Pear blister canker viroid: sequence variability and causal role in pear blister canker disease

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The sequences of several cDNA clones of pear blister canker viroid (PBCVd) P1914T and P47A isolates have been determined. Seven out of eight P1914T clones analysed have a constant sequence which differs at six positions from that of the P2098T isolate reported previously. The remaining P1914T clone (8) has a single nucleotide substitution. The same six changes have also been observed in most of the ten P47A clones sequenced. However, some P47A clones show additional variability in positions on both strands of the central conserved region (CCR) and in another conserved sequence at the left-terminal region. This is the first report of a change affecting the upper strand of a viroid CCR. Reasons why such a change is tolerated are discussed. Infectivity bioassays have demonstrated that PBCVd is the causal agent of PBC disease.

A viroid RNA of 315 nucleotides (nt) has been isolated from pear and characterized (Hernández et al., 1992). Since this RNA is closely associated with pear blister canker (PBC) disease, it has been tentatively called pear blister canker viroid (PBCVd) (Flores et al., 1991). However, a direct causal relationship between PBCVd and PBC disease still remains to be proved. An unusual property of the PBCVd isolate P2098T, sequenced previously, was the absence of sequence variants in the 15 partial or full-length clones analysed, particularly considering that the viroid RNA was obtained directly from pear and not from an experimental host which could have exerted some selection on the distribution of sequences existing in the natural host. In the present communication we report sequence data obtained from two other isolates of PBCVd and provide evidence demonstrating that this viroid is the causal agent of PBC disease.

Purified circular forms of PBCVd RNA were obtained as described previously (Flores et al., 1991). In one set of experiments with PBCVd isolate P1914T, first-strand cDNA was synthesized using reverse transcriptase (RT) and either primers I or II, complementary to nt 167 to 201 or 217 to 250, respectively, of PBCVd isolate P2098T (Fig. 1). Second-strand cDNA was synthesized using the RNaseH method (Gubler & Hoffman, 1983) and the dsDNA was cloned at the SmaI site of pUC18 (Hernández et al., 1992). In a second set of experiments, with PBCVd isolates P1914T and P47A, template RNAs were reverse transcribed and the cDNAs amplified by PCR using two pairs of adjacent primers. PIII was used together with PIV (complementary and homologous to nt 254 to 280 or 281 to 313, respectively, of PBCVd P2098T), and PI (see above) was used together with PV (homologous to nt 201 to 236 of PBCVd P2098T) (Fig. 1). First-strand cDNAs were synthesized as above with either PIII or PI, and PCR amplifications were performed in 50 µl reaction volumes containing 1/20 volume of the RT reaction mixture, 500 ng of each primer, 10 mM-Tris–HCl pH 9-0, 50 mM-KCl, 1.5 mM-MgCl₂, 200 µM each of the four dNTPs and 2.5 U of Taq DNA polymerase. Reactions consisted of 30 cycles (40 s at 94 °C, 30 s at 60 °C and 2 min at 72 °C) followed by a final extension step of 10 min at 72 °C. PCR products were separated by PAGE and full-length DNAs of the expected size were eluted and cloned in linearized and thymidylated pT7Blue (R)T plasmid (Novagen). Inserts were sequenced with chain terminating inhibitors and T7 DNA polymerase. To improve the resolution, in some cases sequencing was performed on single-stranded DNA.

Pear seedlings (Pyrus communis L. ‘Ficudière’) were mechanically inoculated with purified circular forms of PBCVd P2098T and grown as indicated (Flores et al., 1991). Young leaves were collected 8–10 months after...
### Short communication

#### ISOLATES

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<tr>
<th>METHOD AND PRIMERS USED FOR eDNA CLONES</th>
<th>CLONE LOCATION IN PBCVd</th>
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Fig. 1. Sequence variability in PBCVd cDNA clones. The numbering used corresponds to P2098 isolate (Hernández et al., 1992). Deleted bases are indicated by a ◀ symbol.
inoculation and analysed by PAGE and Northern blot hybridization for the presence of PBCVd RNA, which was detected in a significant fraction of the plants (Flores et al., 1991). Six blocks of five pear seedlings were doubly grafted with material from each of six plants assayed as positive for PBCVd in a lower position, and with the indicator pear clone A20 in an upper position. These plants, together with non-inoculated controls, were grown in experimental plots and observed for the appearance of PBC symptoms over 2 years. In a second experiment, four of the pear seedlings mechanically inoculated with purified PBCVd and assayed as positive for this RNA, and four non-inoculated seedlings, were grafted with A20 chip buds 18 months after inoculation. The plants were topped and kept in the greenhouse for 6 months, transferred to a cold chamber at 5 °C for 3 months, pruned to promote new growth of A20, and finally put back in the greenhouse for 6 months. Growth of non-inoculated plants, inoculated plants and the onset of PBC symptoms were monitored during the experiment.

