SHORT COMMUNICATION

PLUM BARK NECROSIS STEM PITTING-ASSOCIATED VIRUS IN DIFFERENT STONE FRUIT SPECIES IN ITALY

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SUMMARY

Different \textit{Prunus} species from Southern Italy with stem pitting symptoms were assayed by RT-PCR for the presence of Plum bark necrosis stem pitting-associated virus (PBNSPaV). DNA of the expected size was amplified from cortical scrapings of almond, peach, plum, apricot, and sweet cherry. The test was repeated monthly on various tissues throughout a year. Different protocols for total nucleic acid extraction and RT-PCR amplification were compared. Virus particles of an Italian apricot isolate (APR-SP1) were purified and used to produce antiserum that reacted in IEM and ELISA with particles of an American PBNSPaV isolate. However, the antiserum did not detect PBNSPaV in crude plant extracts.

Key words: PBNSPaV, stem pitting, stone fruits, molecular detection, serology.

In several countries, cultivated and ornamental \textit{Prunus} species are affected by stem pitting (Lott \textit{et al.}, 1962; Stouffer \textit{et al.}, 1969; Agrios, 1971; Mircetich \textit{et al.}, 1977; Uyemoto \textit{et al.}, 1995), a disease whose agent(s) is still undetermined. In California, high molecular weight double-stranded RNAs (dsRNA) were recovered from symptomatic cherry trees (Zhang \textit{et al.}, 1998) and Black Beaut plums (\textit{Prunus salicina}) (Marini \textit{et al.}, 2002). An apparently novel closterovirus species in the latter host was partially characterized and named Plum bark necrosis stem pitting-associated virus (PBNSPaV) (Marini \textit{et al.}, 2002). A disorder resembling the \textit{P. salicina} disease was observed in 1995 in Apulia (southern Italy) in apricot of cv Tyrinthos grafted on myrobalan. The bark of the trunk and main branches was spongy, much thicker than normal and showed extensive splitting, and the woody cylinder of the scion was marked by pits and grooves (Di Terlizzi and Savino, 1995). Although all symptomatic plants were also infected by \textit{Prunus necrotic ringspot virus} (PNRSV), its involvement in the aetiology of the disease was dismissed as unlikely, because this virus is common in Apulian apricot trees that do not show stem pitting (Savino \textit{et al.}, 1992).

More recently, a 590 bp fragment showing sequence similarity with the HSP70-homologue gene of viruses in the family \textit{Closteroviridae} was amplified from symptomatic plums and apricots using degenerate primers. This amplicon had 97% sequence homology with a comparable region of the American PBNSPaV genome. A set of primers and a cRNA probe were obtained on the basis of this sequence and used successfully as diagnostic tools for PBNSaV detection in Italy (Abou Ghanem-Sabanazdovic \textit{et al.}, 2001). In the present study, an antiserum was raised to a virus isolated from a Tyrinthos apricot (denoted APR-SP1) and it, together with a protocol for total nucleic acid extraction and nested RT-PCR amplification, were used to investigate relationships with the American isolate of PBNSPaV.

Eight trees of five different \textit{Prunus} species: one almond (cv Filippo Ceo), two peaches (cv Springcrest), one plum (cv Black Beaut), two apricots (cv Castelbrite and Tyrinthos), two sweet cherries (cv Ferrovia) were investigated. All plants showed stem pitting symptoms but were also infected by other stone-fruit viruses. The positive control was the Californian isolate of PBNSPaV, kindly provided by Dr. J. Uyemoto (UC Davis, USA) and maintained in \textit{P. salicina} Black beaut. A GF 305 selection was used as a healthy control. Total nucleic acids (TNA) were extracted from leaf tissues according to Foissac \textit{et al.} (2000) or Varveri \textit{et al.} (1987). Some extractions were also done using the RNAeasy column method (Qiagen, Valencia, CA, USA). Standard RT-PCR with the primers described by Abou Ghanem-Sabanazdovic \textit{et al.}, (2001) was done monthly throughout a whole year using the available tissues (flowers, leaves, or cortical scrapings from dormant cuttings) from all stem pitting sources under investigation. Samples of 200 mg were extracted, each from 3 to 5 different parts of the tree. For the optimisation of the two-step RT-PCR protocol, different dilutions of cDNA (1 to 10 µl in a final PCR reaction volume of 50 µl), annealing temperatures (from 46 up to 62°C), final concentrations of MgCl\(_2\) (from 0.5 up to 2.5 mM), and several primer concentrations were tested.
A set of primers was designed within the above reported PCR fragment (290 bp; Abou Ghanem-Sabanazdovic et al., 2001) for a nested-PCR amplification of a 190 bp product. The primer sequences were: ASPn1, 5' - ACG AAT CCG AGT TTC GTC GC - 3', and ASPn2, 5' - AGG CAC TAC TGA CCT GTA GG - 3'. For double-tube nested PCR, 1 µl of the first PCR reaction (done using the external primers) was added to 49 µl of a second PCR reaction mix that contained the internal nested primers. In the single-tube nested PCR reaction, 10 µl of a complete PCR mixture containing the nested primers were deposited under the tube cap and the first reaction mixture was covered with paraffin oil. After the first PCR round with the external primers (25 cycles at an annealing temperature of 52°C), the tubes were centrifuged and the second round was done at 58°C. The ratio between external and nested primer sets was 1:25. All PCR products were analyzed in silver-stained 5% polyacrylamide gels.

