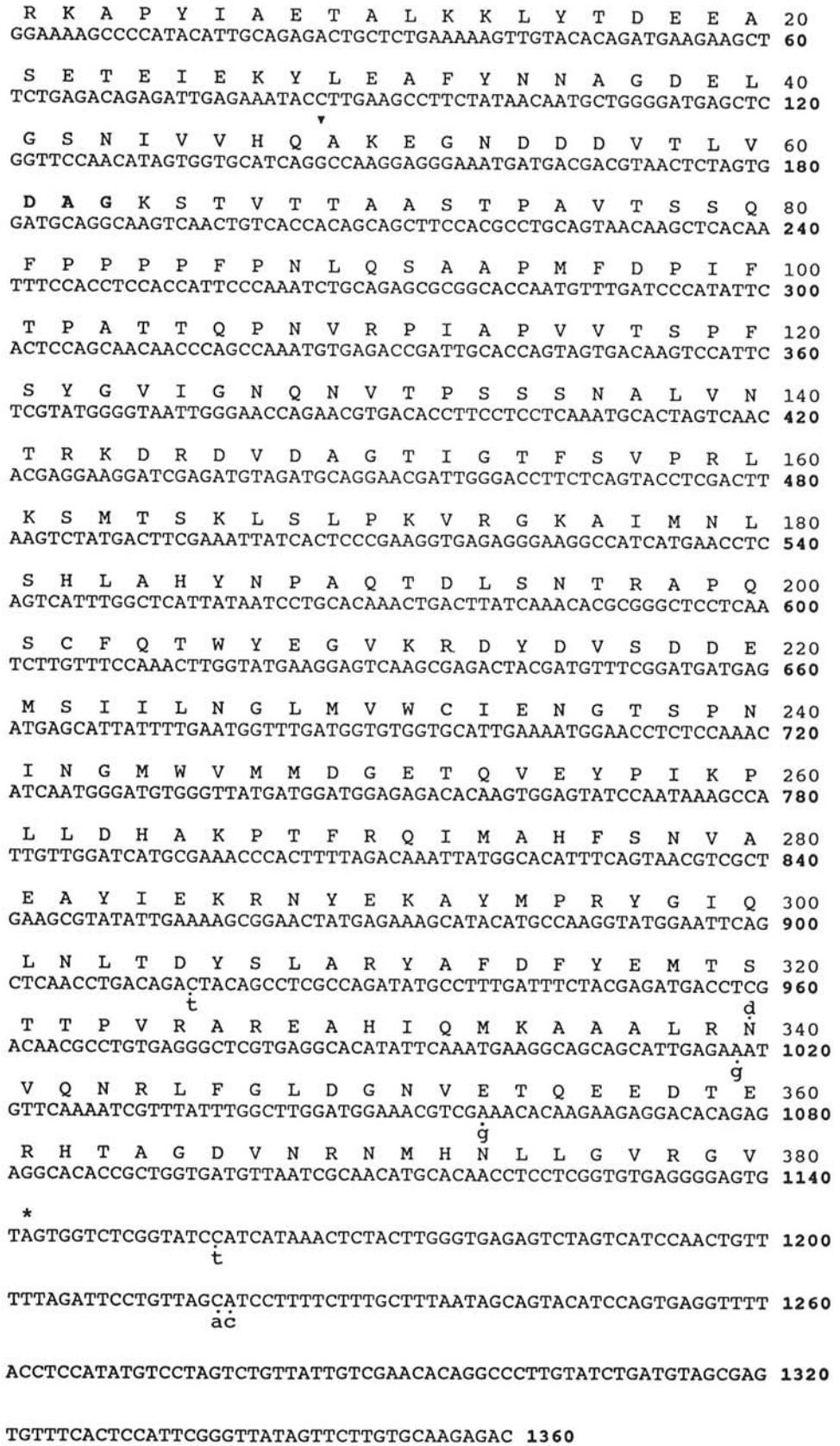

Fig. 1. Nucleotide sequence of the cloned 3′-terminal 1,360 nt of plum pox virus-sour cherry (EMBL accession X97398). The predicted amino acid sequence is shown. The heterogeneities observed in the sequences of independent cDNA clones and the corresponding changes in the coded protein are shown in lowercase letters. The putative cleavage site located between the putative RNA replicase and the coat protein is indicated by a triangle.

substitutions were not silent and caused amino acid changes, especially near the N-terminal end of the CP (Fig. 2). A DAG motif implicated in aphid transmissibility in potyviruses (3) was found in the PPV-SoC CP, despite the low percent identity in this region with other PPV isolates. The nucleotide sequence of the 3′-terminal portion of the PPV-SoC CP-coding region contains neither the conserved *Alu*I restriction site detected in all PPV isolates nor the *Rsa*I restriction site that is common for all isolates of the PPV-D type and is up to 94% identical to the corresponding region of other PPV isolates (21).

