Characterization of the Sweet Cherry Isolate of Plum Pox Potyvirus

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ABSTRACT

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An isolate of plum pox potyvirus from sweet cherry (PPV-SwC) in southern Italy was investigated. The isolate was mechanically or graft transmissible to different *Prunus* and *Nicotiana* spp. but not to *Chenopodium* spp. It was transmitted also by *Aphis fabae* and *Myzus persicae* in a nonpersistent manner. Restriction fragment length polymorphism analysis indicated and nucleotide sequencing confirmed that the isolate lacks *AluI* and *RsaI* sites in the C-terminal region of the coat protein (CP) gene. Western immunoblot results showed that the PPV-SwC CP has an electrophoretic mobility similar to that of strain PPV-D and faster than that of strain PPV-M. Double-antibody sandwich indirect enzyme-linked immunosorbent assay of the CP showed that PPV-SwC, although reacting with universal monoclonal antibodies to PPV, failed to react with antibodies specific to strains M and D. Results indicate that PPV-SwC is different from conventional strains of PPV but closely related to the sour cherry isolate of PPV from Moldova.

Plum pox potyvirus (PPV) is the causal agent of sharka disease of stone fruits, which severely affects sensitive crops in Europe, the Mediterranean region, the Middle East, and South America (17). PPV strains have been classified into two distinct groups on the basis of serological properties, size of coat protein (CP), and restriction profile of the C-terminal region of the CP gene: the D strains have a 36-kDa CP and both an *AluI* and a *RsaI* restriction site; M strains have a 38-kDa CP and only an *AluI* site (16,21). The PPV El Amar strain obtained from apricot in Egypt, although sharing a restriction profile and CP molecular weight similar to the M isolates (16,21), has been proposed to form a third group (4,20). Biologically, PPV strains have been classified into "yellow," "necrotic," and "intermediate," according to the reactions induced in *Chenopodium foetidum* Sc. and *Nicotiana clevelandii* Gray (15).

None of the conventional strains of PPV had been found to infect cherry trees naturally. For this reason, cherry was considered resistant to PPV infection. This was demonstrated by Dosba et al. (7), who

showed that several PPV isolates obtained from peach, apricot, and plum failed to infect sweet cherry (*Prunus avium* L.) after graft-inoculation. The virus remained localized after aphid transmission. Recently, PPV was isolated from naturally infected trees of sour cherry (*P. cerasus* L.) in Moldova (8,14) and sweet cherry in southern Italy (5,6).

In this report, the sweet cherry isolate of PPV (PPV-SwC) has been characterized biologically by graft, mechanical, and aphid transmission. Double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) with PPV-specific monoclonal antibodies (MAbs) and Western immunoblot using a polyclonal antiserum were performed for serological characterization. Reverse-transcription polymerase chain reaction (RT-PCR) utilizing PPV-specific primers for the C-terminal region of the CP gene, in combination with restriction fragment length polymorphism (RFLP), molecular hybridization, and nucleotide sequencing analysis, was used to characterize PPV-SwC at the molecular level. The results show that PPV-SwC is different from the conventional strains of PPV but is closely related to the sour cherry isolate (PPV-SoC) from Moldova.

MATERIALS AND METHODS

Biological assays. PPV-SwC was mechanically transmitted from sweet cherry to *N. benthamiana* Domin. and maintained in

the same species. For mechanical-transmission tests, infected leaves were ground in 20 mM phosphate buffer, pH 7.2, containing 0.1% 2-mercaptoethanol, and the sap was mechanically inoculated onto leaves of herbaceous and woody plants previously dusted with celite. For graft transmission, buds from naturally infected sweet cherry cv. Ferrovia trees were grafted onto 3-year-old healthy sweet cherry of the same cultivar, and onto peach (*P. persica* L.) cv. GF 305 seedlings. Aphid transmission was carried out with broadbean aphids (*Aphis fabae* Scopoli) and green peach aphids (*Myzus persicae* Sulzer). Aphids were reared on healthy *Vicia faba* L. plants, starved overnight, allowed to feed for 30 to 60 s on infected *N. benthamiana*, then transferred to healthy plants of the same species. Insects were killed 1 h later with an insecticide. Infection of tested plants was checked by RT-PCR as described by Crescenzi et al. (6) or by ELISA tests, using a commercial kit (Loewe Biochemica, Munich).

