Identification and Molecular Characterization of *Pear Blister Canker Viroid* Isolates in Campania (Southern Italy)

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Abstract

Pear blister canker disease is a bark disorder induced by *Pear blister canker viroid* (PBCVd), a member of genus *Apsacaviroid* within family *Pospiviroidae*, on the pear indicators 'A 20' and 'Fieud 37', whereas infections of most commercial pear cultivars are latent. Only a few isolates of this viroid have been characterized so far. To evaluate the presence and spread of PBCVd in Campania (Southern Italy), fifty sources of different pear cultivars were collected in the summer of 2002 and analyzed by polyacrylamide gel electrophoresis and molecular hybridization with a PBCVd-specific riboprobe. The results of this analysis revealed that 10% of the tested trees were infected, with PBCVd being detected on cultivars 'Coscia' and 'Conference'. RT-PCR amplification and sequencing revealed in isolate O3 of cultivar 'Coscia' 18 polymorphic positions when compared with the standard reference sequence of PBVCd isolate P2098T, 12 of which previously unreported. Ten of these new changes were also found in isolate DF9 of cultivar 'Conference'. Isolates G29, G30 and G31 of cultivar 'Coscia' had essentially the same sequence, differing only in a single nucleotide substitution.

INTRODUCTION

Pear blister canker viroid (PBCVd) belongs to the genus *Apscaviroid,* within the family *Pospiviroidae,* and consists of a 315-316 nucleotide RNA that adopts a quasi-rod-like conformation in the proposed secondary structure of minimum free energy (Hernández, et al., 1992; Ambrós et al., 1995; Loreti et al., 1997) (Fig. 1). PBCVd has been reported as the causal agent of pear blister canker (PBC) disease, consisting exclusively of bark disorders in the pear indicators (*Pyrus communis* L.) 'A 20' and 'Fieud 37' (Flores et al., 1991; Ambrós et al., 1995; Desvignes et al., 1999), whereas infection of most commercial pear varieties occurs without symptoms (Ambrós et al., 1995; Desvignes, 1970; Desvignes et al., 1999). The viroid has been so far reported in France, Spain and Italy (Desvignes, 1970; Hernández et al., 1992; Ambrós et al., 1995; Loreti et al., 1997), but it might be present in other areas of the world (Flores et al., 2003). The sequence variability reported among certain European isolates might explain the differences in bark symptom severity induced by PBCVd on the pear indicator 'A 20' (Desvignes, 1970; Hernández et al., 1992; Ambrós et al., 1995; Loreti et al., 1997).

We report in this work the occurrence of PBCVd in commercial pear orchards of Campania (Southern Italy) together with the molecular characterization of the identified isolates.

MATERIALS AND METHODS

Biological Sources

50 samples of the pear cultivars 'Coscia', 'Spadona', 'Conference', 'Spadoncina', 'Vernina' and 'Butirra', as well as of two additional local cultivars poorly characterized, were collected from several commercial orchards in Campania during the summer of

2002.

RNA Extraction and Dot-blot Hybridization

Samples of pear leaves (5 g) were powdered with liquid nitrogen and total RNAs were extracted with buffer-saturated phenol and fractionated by chromatography on non-ionic cellulose (Pallás et al., 1987; Flores et al., 1991). Aliquots (5 µl) were applied to nylon membranes, hybridized overnight at 70°C (in the presence of 50% formamide) with a full-length digoxigenin-labeled PBCVd riboprobe, and detected as recommended by the supplier (DIG Luminescent Detection Kit for Nucleic Acids, Boehringer).

Electrophoretic Analysis and Northern-blot Hybridization

PBCVd extracts were separated by two consecutive polyacrylamide gel electrophoreses (PAGE) under non-denaturing and denaturing conditions (Flores et al., 1985, 1991), using *Citrus exocortis viroid* (CEVd, 371 nt) and *Avocado sunblotch viroid* (ASBVd, 247 nt) as standards to delimit the portion of the first gel that was cut and applied on top of the second gel which was stained with silver (Igloi, 1983). Alternatively, the RNAs in the second gel were electrotransferred to nylon membranes (Zeta-Probe, Bio-Rad) by means of a 2500 Transfor (LKB) electroblotting unit using the conditions of Mühlbach et al. (1983). After a prehybridization at 70°C for 2 h, the membranes were hybridized under the same conditions as those for dot-blot analysis.

