MOLECULAR DETECTION OF A CLOSTEROVIRUS ASSOCIATED WITH APRICOT STEM PITTING IN SOUTHERN ITALY

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SUMMARY

Electrophoretic analysis of cortical and leaf tissue extracts from apricot trees of cv. 'Tyrinthos' from Apulia (southern Italy) affected by stem pitting, contained multiple double-stranded RNA (dsRNA) species, the largest with an estimated size of 15 kbp. A segment of 590 nucleotides, showing sequence homology with the HSP70 homologue gene of members of the family *Closteroviridae* was amplified by RT-PCR from symptomatic trees using degenerated primers designed on the conserved phosphate 1 and 2 motifs of the HSP70 gene sequence. Computer-assisted analysis showed that the 590 nt fragment from Italian apricot trees had 97% sequence homology with Plum bark necrosis stem pitting-associated virus (PBNSPaV), a recently reported closterovirus infecting Black Beaut plum (*Prunus salicina*) in California. A set of primers that amplified a 290 nt virus-specific fragment was designed and a cRNA probe was generated. This riboprobe and RT-PCR assays with these primers detected PBNSPaV in diseased but not in healthy apricot trees.

Key words: apricot, stem pitting, closterovirus, diagnosis, RT-PCR, HSP70-like protein.

INTRODUCTION

In Europe and elsewhere, cultivated and ornamental *Prunus* species are affected by a stem pitting condition (Lott *et al.*, 1962; Barrat *et al.*, 1968; Ragozzino and Caia, 1968; Mircetich and Fogle 1969; Stouffer *et al.*, 1969; Agrios, 1971; Aldwinkle and Anderson, 1972; Mircetich *et al.*, 1977; Uyemoto *et al.*, 1995), whose agent is still unknown.

Stem pitting symptoms were reproduced in peach seedlings following inoculation with the nepovirus *Tomato ringspot virus* (ToRSV) (Smith *et al.*, 1973). More recently, however, high molecular weight double-stranded RNAs (dsRNA) were observed in California in cherry trees with stem pitting (Zhang *et al.*, 1998), and in Black Beaut plums (*Prunus salicina*) with severe bark necrosis and stem pitting (Marini *et al.,* 2001). An apparently novel closterovirus species was detected in *P. salicina*, which was named Plum bark necrosis stem pitting-associated virus (PBNSPaV) (Marini *et al.*, 2001).

In 1995, a disorder was observed in Apulia (southern Italy) in apricot (*P. armeniaca*) cv. 'Tirynthos' grafted on myrabolan, which somewhat resembled the Californian disease of *P. salicina*. The bark of trunk and main branches showed extensive splitting, was spongy and much thicker than normal, and the woody cylinder of the scion was marked by pits and grooves (Di Terlizzi and Savino, 1995) (Fig. 1). Although all symptomatic plants were infected by the ilarvirus *Prunus necrotic ringspot virus* (PNRSV) the involvement of PNRSV in the aetiology of the disease was regarded as unlikely, for this virus is common in Apulian apricot trees with no signs of stem pitting (Savino *et al.*, 1992).

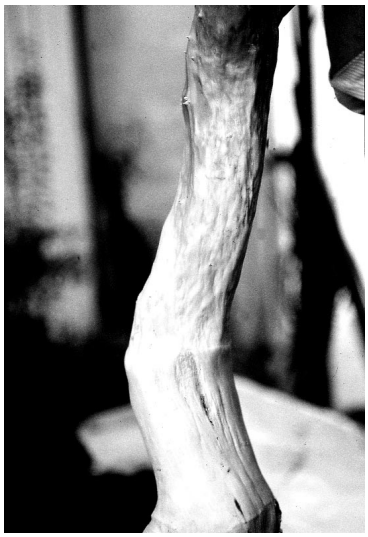

Fig. 1. Stem pitting symptoms on the trunk of a diseased apricot.