RT-PCR and RFLP analysis. Total nucleic acid (TNA) was extracted from naturally and artificially infected cherry leaves and from artificially infected peach leaves, as described by Levy and Hadidi (11). TNA was purified, using RNase-free ELUTIP R minicolumns (Schleicher & Schuell, Keene, NH), following the manufacturer's instructions. RT-PCR was performed as described by Crescenzi et al. (6) with primers designed for the C-terminal region of the PPV CP gene (21). Antisense and sense primers for the CP gene were 5'-CAGAC-TACAGCCTCGCCAGA-3' and 5'-ACC-GAGACCACTACACTCCC-3', respectively. Amplified products were electrophoresed onto 5% polyacrylamide gels (PAGE) at 150 V for 90 min and visualized by silver staining. For RFLP analysis, the PCR-amplified products were purified by phenol/chloroform extraction, followed by ethanol precipitation, and then incubated with 5 units of *RsaI* or *AluI* restriction enzyme for 1 h. Digestion products were analyzed on 7% PAGE and visualized by silver staining. DNA molecular weight markers (Bio Ventures, Murfreesboro, TN) were included in the gel to estimate the size of amplified DNA fragments.

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Southern hybridization. PAGE-fractionated RT-PCR products were denatured and electroblotted onto Nytran (Schleicher & Schuell, Keene, NH) membranes (18) and hybridized with a digoxigenin-labeled cRNA probe corresponding to the C-terminal region of the CP gene of PPV-SoC (14), using a Genius nonradioactive labeling system (Boehringer Mannheim, Indianapolis, IN). Hybridized products were visualized with the Genius nonradioactive detection kit, using Lumi-Phos 530 substrate (Boehringer), and were exposed to Hyperfilm (Amersham Corp., Arlington Heights, IL).

Cloning and sequencing. Amplified 243-bp products were recovered from the gel following the crush and soak method (18) and directly cloned into the pCR vector, using the TA cloning kit (Invitrogen, San Diego, CA), according to the manufacturer's directions. Insertion of the fragments into the vector was checked by colony PCR (10). Nucleotide sequence

was determined by the dideoxy-sequencing method (19).

ELISA. DASi-ELISA was performed as described by Cambra et al. (3). Plates were coated with immunoglobulin Gs (IgGs) from a polyclonal antiserum to an Albanian isolate of PPV (PPV-AL) (12). Extracts from PPV-SwC-infected *N. benthamiana* or sweet cherry were compared with those from PPV-SoC- or PPV El Amar-infected *N. benthamiana* and peach cv. GF 305 seedlings, respectively. Positive controls were an Italian isolate of PPV-D (ISPAVE 31) and a French isolate of PPV-M. The samples were tested comparatively with three MAbs: universal MAb5B, which recognizes all known PPV serotypes; MAb4DG5, which is PPV-D specific (2); and MAbAL, which is PPV-M specific (1). Positive reactions were revealed by alkaline phosphatase-conjugated goat anti-mouse IgGs (Sigma Chemicals, St. Louis), using *p*-nitrophenyl phosphate as substrate.