RT-PCR Amplification and Sequencing

Reverse transcription (in 20 µl) was performed with Superscript^{TM} RNase H$^-$ reverse transcriptase and primer PIII (5'-ACTTCCACCCTCGCCGCGAAGCCAAGC-3'), complementary to positions 254-280 of the PBCVd reference sequence (Hernández et al., 1992). Aliquots (2 µl) of the cDNA preparation were then PCR-amplified (in 50 µl) with primers PIII and PIV (5'-TTACCGCGGACCCCCGAGAGGAGGCCCTCGGGT-3'), homologous to positions 281-313 of the reference sequence (Hernández et al., 1992) and *Taq* DNA polymerase. Reverse transcription and PCR amplification were carried out as recommended by the supplier (Invitrogen), and the PCR cycling profile was as reported previously (Ambrós et al., 1995). RT-PCR amplified products were eluted and purified with a commercial kit according to the instructions of the manufacturer (Nucleospin Extract Kit, Macherey-Nagel GmbH), and sequenced in both directions automatically (IRBM Sequencing Service, Pomezia, Italy). Sequence alignments were performed using the CLUSTAL W program, version 1.5 (Thomson et al., 1994).

RESULTS AND DISCUSSION

Dot-blot analysis with a PBCVd-specific riboprobe of the 50 pear samples collected in different areas of Campania (Southern Italy) revealed the presence of PBCVd in four pear trees (G29, G30, G31 and O3) of cultivar 'Coscia' and in one (DF9) of cultivar 'Conference' (Fig. 2). The PBCVd-infected trees exhibited mild bark alterations, but these alterations were also observed in PBCVd-free trees and, therefore, could not be associated with the presence of the viroid.

Analysis by double PAGE and northern-blot hybridization confirmed the data obtained by the dot-blot hybridization, showing in these five plants the existence of a circular RNA with the mobility expected for PBCVd (Fig. 3 and 4). Moreover, RT-PCR with a pair of PBCVd-specific and adjacent primers led to the amplification of DNA products of the same size as those resulting from a PBCVd positive control (data not shown). Sequencing of the PCR-amplified DNAs showed that O3 and DF9 isolates were similar, differing only in three polymorphic positions, and that G29, G30 and G31 isolates (collected from the same orchard) had essentially identical sequences, varying only in a single nucleotide substitution. The five isolates had a uniform size of 316 nucleotides. When compared with the standard P2098T isolate (Hernández et al., 1992), the sequence of O3 and DF9 isolates showed 19 (6.0%) and 17 (5.4%) changes, respectively. More specifically, 12 of the 19 changes (O3 isolate) and 10 of the 17 changes (DF9 isolate),

have not been previously reported (Table 1.). These changes are mainly found in the upper and lower strands of the variable domain, located between the central and the right terminal domains (Fig. 1.), and do not affect the palindromic or the stem-loop structures proposed to mediate processing of oligomeric intermediates of members of family *Pospiviroidae* (Ambrós et al., 1995; Diener, 1986; Visvader et al., 1985). No new polymorphic positions were detected in G29, G30 and G31 isolates when compared with PBCVd sequences previously reported.

These results provide new data on the sequence variability of PBCVd and indicate that the spread of PBCVd has been probably underestimated because most pear cultivars are tolerant.

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Tables

Table 1. Polymorphic positions observed in two PBCVd isolates with respect to the reference sequence of isolate P2098T (Hernández et al., 1992). Nucleotide insertions and deletions are indicated by (+) and (-). New detected changes are highlighted with bold characters.