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The present study was carried out for determining the aetiology of the Apulian apricot disease and identifying its possible causal agent.

MATERIALS AND METHODS

Virus source. Virus sources used in this study were two naturally infected cv. 'Tyrinthos' trees (F1P24 and F1P27), and two *P. salicina* indicators which had been graft-inoculated with bud wood from an infected Italian apricot tree (T5P2) and with a PBNSPaV source from California, respectively. Both sources induced stem pitting in *P. salicina*, but only the Californian isolate induced also necrosis of the bark.

Mechanical transmission. Virus recovery was attempted by inoculating herbaceous hosts either with sap expressed from leaves and cortical tissues of naturally infected and graft-inoculated plants, ground in 0.1 M phosphate buffer pH 7.2, containing 2% nicotine, or 0.5 M Tris-HCl pH 8.2, containing 0.01 M $MgSO_4$ and 2% nicotine, or with concentrated partially purified leaf tissue extracts. The experimental host range was: *Nicotiana benthamiana, N. occidentalis, N. clevelandii, N. rustica, Chenopodium quinoa, C. amaraticolor, Cucumis sativus, Vigna unguiculata*, and *Gomphrena globosa.*

Double-stranded RNAs. dsRNAs were recovered by phenol extraction and chromatography through cellulose CF-11 columns (Dodds, 1993) from symptomatic and apparently healthy apricot trees. After enzymatic digestion with RNase-free DNase (60 mg ml^{-1}) and DNase-free pancreatic RNase (0.5 mg ml^{-1}) (Saldarelli *et al.*, 1994), preparations were analyzed in 6% polyacrylamide gel electrophoresis (PAGE) in comparison with dsRNAs from *N. benthamiana* infected by the closterovirus *Grapevine leafroll-associated virus 2* (GLRaV-2) (Abou-Ghanem *et al.*, 1998). Gels were stained with silver nitrate.

Reverse transcription polymerase chain reaction (RT-PCR). The degenerate oligonucleotide primers used (HSP1 and HSP2), were the same designed by Tian *et al.* (1996) on the conserved phosphate 1 and 2 motifs of the HSP70 homologue gene of members of the family *Closteroviridae.* Reverse transcription was performed on dsRNA preparations extracted from F1P24, T5P2, and GLRaV-2-infected *N. benthamiana*. Briefly, dsRNA preparations were denatured with 20 mM methyl mercuric hydroxide, primed with 1 µg of random DNA hexanucleotides (Roche Molecular Biochemicals) and reverse transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) in a 50 ml reaction for 1 h at 39°C.

Five ml of cDNA were mixed with 95 µl of the amplification mixture [1x Taq Promega buffer, 3 mM $MgCl_2$, 2 mM of each dNTP, 200 ng of each primer and one unit of Taq DNA Polymerase (Promega Corporation, Madison USA)]. Initial denaturation was at 94°C for 2 min and cycling was as follows: denaturation for 30 sec at 94°C, annealing for 30 sec at 40°C, extension for 1 min at 72°C for 35 cycles, and final extension for 7 min at 72°C. PCR amplified products were analyzed by electrophoresis in 1.2% agarose gel in 1x TBE buffer and visualized by staining with ethidium bromide (Sambrook *et al.*, 1989).

cDNA cloning and sequencing. Three µl aliquots of PCR products were ligated to a pGEM-T Easy vector (Promega Corporation, Madison USA) immediately after electrophoresis. The resulting recombinant plasmids were cloned in *Escherichia coli* DH5α and transformed cells were plated on Luria-Bertani medium containing 75 mg ml^{-1} ampicillin. Recombinant colonies were selected, plasmids were purified, digested with *Eco*RI, and analyzed by agarose gel electrophoresis (Sambrook *et al.*, 1989). Plasmids containing cDNAs of the predicted size (*ca* 600 bp) were used to determine the nucleotide sequence of the amplified product in both cDNA strands by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using 35S-ATP and the Thermo Sequenase cycle sequencing kit (Amersham).