Western immunoblot. Leaves of *N. benthamiana* infected with PPV-SwC, PPV-SoC, PPV-D (ISPAVE 31), and PPV-M (French isolate) were homogenized in 50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 30% glycerol, 2% sodium dodecyl sulfate, and 0.1% bromophenol blue, and the extracts were electrophoresed under denaturing conditions following the discontinuous system of Laemmli (9). Extracts from leaves of healthy *N. benthamiana* were used as controls. The stacking and separating gels were at 5 and 12% acrylamide, respectively. Electrophoresis was carried out at 100 V for 30 min, followed by 150 V (from 31 to 17 mA) for 15 h at 15°C. After electrophoresis, proteins were electroblotted onto nitrocellulose membrane (0.45 µm; Sartorius, Göttingen, Germany) at 0.2 A for 8 h at 4°C. The membrane was saturated with gelatin and incubated with anti-PPV conjugated alkaline phosphatase (Loewe) and diluted 1:2,000. The reaction was visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium Sigma Fast (Sigma).

RESULTS

Biological assays. After mechanical inoculation, PPV-SwC induced yellow vein banding and necrosis, starting from the leaf edges in *P. avium* (Fig. 1) and *P. cerasus*. In peach cv. GF 305, PPV-SwC induced vein yellowing and necrosis associated with leaf distortion, and in *P. mahaleb* L. and *P. cerasifera* Ehrh., PPV-SwC induced strong systemic mosaic. Symptoms on woody plants appeared 12 to 15 days after inoculation. PPV-SwC induced systemic chlorotic mottling in *N. benthamiana*, *N. glutinosa* L., and *N. occidentalis* Wheeler, faint systemic mosaic in *N. tabacum* L. 'Burley 64,' local chlorotic and necrotic lesions followed by systemic mottling in *N. clelandii*, and latent infection in *N. sylvestris* Speg. & Comes. *C. foetidum*, *C. quinoa* Willd., *C. murale*, and *C. amaranticolor* Coste & Reyn. were not infected. The isolate also was transmitted by graft-inoculation to cherry and peach plants. Both *A. fabae* and *M. persicae* transmitted the virus under laboratory conditions.

RFLP and sequence analyses. RT-PCR amplification with primers for the C-terminal region of the PPV CP gene produced amplified fragments of the expected 243-bp size from either naturally or artificially infected tissue but not from the healthy control (Fig. 2A). RT-PCR amplification products hybridized with the cRNA probe corresponding to the C-terminal region of the PPV-SoC CP gene (Fig. 2B). In RFLP analysis of the 243-bp amplified products, both *RsaI* and *AluI* enzymes failed to digest the amplified fragment from PPV-SwC (Fig. 2C). After cloning, three recombinant colonies were tested for the presence of the 243-bp fragment amplified either from naturally or artificially infected sweet cherry and from artificially infected peach cv. GF

Fig. 1. Systemic yellow vein banding on leaves of sweet cherry plants mechanically inoculated with an isolate of plum pox virus from sweet cherry.

305. All clones (nine colonies) displayed an insert of the expected size. The nucleotide sequence (EMBL accession no. Y09851) of the fragment showed no *RsaI* and *AluI* sites, confirming the results of the RFLP analysis (data not shown).

Serological analyses. The PPV-SwC CP clearly reacted with the polyclonal antiserum to PPV in Western immunoblot. Proteins of the two cherry isolates and PPV-D showed a very similar electrophoretic mobility, but their mobility was clearly faster than that of the PPV-M CP (data not shown). In DASi-ELISA (Fig. 3), PPV-SwC from either *N. benthamiana* or artificially infected sweet cherry reacted with universal MAb5B, whereas it failed to react with MAb4DG5 or MAbAL. PPV-SoC and PPV El Amar gave the same results as PPV-SwC.