Analysis of the phylogenetic interrelationships among several PPV strains, based on predicted amino acid sequences of their coat proteins, revealed that PPV-SoC forms a phylogenetically distinct group that does not fall into any existing cluster of PPV strains (Fig. 3).

Western blot analysis of the PPV-SoC CP. Western blot analysis revealed that the PPV-SoC CP has an estimated molecular mass of about 36 kDa (data not shown). The predicted molecular mass of PPV-SoC CP is 36,801 Daltons.

Construction and development a cRNA probe specific for PPV-SoC. Because the 5′-terminal region of the PPV-SoC CP-

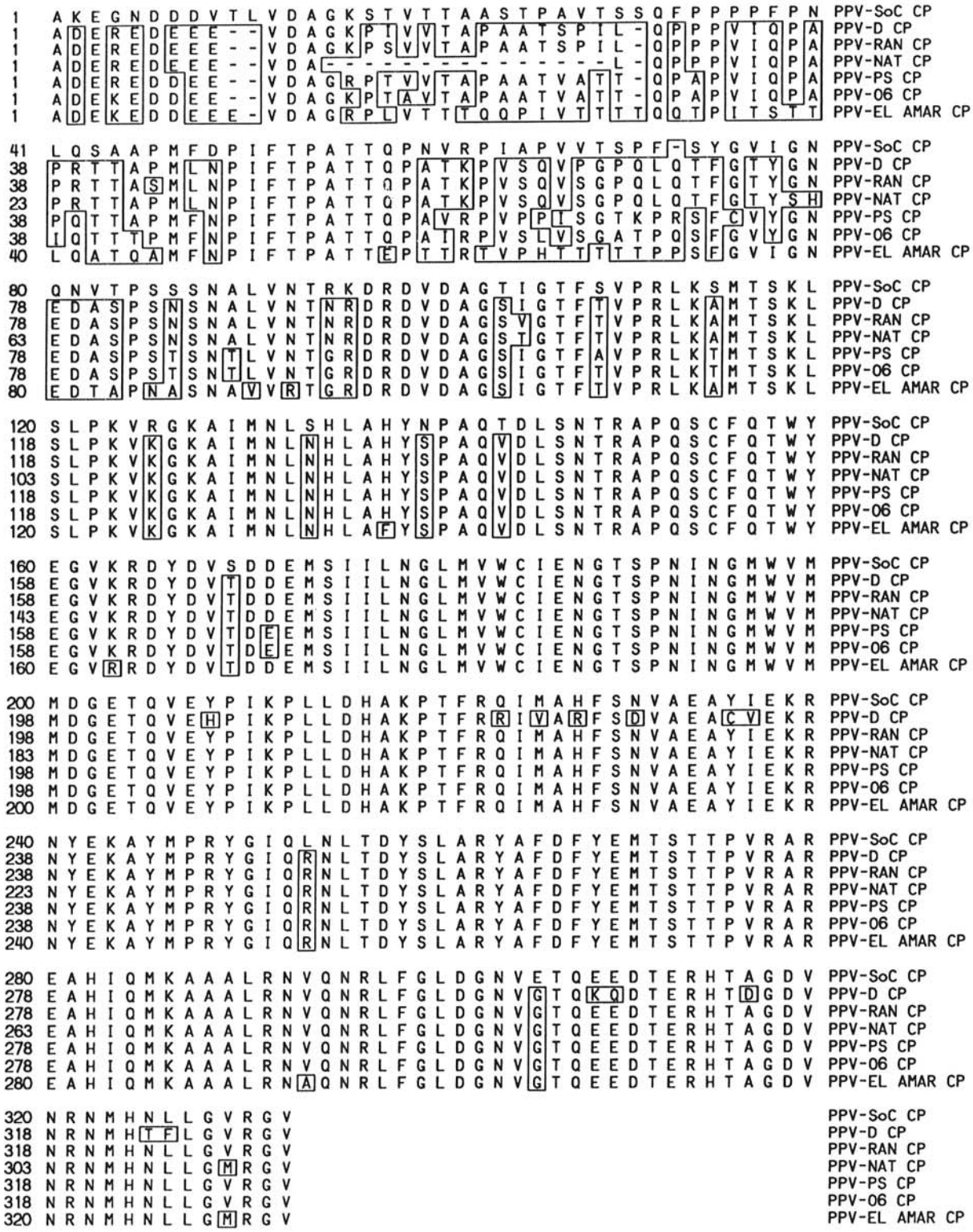

Fig. 2. Multiple alignment of coat protein amino acid sequences of seven plum pox virus (PPV) strains, including PPV-sour cherry (PPV-SoC). Residues that differ from PPV-SoC are boxed.