RESIDUE POSITION	CHANGE IN O3	CHANGE IN DF9
4-5	**C(+)**	
49	**G→A**	
54-55	A(+)	
66	**U→C**	
71-72	**G(+)**	
112	**C→U**	
113	**G→(-)**	
115	**U→G**	
119	U→G	
149	U→C	
175	**C→U**	
176	U→C	
181	**G→C**	
182	**A→U**	
183	**G→U**	
234	C→G	
235	G→C	
248	**A→(-)**	**A→G**
249	U→A	

Figures

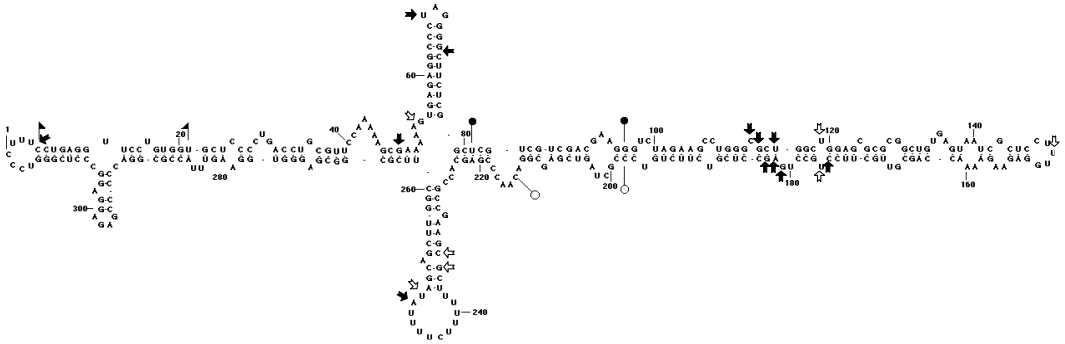

Fig. 1. Secondary structure proposed for the PBCVd reference isolate P2098T (Hernández et al., 1992). The terminal conserved region (TCR) is delimited by flags and the upper and lower strands of the central conserved region (CCR) by black and white circles, respectively. New changes in the PBCVd isolates reported here are denoted by black arrows and changes already reported (Ambrós et al., 1995) by white arrows.

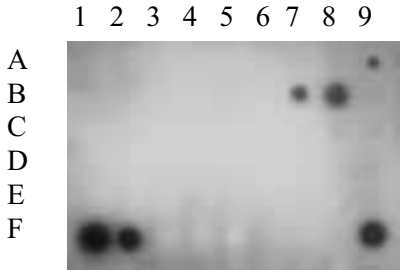

Fig. 2. Dot-blot analysis with a PBCVd-specific digoxigenin-labeled riboprobe of RNA extracts from 50 pear samples. F6 to F8 are healthy controls and F9 a PBCVd-infected control.

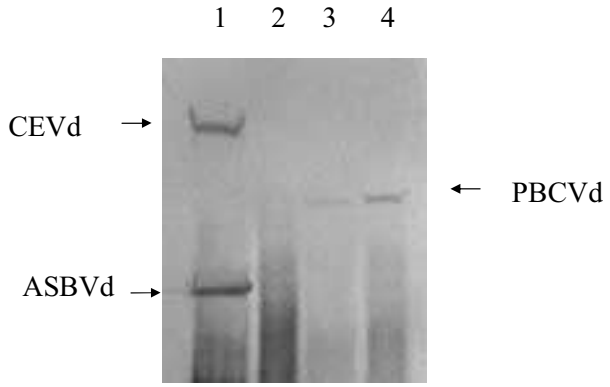

Fig. 3. Detection of the PBCVd circular forms by double PAGE and silver staining. Lane 1, CEVd and ASBVd standards. Lane 2, healthy control. Lane 3, G30 isolate. Lane 4, PBCVd-infected control.

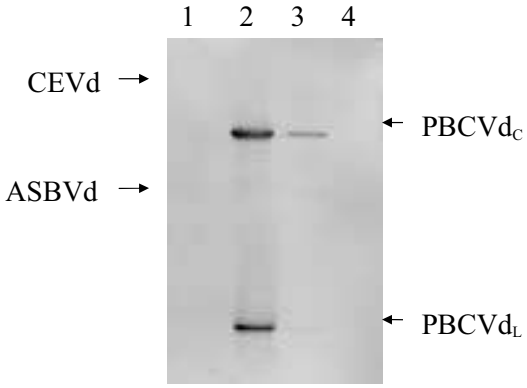

Fig. 4. Analysis by double PAGE and northern-blot hybridization with a PBCVd-specific digoxigenin-labeled riboprobe. Lane 1, CEVd and ASBVd standards. Lane 2, PBCVd-infected control. Lane 3, G30 isolate. Lane 4, healthy control. The position of the circular (C) and linear (L) forms of PBCVd RNAs are shown.