Computer-assisted analysis of nucleotide and protein sequences. Nucleotide and protein sequences were analysed using the Strider 1.1 program (Marck, 1988). Protein sequences were aligned with CLUSTAL W (Thompson *et al.*, 1994). Search for homologies with proteins from the Protein Information Resources (PIR, release 47.0), was done with the FASTA (Pearson and Lipman, 1988) and BLASTA (Altschul *et al.*, 1990) programs. Tentative phylogenetic trees were constructed and bootstrap analysis made with the NEIGHBOR, SEQBOOT, PROTDIST, and CONSENSE programs of the PHYLIP package (Felsenstein, 1989).

Total nucleic acid (TNA) extraction. Approximately 0.5 to 0.6 g of bark shavings or leaf tissue were placed in a plastic bag with gauze (Bioreba) and ground with a rolling grinder in the presence of 5 ml of sterile phosphate buffered saline (PBS 1x) containing 0.5% Tween 20, 2% polyvinylpyrrolidone (PVP) and 20 mM diethyldithiocarbamate (DIECA). Aliquots (1 ml) were placed in an eppendorf tube and incubated for 15 min

at 55°C with 1% of sodium dodecyl sulfate (SDS), followed by the addition of 250 µl of 5 M potassium acetate and further incubation at 0°C for 20 min. Tubes were centrifuged at 13,000 rpm for 10 min, 500 µl of the supernatant liquid were recovered, to which 300 µl of a 6 M sodium iodide solution, 200 µl ethanol, and 25 µl of a silica particles solution were added (Boom *et al.*, 1990). The mixture was incubated at room temperature for 15 min with intermittent shaking, then the eppendorf tubes were centrifuged at 13,000 rpm for 2 min. The supernatant liquid was discarded and the silica pellet washed twice with 1 ml of washing buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 50 mM NaCl and 50% ethanol). Nucleic acids were eluted with 100 µl RNase-free water and used as template for cDNA synthesis as described (Minafra and Hadidi, 1994).

Virus-specific PCR detection. Based on the sequenced portion of the HSP70 cistron, virus-specific sense and antisense primers, denoted as ASP1 (5'-CGGTAGGGCTGTGACTACCG-3') and ASP2 (5'-GTAGTCCGCTGGTACGCTACAAG-3'), intended to amplify a genome fragment of 290 bp, were designed by computer analysis (Primer Selection Program, Henry M. Jackson Foundation, Bethesda), synthesized by GENENCO-Life Science (Florence, Italy), and used for PCR assays from crude plum and apricot extracts (Wetzel *et al.*, 1991), or purified total nucleic acids.

The RT-PCR protocol was as described above, except that annealing was at higher stringency (52°C instead of 40°C) and $MgCl_2$ concentration was 1 mM instead of 3 mM. PCR amplified products were analyzed by electrophoresis in 5% PAGE in 1x TBE buffer, and visualized by silver staining.

Riboprobe synthesis and hybridization. A non radioactive, DIG-labeled cRNA probe (pASP) was generated by SP6 RNA polymerase from the pGEM-T easy cloning vector containing virus-specific sequences, using the SP6/T7 DIG RNA Labeling kit (Roche MolecularBiochemicals) according to the manufacturer's instructions. TNAs from virus-infected apricot, *P. salicinia*, and healthy controls and purified dsRNAs were spotted onto nylon membranes (Hybond N+, Amersham) after denaturation with 50 mM NaOH, 5 mM EDTA at room temperature for 5 min. Membranes were then hybridized overnight at 55°C using the labelled cRNA probe pASP and hybridization signals were detected by chemiluminescence according to manufacturer's instructions (DIG chemiluminescent detection kit, Roche Molecular Biochemicals).