Fig. 2 summarizes the changes found in the primary structure of PBCVd isolate P1914T with respect to that of the reference isolate P2098T sequenced previously (Fig. 2). Three of the changes, located at positions 50, 119 and 149, were present in the four partial-length clones (1-4) obtained by the RNaseH method and primers PI or PII. The remaining changes U176 → C, loss of C2a4, and C236 → CC were not observed in all clones because of their incomplete length or to the fixation of these positions by the two primers used (nt 167-201 and 217–250). To obtain additional information on the variability of this isolate, particularly between positions 217–224, which were not covered by the previous clones, adjacent primers PIII and PIV were synthesized and used to produce full-length clones by RT–PCR. Sequencing of four of these clones (5-8) confirmed the presence in all of them of the six changes mentioned above, indicating that the PBCVd isolate P1914T has a constant sequence of 315 nt (the only exception was clone 8 where the substitution G310 → U was observed).

On the other hand, ten full-length RT–PCR clones of P47A, a second PBCVd isolate, obtained using two pairs of adjacent primers, were selected and sequenced (Fig. 1). Two out of the four clones derived from primers PI and PV, clones 1 and 2, were identical. With respect to the standard isolate, P2098T, they had the four changes observed in P1914T isolate. It was not possible to know whether these two clones also contained U176 → C and the loss of C234 since both positions were fixed by primer PI. Clone 3 was very similar to clones 1 and 2 (a U108 → C substitution was the only difference) whereas clone 4 was more divergent (Fig. 1).

The second set of six P47A clones, obtained with primers PIII and PIV, all had a size of 315 nt and the same six changes detected in isolate P1914T (Fig. 1), indicating a closer relationship between these two isolates than either has with isolate P2098T.

In addition, four of the last six P47A clones had specific changes, prominent among which were: A90 → G (clone 9) and G231 → U (clone 10) in the upper and lower strands, respectively, of the central conserved region (CCR) characteristic of the members of the apple scar skin viroid (ASSVd) group (Hashimoto & Koganezawa, 1987; Koltunow & Rezaian, 1988), and G11 → U (clone 7) affecting the terminal conserved region (TCR) present...
in two viroid groups whose type members are ASSVd and potato spindle tuber viroid (PSTVd) (Gross et al., 1978). Substitutions in the lower strand of the CCR have been found in some PSTVd isolates (Owens et al., 1992), but not in viroids of the ASSVd group. On the other hand, no change has been reported so far in the upper strand of any member, or sequence variant, of the viroid groups whose type members are PSTVd (Schönlezer et al., 1985; Visvader & Symons, 1985; Puchta et al., 1990a; Góra et al., 1994) and ASSVd (Koltunow & Rezaian, 1989; Puchta et al., 1990b), or the more recently described Coleus blumei viroid 1 (Spieler et al., 1990), showing that the \( A_{90} \rightarrow G \) substitution is unique in this regard.

Evidence that a sequence variant with the \( A_{90} \rightarrow G \) change probably exists in the initial viroid population is based on the observation that this change affects neither the minimum free energy secondary structure proposed previously for the PBCVd (Fig. 2), nor the palindromic structure which could be formed in PBCVd multimers (Fig. 2; right inset), since in both cases an AU base pair is replaced by a GU base pair. Moreover, the stem-loop structure which could form the upper strand of the PBCVd CCR is not affected, since position 90 is located in the loop (Fig. 2; left inset). Both the palindromic and stemloop structures have been suggested to participate in the processing of oligomeric viroid intermediates (Diener, 1986; Visvader et al., 1985), and probably only base changes that do not affect these structures can be tolerated. Also worthy of note is that these two structures, as well as the proposed secondary structure of minimum free energy, are not affected by changes \( A_{103} \rightarrow G \) and \( U_{108} \rightarrow C \) located in the central domain but out of the CCR (Fig. 2).

The substitution found in one of the clones within the TCR is also the first reported to affect this conserved region which seems able to accommodate minor changes. Therefore, although PBCVd isolates P2098T and P1914T have almost constant sequence, the variability observed in P47A indicates that the concept of quasispecies (Holland et al., 1982) can be extended to PBCVd.

We have shown that PBCVd can be recovered from pear seedlings 'Fieudiere' inoculated with purified viroid (Flores et al., 1991). Although this observation suggests that PBCVd is the causal agent of PBC disease, definitive proof requires that PBCVd is able to induce the disease. Since symptoms are not expressed in young pear seedlings, material from seedlings in which replication was detected after mechanical inoculation with PBCVd RNA was assayed with the pear indicator A20 by two different protocols. When chip buds of A20 were grafted on 'Fieudiere' seedlings that were simultaneously graft-inoculated with PBCVd-infected material, the characteristic syndrome of PBC disease was observed in all plants 2 years after inoculation and PBCVd RNA was isolated from A20 plants. When chip buds of A20 were grafted on 'Fieudiere' seedlings that had been inoculated previously with purified PBCVd RNA, growth of A20 was significatively reduced, the typical symptoms appeared approximately 1 year post-inoculation and the plants then died in a period of 2–4 months. The non-inoculated controls remained symptomless and showed normal growth. Together, these results demonstrate that PBCVd is the causal agent of PBC disease.

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References


Hernández, C., Elena, S. F., Moya, A. & Flores, R. (1992). Pear blister canker viroid is a member of the apple scar skin subgroup (aps-caviroides) and also has sequence homology with viroids from other subgroups. Journal of General Virology 73, 2502–2507.


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