coding sequence has a low percent identity with that of other PPV isolates, this region was used to design a cRNA probe that is specific for PPV-SoC and that differentiates this strain from other conventional isolates of PPV. A 447-bp cDNA fragment containing 303 bp from the 5′ terminal of the CP-coding region and 144 bp from the 3′ terminal of the NIb-coding region was amplified from a full clone previously sequenced and identified as containing the 1,360 nt of the 3′-terminal fragment of the PPV-SoC genome.

The amplified DNA fragment was subcloned into a plasmid vector containing a promoter for DNA-dependent RNA polymerase and sequenced again to confirm its origin. This cDNA fragment does not have any continuous sequence homology (more than 10 to 15 nt) with the corresponding regions of other PPV isolates. A nonradioactive DIG-labeled cRNA probe was generated from the subclone and used in a dot-blot hybridization assay with several PPV isolates, including PPV-D and PPV-M. Additionally, a DIG-labeled cRNA probe derived from the conserved C-terminal region of the PPV CP was used for hybridization to ascertain that samples were infected with PPV. The PPV-SoC cRNA probe hybridized to samples from PPV-SoC–infected tissue but not to samples from tissues infected with other isolates of PPV (Fig. 4B). In contrast, the PPV cRNA probe for the conserved region of PPV CP hybridized to samples of all PPV isolates, including those of PPV-SoC (Fig. 4A).

Serological characterization of PPV-SoC. Table 1 shows that PPV-SoC reacted positively with MAb 5B. This MAb has been described as representing a common and frequent epitope in the CP of all PPV isolates tested (2,5). PPV-SoC reacted positively also with MAb 4DB7, which represents an epitope not well conserved in PPV isolates. The highest absorbance value for the PPV-SoC assay was obtained with this MAb. PPV-SoC, however, did not react against MAb 4DB12 and 1EB6, which represent two different epitopes well conserved in the majority of the tested isolates of PPV (5,19). In addition, PPV-SoC failed to react against MAbs 4DG5 and 4DG11, described as D-type specific (2,5), and was not recognized by MAb A, described as M-type specific (4).

Aphid transmission of PPV-SoC. Distinctive leaf symptoms (mild mosaic) appeared on *N. benthamiana* or *N. clevelandii* and on Stanley plum plants 2 to 3 and 6 to 7 weeks, respectively, after aphid inoculation. Eleven of twenty-seven aphid-inoculated herbaceous plants (1/3 *N. clevelandii* and 10/24 *N. benthamiana*) and two of three Stanley plum plants became infected. The virus was successfully transmitted at an AAP of 5 min to 16 h. Molecular hybridization of tissue extracts from leaves with mosaic symptoms to a DIG-labeled cRNA probe specific only for members of the PPV-C subgroup or to a cRNA probe specific for all PPV isolates revealed strong hybridization signals in both cases (data not shown). RT-PCR assays with primers for the 3′-noncoding region of PPV genome (13,17) were positive. Thus, PPV-SoC is aphid transmissible.

DISCUSSION

We demonstrated, using sequencing and serological analyses, that the PPV-SoC CP is significantly different from other isolates of PPV and that the virus represents a unique strain of PPV and is the type member and prototype of a new subgroup of PPV strains termed PPV-C. Potyvirus CP molecules differ in the length and sequence of the N-terminal region, whereas approximately two-thirds of the C-terminal sequence is conserved (33). The C-terminal region of the PPV-SoC CP and adjacent 3′NCR are very similar to those of other PPV isolates; however, the N-terminal region is significantly