RESULTS

Mechanical transmission. All attempts to transmit virus to herbaceous hosts failed. Virus particles were not seen with electron microscope (Philips 201C) observations of leaf dips from inoculated herbaceous hosts or concentrated preparations of apricot tissues.

dsRNA analysis. Electrophoretic analysis showed the presence of multiple dsRNA bands in extracts of cortical scrapings and leaves of symptomatic but not healthy plants (not shown). The band with the largest molecular weight had the apparent same size as the replicative form of the entire viral genome of GLRaV-2, estimated to be *ca* 15 kbp (Zhu *et al.*, 1998).

RT-PCR. Primers HSP1 and HSP2 (Tian *et al.*, 1996) amplified a single DNA fragment of about 600 nucleotides from reverse-transcribed cDNA, synthesized on dsRNA extracts from a symptomatic apricot tree, from GLRaV-2-infected *N. benthamiana,* and from BNSPaV-infected *P. salicina* (Fig. 2, lanes 3 to 5). No amplification was obtained from cDNA synthesized from healthy 'GF305' seedling extracts (Fig. 2, lane 2).

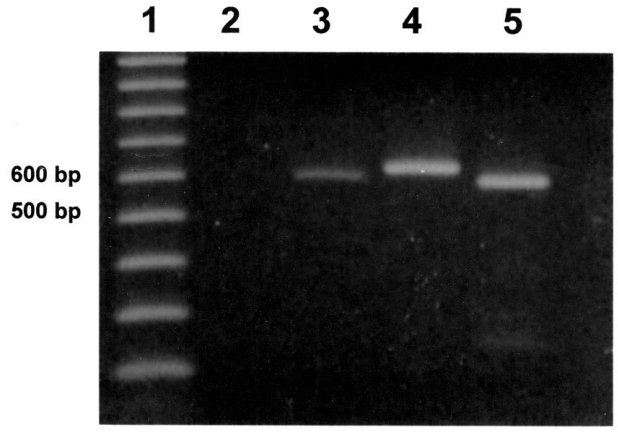

Fig. 2. RT-PCR products amplified from dsRNA extracts using Tian *et al.* (1996) HSP70 degenerate primers HSP1 and HSP2. Extracts are from a cv. 'Tirynthos' apricot (lane 5) and a PBNSPaV-infected *P. salicina* (lane 3). Lane 4 contains a dsRNA extract from a GLRaV-2-infected *N. benthamiana* (positive control) and lane 2 an extract from a healthy GF305 seedling (negative control). Markers (Sigma PCR MW ladder) are in lane 1.

Sequence analysis. The sequence of the cloned cDNA fragment (EMBL accession number AJ305307) consisted of 590 nt, encoding a polypeptide of 196 amino acids that contained consensus amino acid residues present in the HSP70-like proteins of closteroviruses. This provided evidence that this clone was indeed a fragment of a viral genome containing the HSP70 coding region. Computer-assisted comparison of deduced amino acid sequence showed that the 590 nt polypeptide had an average 30-32% similarity with the HSP70-like proteins of a number of members of the family *Closteroviridae.* However, similarity was about 97% with PBNSPaV (Fig. 3), indicating that the putative closterovirus associated with apricot stem pitting in southern Italy is an isolate of PBNSPaV. Comparative phylogenetic analysis of the HSP70 sequence of PBNSPaV from apricot with those of other sequenced members of the family *Closteroviridae,* showed that PBNSPaV stands on its own, in a clade that includes viruses transmitted by mealybugs (Fig. 4). These viruses, as recently suggested (Karasev, 2000), may belong to a third genus in the family *Closteroviridae,* for which the name *Mealyvirus* has been proposed (G.P. Martelli, unpublished information).