DISCUSSION

PPV-SwC when graft or mechanically transmitted to sweet cherry induced leaf symptoms similar to those observed under natural infection. The isolate shows a peculiar biological behavior, because it is able to systemically infect several *Prunus* species considered resistant to PPV infection (17). Moreover, it is remarkable that PPV-SwC infects peach and myrobalan plants, whereas, conversely, PPV isolates commonly obtained from these hosts cannot infect cherry plants (7). These findings, as well as the transmissibility by different

Fig. 3. Comparative double-antibody sandwich indirect enzyme-linked immunosorbent assay of five isolates of plum pox virus (PPV), with three monoclonal antibodies: Mab5B, Mab4DG5, and MAbAL. SwC: PPV-SwC from *N. benthamiana*; SwC*: PPV-SwC from sweet cherry; SoC: PPV-SoC from *N. benthamiana*; El Am: PPV El Amar from peach cv. GF 305; M: PPV-M (French isolate) from peach cv. GF 305; and D: PPV-D (ISPAVE 31) from peach cv. GF 305. Healthy controls are *N. benthamiana* (Nb), peach cv. GF 305 (GF), and sweet cherry (Ch).

Fig. 2. Comparison of a sweet cherry isolate of plum pox virus (PPV-SwC) with other strains of PPV. (A) Polyacrylamide gel electrophoresis (PAGE) of the reverse-transcription polymerase chain reaction-amplified products of the C-terminal region of the PPV-SwC coat protein gene. Lane M, size markers: 1,000, 700, 525, 500, 400, 300, and 200 bp (from top to bottom); lane 1, naturally infected cherry; lane 2, artificially infected cherry; lane 3, artificially infected peach; and lane H, healthy control. (B) Southern hybridization of the amplified fragments from lane 1, naturally infected cherry; lane 2, artificially infected cherry; and lane 3, artificially infected peach tissues. Lane H, healthy control. (C) PAGE of the restriction fragment length polymorphism products of the PPV-SwC 243-bp amplified fragment. Lane 1, untreated; lane 2, *RsaI* treated; lane 3, *RsaI*-treated control (PPV-D amplified fragment); lane 4, *AluI* treated; and lane 5, *AluI*-treated control (described above). Arrowheads indicate digestion products. Lane M, size markers: 2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234, 220, and 154 bp (from top to bottom).

aphid species, suggest that cherry plants can act as a source of PPV for other *Prunus* species used as crops or rootstocks. These facts should be considered in quarantine schemes aimed at limiting the spread of PPV. Symptoms induced by PPV-SwC in peach cv. GF 305 and *N. benthamiana* are typical for known PPV isolates, whereas those induced in *N. clevelandii* resemble symptoms described for the "necrotic" strain. Because PPV-SwC failed to infect several *Chenopodium* species, attempts to determine which group of PPV—yellow, intermediate, or necrotic (15)—this isolate is related to is not feasible.

DASI-ELISA with virus- and strain-specific MAbs indicated that the PPV-SwC, PPV-SoC, and PPV El Amar strains, although they react with virus-specific MAb5B, are serologically distinct from PPV-D and PPV-M. Western immunoblot analysis showed that the PPV-SwC and PPV-SoC CPs have similar electrophoretic mobility, which is also similar to that of the D strain. However, both cherry isolates display an electrophoretic mobility clearly distinct from that of the M strain. Southern hybridization assay showed that primers designated for the C-terminal region of the PPV CP gene were helpful in amplifying a 243-bp fragment corresponding to the same region of PPV-SwC. The fragment lacks the *AluI* site typical of the known isolates of PPV and the *RsaI* site typical of the D isolates. This agrees with the findings that PPV-SoC lacks such sites as well (13).

Most isolates of PPV reported in Italy belong to the D group (16), which is mainly present in western Europe, whereas the M group is more common in eastern Europe (21). Our results indicate that PPV-SwC does not belong to either the PPV-M or the PPV-D group, because it displays several unusual features. Our data show that PPV-SwC is related to PPV-SoC, supporting the proposed hypothesis that cherry isolates of PPV are members of a different strain group of PPV, for which the name PPV-C (cherry)

has been proposed (13). The relation of the cherry isolates to the atypical PPV El Amar isolate, which displayed the same reaction in ELISA but shows an *AluI* site in the C-terminal region of the CP gene (21), is unclear, raising questions about the complexity of PPV molecular differentiation.

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