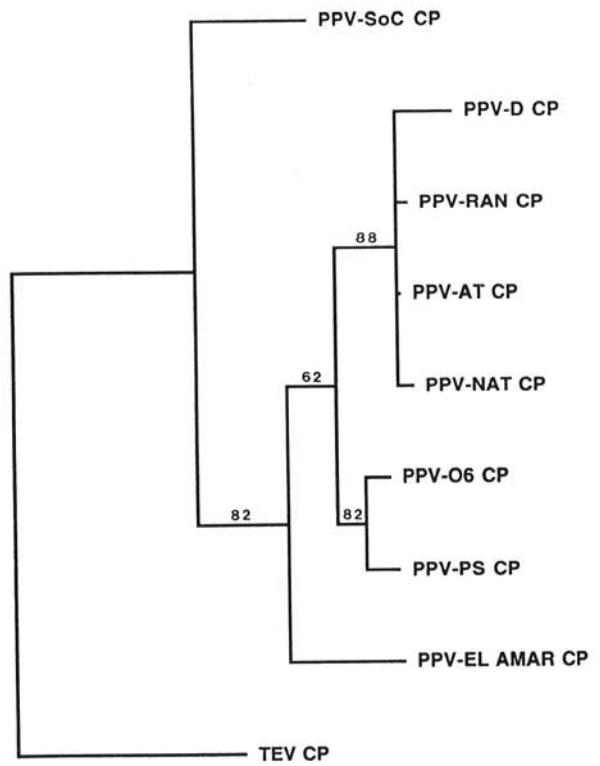

Fig. 3. Consensus phylogenetic tree constructed by parsimony analysis of coat protein amino acid sequences of several plum pox virus (PPV) strains. Values at the nodes indicate significance in a bootstrap analysis with 100 replicates. Tobacco etch virus (TEV) was defined for outgrouping. PPV-D (32); PPV-RAN (16); PPV-NAT and PPV-AT (20); PPV-EL-AMAR (34); and PPV-PS and PPV-o6 (7).

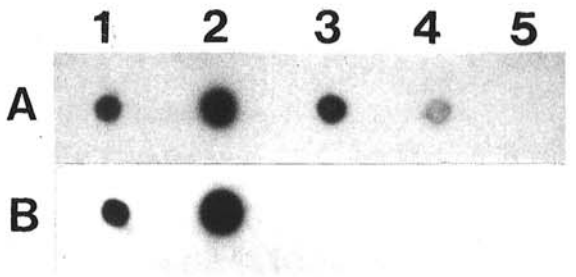

Fig. 4. Dot-hybridization of plum pox virus (PPV)-infected tissue extracts with **A,** a digoxigenin-labeled cRNA probe either universal for PPV or **B,** one specific for PPV-SoC. Lane 1, PPV-SoC–infected *Prunus avium*; lane 2, PPV-SoC–infected *Nicotiana benthamiana*; lane 3, PPV-D–infected *N. benthamiana*; lane 4, PPV-M–infected *N. benthamiana*; and lane 5, uninfected tissue of *N. benthamiana*.

TABLE 1. Absorbance values obtained in indirect double-antibody sandwich enzyme-linked immunosorbent assay (indirect DAS-ELISA) with 10 monoclonal antibodies (MAbs) against two plum pox virus (PPV)-positive controls representing D and M serotypes, the isolate PPV-SoC, and healthy controls

	PPV isolate[b]			HC[c]		
MAb[a]	NAT (D)	Marcus/ Mp (M)	SoC	*N. benth.*	Peach GF 305	Sour cherry
5B	2.350[d]	3.432	0.935	0.080	0.070	0.082
4DB12	0.218	3.370	0.081	0.085	0.082	0.090
1EB6	3.216	3.600	0.129	0.094	0.085	0.091
3C6	1.024	0.702	0.186	0.091	0.077	0.087
4F4	0.602	0.270	0.132	0.114	0.101	0.092
4DB7	3.387	1.125	1.225	0.088	0.090	0.101
4CB1	0.602	0.297	0.170	0.092	0.099	0.105
4DG5	3.240	0.120	0.077	0.099	0.078	0.069
4DG11	3.136	0.119	0.076	0.075	0.082	0.073
A	0.114	3.153	0.111	0.106	0.093	0.102

[a] PPV-specific MAbs.
[b] PPV isolates tested in comparison.
[c] HC: healthy controls; *Nicotiana benthamiana* (*N. benth.*), peach GF 305, and sour cherry were controls of PPV-D, PPV-M, and PPV-SoC, respectively.
[d] Values are the average of four ELISA wells.

different. The variable N-terminal domain is reported to be an immunodominant region (30,31,33) involved in virus-specific immunochemical properties. In addition, it is important for virus-vector interactions.