Virus detection by molecular hybridization and RT-PCR. A DNA fragment of the expected size (290 bp) was amplified from cortical scraping extracts of cv. 'Tirynthos' apricots in which the virus was originally found (Fig. 5A, lanes 2 and 3), of *P. salicina* infected with a Californian isolate of BPNSPaV (Fig. 5A, lane 4) and of *P. salicina* graft-inoculated with an Apulian apricot stem pitting source (Fig. 5A, lane 5). No product was amplified from the negative (healthy 'GF305' seedlings) and water controls (Fig. 5A, lanes 1 and 6, respectively).

In dot spot assays, the cRNA probe pASP specifically hybridized dsRNA extracts from apricot tissue (Fig. 5B, row 3, lane a), and TNA extracts from infected apricot (Fig. 5B, row 1, lane c) and *P. salicina* (Fig. 5B, row 2 lane c). No hybridization was obtained with TNA extracts from healthy 'GF305' (Fig. 5B, rows 1, 2, 3 lane b).

DISCUSSION

The high homology of HSP70 sequences suggests that an isolate of PBNSPaV occurs in southern Italy, where it is associated with stem pitting of apricot. The pathology of PBNSPaV in apricot is unknown. In this regard, a comparative host range study involving various *Prunus* species graft-inoculated with T5P2 and PBNSPaV is in progress. The availability of molecular tools (virus-specific PCR primers and riboprobe) presently developed, makes it now possible to conduct surveys for the assessment of disease incidence, and to investigate the aetiology of undetermined graft-transmissible diseases of other stone fruit species, *e.g.* almond (*P. amygdalus*) and cherry (*P. avium*), which

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PBNSPaV  -LDFGTTFSSVCFSPANKSNGCTEESDSIFIPTILGLRKDGTFCIGRAVTTEEGLDVYRD
Apricot  GFDFGTTFSSVCFSPANKSNGCTEESDSIFIPTILGLRKDGTFCIGRAVTTEEGLDVYRD
          :************************************************************

PBNSPaV  IKRWVGCNHINESEFRRKLKPEYTVIVDRYNVSIGPVSGNITRVMPVVDLIYLYIKGLVQ
Apricot  IKRWVGCNHINESEFRRKLKPEYTVIVDKYNVSIGPVSGNLTRVMPVVDLIYLYIKGLVQ
         ****************************:***********:********************

PBNSPaV  LTISQTNLQVSSASCSVPADYYSFKRSFVYTACNALGIGVRAVINEPTAAGFCSLLEKTG
Apricot  LTISQTNLQVSSASCSVPADYNSFKRSFVYTACNALGIGVRAVINEPTAAGFCSLLEKTG
         ********************* ************************************

PBNSPaV  GATSYTLVYDFGGGTFDVSLLAVSNNVIVVVDSRGDNLLGGGTF
Apricot  GATSYTLVYDFGGGTF--------------------------
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Fig. 3. Alignment of deduced amino acids encoded by the sequence comprised between phosphate motives 1 and 2 of the HSP70 gene of the apricot isolate of PBNSPaV and PBNSPaV from California. Asterisks and dots indicate identical and similar residues, respectively. Note the high degree of identity between the two sequences.