Qiagen kit (Qiagen, Valencia, CA, USA) and silica particle methods (Foissac et al., 2000) were equally efficient for TNA extraction (Fig. 1A, lanes 1-2 and 5-6). The latter protocol in particular, when used in winter on cortical scrapings, removed most of the polyphenols. Crude extraction with sodium citrate buffer (Varveri et al., 1987) gave a percentage of positive detection lower than 70%, when compared with the silica method. Positive amplification was obtained by standard RT-PCR from July to October from cortical scrapings and leaf tissues of all tested stone fruit species. In almond, Black Beaut plum and Castelbrite apricot, positive detection started in May, although only in phloem scrapings. The latter material yielded the best intensities of amplified band, while flowers were always negative. As shown in Fig. 1A, dilution of crude extract in citrate buffer affected amplification: a 1:1 dilution did not yield any visible band because of the inhibition of enzymatic reactions by plant sap components (Fig. 1A, lane 3). On the contrary, when the same extract was diluted 1:5, a strong band appeared (Fig. 1A, lane 4). Dilution had less effect in extracts made by other methods. For an optimized PCR reaction, the best concentration of both primers was 200 pM, the MgCl₂ concentration was 1.5 mM, and the annealing temperature was 58°C (not shown).

When lower concentrations of primers or MgCl₂ were used, the amplified bands were less intense. To assess a sensitivity threshold, the originally cloned 590 bp fragment of the viral HSP70 gene (Abou Ghanem-Sabanazdovic et al., 2001) was transcribed, mixed at different dilutions with 200 ng of silica-purified TNA from healthy plum and used as amplification control in standard RT-PCR: the lowest quantity that gave a detectable band was 50 fg (not shown). Double-tube nested PCR amplified virus-specific sequence (190 bp) from extracts of dormant cuttings (Fig. 1B, lanes 4-6) in periods of the year (winter, early spring) when standard RT-PCR did not yield any visible amplification product (Fig. 1B, lanes 1-3). The single-tube nested PCR gave positive results when concentrated template was used (Fig. 1C, lanes 1 and 3). A more careful definition of cycling steps and reagents concentration in first and second round may avoid the co-existence of both amplified products (Fig. 1C, lanes 1 and 3).

Partially purified preparations from cortical tissues of plants infected either by APR-SP1 or the American...
isolate of PBNSPaV contained closterovirus-like particles, up to ca. 1,500 nm in length (Fig. 2 A). Virus particles were purified from isolate APR-SP1 essentially as described by Namba et al., (1991) for grapevine closteroviruses. About 500 g of cortical scrapings from dormant cuttings were ground in liquid nitrogen and extracted with phosphate buffer, pH 7.2 (instead of Tris-HCl, pH 8.2). Fractions from the final centrifugation on a Cs₂SO₄-sucrose cushion.step gradient were examined in the electron microscope and those containing the highest concentration of virions were injected into a New Zealand rabbit three times at weekly intervals. Antiserum was collected a week after the last injection. Its titre, determined by IEM decoration (Milne and Luisoni, 1977) on partially purified preparations of the homologous antigen (Fig. 2B) was 1:40. Particles of the American PBNSPaV isolate were decorated to the same dilution (Fig. 2C). DAS-ELISA (Clark and Adams, 1977) was done with the antiserum after absorption with extracts from cortical tissues of a healthy plum. Immunoglobulins (IgGs) were recovered by fractionation on Protein A-Sepharose and conjugated with alkaline phosphatase. Positive results were obtained with partially purified preparations of either virus. However, the antiserum failed to detect APR-SP1 when crude sap from leaves or cortical scrapings from infected Tyrinthos and Black beauty plants were tested by DAS-ELISA.

The Italian isolate APR-SP1 is serologically very close, but not identical, to the Californian isolate of PBNSPaV. Thus, since in Italy PBNSPaV is associated with stem pitting of almond, peach and cherry, its role in the aetiology of this disease of *Prunus* appears very likely. As with other closteroviruses, e.g. those infecting grapevines (Martelli et al., 1997), PBNSPaV particles are present in infected tissues at low concentrations in the early phases of growth but its titre increases progressively during the growing season, reaching a maximum at the end of summer. Although fresh phloem tissue is a desirable material for standard RT-PCR detection, nested PCR can also give satisfactory results with dormant cuttings. The availability of a sensitive test for PBNSPaV detection in commercial stone fruit orchards represents a valuable tool for use in sanitation and certification programmes.

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Fig. 2. Electron micrographs of PBNSPaV particles. A) Particles of the Italian virus isolate APR-SP1 stained with uranyl acetate. B) Virus particle as above decorated by the homologous antiserum, diluted 1:40. C) Part of a virus particle of American PBNSPaV isolate decorated with the antiserum to APR-SP1 at 1:40 dilution. Bar=100 nm.

REFERENCES


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