All aphid-transmissible PPV isolates contain a conserved DAG motif close to the N terminus of the CP (7,16). PPV-SoC contains a DAG motif in its CP amino acid sequence and is aphid transmissible, which supports the involvement of this triplet in aphid transmission of the virus. PPV-SoC was transmitted by *M. persicae* at an AAP of 5 min to 16 h with a 24-h IAP. The transmission type was stylet-borne, and the long AAP was successful because leaf pieces were transferred and aphids were allowed to walk off the infected leaves at their volition. Presumably, only the aphids that made short probes before moving were actually viruliferous. For plum pox virus, *M. persicae* is the most prominent and efficient vector (27). Aphid transmission of PPV-SoC from one *Prunus* species (sweet cherry) to another (plum) suggests a possible role for this vector in the epidemiology of the disease among stone fruit trees in nature.

The biological significance of the changes described in the N-terminal portion of the PPV-SoC CP is currently uncertain. However, we suggest that these changes may provide a molecular basis for the extension of the typical host range of plum pox virus by activating the viral movement function in the unusual host, *P. cerasus*. TEV capsid protein has been shown to be involved in the cell-to-cell and long-distance movement of TEV (9,10); these two functions require N- and C-terminal regions of the CP. Although the N-terminal domain appeared to play only an accessory role in the cell-to-cell movement (10), long-distance movement of the TEV mutant that lacked part of the N-terminal domain was completely inhibited (10).

The boundaries separating strains of individual viruses and distinct viruses are not strictly defined (31,33). A study of the frequency distribution of the amino acid sequence similarity for CPs of 17 strains of 8 distinct potyviruses (31) showed that distinct members exhibit sequence identities of 38 to 71%, and strains of individual viruses have identities of 90 to 99%. Thus, on the basis of CP amino acid similarity with other strains of PPV, PPV-SoC represents at least a new strain of PPV and might be placed in the intermediate zone (71 to 85%) dividing independent members of the genus *Potyvirus* and strains of PPV.

Our study of the phylogenetic relationships among major known PPV isolates confirms this finding, revealing that PPV-SoC forms a distinct cluster in the consensus, most parsimonious phylogenetic tree (Fig. 3). The high level of nucleotide and amino acid divergence at the N-terminal region of the PPV-SoC CP as well as its unusual natural host may suggest that PPV-SoC originated from an RNA recombination event involving an isolate of PPV and an unknown virus, hypothetically closely related to PPV. This is because database searches have shown that no sequence more similar to the N-terminal region of the CP of PPV-SoC than those of other PPV isolates have been found. Evidence for RNA recombination within potyviruses has been reported recently (7), and a new potyvirus other than PPV has been detected in *Prunus* spp. (13). Hence, PPV-SoC may represent an evolved isolate of the existing PPV that went through certain gradual changes that gave it a selective advantage and led to its ability to systemically infect sour cherry trees.

The distinct features of the N-terminal region of the PPV-SoC CP allowed us to develop a cRNA probe specific for identification of the virus and differentiated PPV-SoC from other isolates of PPV. This probe may be useful for the study of identity, epidemiology, and geographic distribution of members of the PPV-C subgroup in different species of stone fruits. As an example, preliminary results with this highly specific probe with the Italian sweet cherry isolate of PPV (PPV-SwC) (8) have shown that this isolate is indeed a member of the PPV-C subgroup (L. Nemchinov, A. Hadidi, and A. Crescenzi, *unpublished data*).

Cambra et al. (5) established 21 PPV serogroups on the basis of the reaction pattern of 81 PPV isolates against 9 MAbs, and Asensio et al. (2) described 23 serogroups when using 85 PPV isolates against the same MAbs. PPV-SoC gave a positive reaction only against MAbs 5B and 4DB7 (Table 1). Among four MAbs with very broad specificity, namely 5B (which recognizes all known isolates of PPV) and 1EB6, 3C6, and 4DB16 (which recognize the vast majority of PPV isolates), only MAb 5B reacted positively with PPV-SoC. Furthermore, PPV-SoC failed to react against MAbs 4DG5 and 4DG11, which are described as D-type specific (2,5), and it was not recognized by MAb A, which is described as M-type specific (4). This serological evidence indicates that PPV-SoC is significantly different from all previously described PPV isolates and supports the hypothesis that PPV-SoC forms a new serotype that is neither D or M. Very similar serological reactivity to PPV-SoC was demonstrated recently for the Italian PPV-SwC (A. Crescenzi, *unpublished data*).

In summary, PPV-SoC is a unique strain of PPV that is the type member and prototype of a new subgroup, PPV-C, that differs from other isolates of PPV by its unusual coat protein, serological reactivity, and biological properties.

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