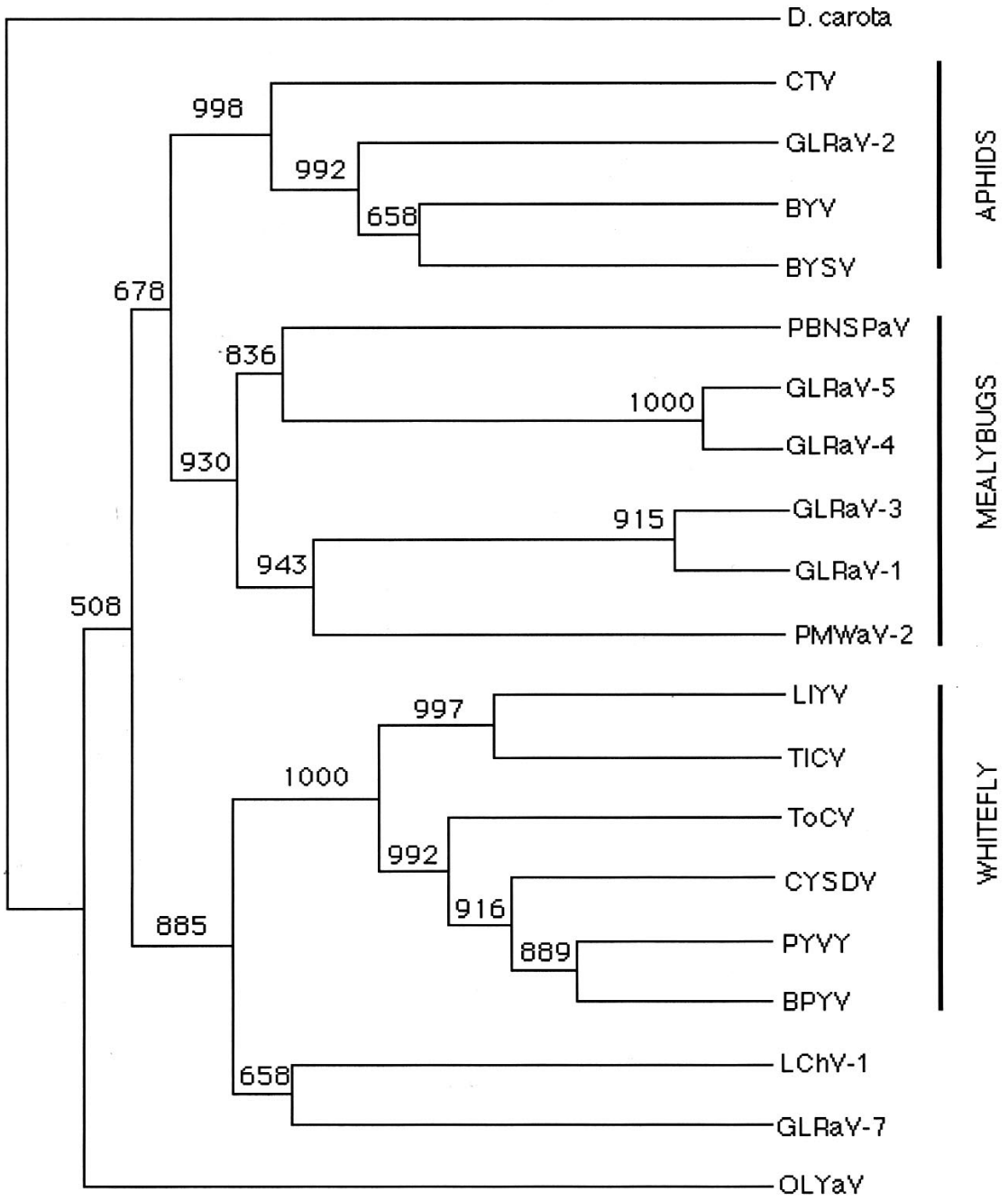

Fig. 4. Comparative phylogenetic analysis of the phosphate 1 and 2 motifs of the HSP70 protein of the PBNSPaV isolate from apricot, with comparable proteins of members of the family *Closteroviridae*. This tree was generated from an alignment of the amino acid sequences between the motifs A and C. EMBL accession numbers of the sequences used are: *Beet yellows virus* (BYV) X73476; *Citrus tristeza virus* (CTV) U02547; *Beet yellow stunt virus* (BYSV) U51931; *Lettuce infectious yellow virus* (LIYV) U05242; Little cherry virus-1 (LChV-1) Y10237; Beet pseudoyellows virus (BPYV) U67447; *Tomato infectious chlorosis virus* (TICV) U67449; *Cucurbit yellow stunt disorder virus* (CYSDV) U67448; *Grapevine leafroll-associated virus 1* (GLRaV-1) AF195822; *Grapevine leafroll-associated virus 2* (GLRaV-2) AF039202; *Grapevine leafroll-associated virus 3* (GLRaV-3) Y1589; Grapevine leafroll-associated virus 4 (GLRaV-4) AF039553; Grapevine leafroll-associated virus 5 (GLRaV-5) AF039552; Grapevine leafroll-associated virus 7 (GLRaV-7) Y15987; *Tomato chlorosis virus* (ToCV) AF024630; Olive Leaf yellowing-associated virus (OLYaV) Y18128; Plum bark necrosis-stem pitting associated virus (PBNSPaV) AF195501; *Pineapple mealybug wilt-associated virus 2* (PMWaV-2) AF283103; Potato yellow vein virus (PYVV) AF150984. The HSP70 sequence from carrot (*Daucus carota*) was used as outgroup.

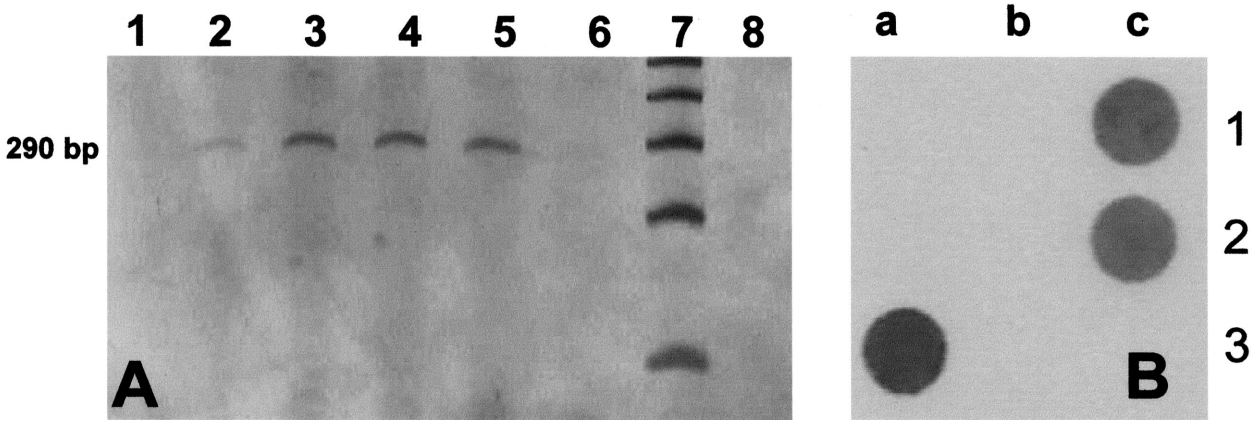

Fig. 5. A. RT-PCR of purified TNAs using primers ASP-1 and ASP-2 specific to the apricot isolate of PBNSPaV. Amplified DNA products of the expected size (290 bp) are present in samples from cv. 'Tyrinthos' (lanes 2, and 3), *P. salicina* (lane 4). Lane 5 contains the product amplified from a dsRNA extract from a symptomatic, graft-inoculated *P. salicina* (positive control). No amplification was obtained with samples from healthy 'GF305' seedling (negative control, lane 6) and the water control (lane 1). Markers are in lane 7. **B**. Dot spot hybridization with molecular probe to the apricot isolate of PBNSPaV. The cRNA probe hybridized specifically with viral RNA present in dsRNA extracts from apricot tissue (row 3, lane a), and TNA extracts from infected apricot (row 1, lane c) and *P. salicina* (row 2, lane c). No hybridization was obtained with TNA extracts from healthy 'GF305' (Fig. 5B; rows 1, 2, 3 lane b). Rows 1 and 2 of lane a are blanks.

occur in southern Italy and are characterized by decline and wood pitting (Quacquarelli and Savino, 1980; Di Terlizzi and Savino, 1997 and unpublished observations).

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Received 7 February 2001
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