DETECTION BY TISSUE-PRINTING OF POME FRUIT VIROIDS AND CHARATERIZATION OF *PEAR BLISTER CANKER VIROID* IN BOSNIA AND HERZEGOVINA

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SUMMARY

A tissue printing hybridization (TPH) method for detecting pome fruit viroids was developed and used for a survey in Bosnia and Herzegovina. A total of 310 samples, representative of 65 apple and 51 pear cultivars and of several rootstocks were tested for the presence of *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd) and *Pear blister canker viroid* (PBCVd). Whereas ASSVd and ADFVd were not found, 13% of the pear samples tested by TPH gave positive signals for PBCVd that were confirmed by dot-blot and northern-blot hybridization assays. These data show for the first time that PBCVd infects pear trees (belonging to at least 10 different cultivars) in Bosnia and Herzegovina and validate the use of TPH for surveys of viroid infections in pome fruit trees. Molecular characterization of the PBCVd population infecting the native pear cv Rancica identified five new polymorphic positions in the viroid genome, two of which are shared by all the sequenced variants of this Bosnian isolate. Apple cv Spy 227 was shown to be an experimental host of PBCVd.

Key words: Tissue printing, viroid, pome fruits diagnosis, PBCVd.

INTRODUCTION

Viroids are infectious agents with genome of small (246-401 nt), circular, non-coding RNA, that induce diseases in several plants (Flores *et al.*, 2005a). Pome fruit trees in the Mediterranean area have been reported as natural hosts of a number of viroids, some of which are responsible of particular diseases (reviewed by Koganezawa *et al.*, 2003; Kyriakopoulou *et al.*, 2001, 2003; Di Serio *et al.*, 2003; Flores *et al.*, 2003). However, pathogenic properties have been demonstrated only for *Apple scar skin viroid* (ASSVd) (Hashimoto and Koganezawa 1987; Puchta *et al.*, 1990; Yang *et al.*, 1992) and *Apple dimple fruit viroid* (ADFVd) (Di Serio *et al.*, 1996), which elicit fruit disorders on certain apple cultivars (Koganezawa 1986; Desvignes *et al.*, 1999a; Di Serio *et al.*, 2001), and for *Pear blister canker viroid* (PBCVd) (Hernàndez *et al.*, 1992a), the agent of bark alteration of the pear indicator A20 (Hernàndez *et al.*, 1992b; Ambròs *et al.*, 1995a). These viroids share structural properties, including a central conserved region, and are classified in the genus *Apscaviroid*, family *Pospiviroidae* (Flores *et al.*, 2005b).

ASSVd and ADFVd can infect both apple (*Malus pumila* L.) and pear (*Pyrus communis* L.), in which they may or may not induce symptoms (Di Serio *et al.*, 2001; Kyriakopoulou *et al.*, 2003). Natural infections by PBCVd were reported in pear and quince *(Cydonia oblonga* Mill.), nashi (*Pyrus serotina* Rehd.) (Joyce *et al.*, 2006), and wild pear (*P. amygdaliformis* Vill.) (Kyriakopoulou *et al.*, 2001), but not in apple (*Malus pumila* L.). Although PBCVd was experimentally transmitted to several species of the genus *Malus*, attempts to graft-transmit this viroid to *M. pumila* were unsuccessful (Desvignes *et al.*, 1999b).

PBCVd has been recorded from several Mediterranean countries including France, Spain, Italy and Tunisia (Desvignes, 1970; Hernández *et al.*, 1992b; Ambrós *et al.*, 1995b; Loreti *et al.*, 1997; Malfitano *et al.*, 2004; Hassen *et al.*, 2006) whereas ASSVd has never been detected in commercial orchards in Italy or France (Loreti *et al.*, 1998; Desvignes *et al.*, 1999a), and ADFVd has been reported sporadically and only from Italy (Di Serio *et al.*, 1996) and Lebanon (Choueiri *et al.*, 2007).

Therefore, these pome fruit viroids are still not prevalent in the Mediterranean area and their spread could be efficiently controlled by using viroid-free propagation material. The availability of reliable detection methods is important for achieving this objective because of the need to detect symptomless infections that occur frequently (Desvignes *et al.*, 1999a and 1999b; Ambros et al., 1995a; Di Serio *et al.*, 2001). Such methods would be instrumental for large-scale surveys in an area like the Mediterranean basin, which has not been extensively investigated.

Efficient methods for detecting pome fruit viroids, based on RT-PCR and nucleic acid hybridization have

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been reported previously (Ambros *et al.*, 1995b; Faggioli *et al.*, 2001; Di Serio *et al.*, 2001; 2002). However, these technologies need skilled technical expertise for nucleic acid preparation, which is time consuming and expensive. An alternative and faster detection method useful for large-scale field indexing is tissue printing hybridization (TPH), which was used successfully for detecting several viroids in fruit trees, including *Hop stunt viroid* (Astruc *et al.*, 1996; Amari *et al.*, 2001) and *Peach latent mosaic viroid* (Loreti *et al.*, 1999; Torres *et al.*, 2004). However, a limitation to this technique is the seasonal fluctuation in the titer of citrus viroids (Duran-Vila *et al.*, 1993). In the case of pome fruit viroids, TPH has been used previously only for the detection of ASSVd in apple plants graft-inoculated with infected material (Podleckis *et al.*, 1993; Hurtt *et al.*, 1996). We have now exploited TPH to develop a reliable method for detecting ASSVd, ADFVd and PBCVd in the field, and to carry out a survey in Bosnia and Herzegovina.

MATERIALS AND METHODS

Plant material. Three one-year-old self-rooted apple seedlings of cv. Spy 277 were separately graft-inoculated with ASSVd or PBCVd, kindly supplied by F. Faggioli (CRA, Centro di Ricerca per la Patologia Vegetale, Rome, Italy) and with ADFVd kindly supplied by A. Ragozzino (Università degli Studi di Napoli, Italy) and grown in pots. One year post-inoculation, these plants were used for testing TPH.

Field surveys and sample collection. Surveys for symptoms and sample collections were made in a varietal collection, 10 commercial orchards and two nurseries. The varietal collection was located in Sarajevo and comprised 25 apple and 26 pear cultivars, most of which were of local origin. Surveyed orchards were located in the northern and central parts of Bosnia and Herzegovina (Banjaluka, Sarajevo, Gradiska, Srbac, and Magla), which are the main pome fruit-growing areas of the country. Nurseries were in Prijedor and Banjaluka. Fifty one pear and 65 apple cultivars and several rootstocks were tested by TPH using no less than two plants per accession for a total of 310 samples (130 pear, 178 apple and two quince trees).

Nucleic acid preparations and RT-PCR detection. Total nucleic acid preparations (TNA), made by phenol-extraction as reported by Dalmay *et al.* (1993) from 100-200 mg of leaf tissue, were used directly for dot-blot (DBH) and Northern blot hybridizations or were further purified by a modified silica gel capture system (Foissac *et al.*, 2001) for RT-PCR. To detect ASSVd and ADFVd in the graft-inoculated apple trees, a multiplex fluorescent RT-PCR was used (Di Serio *et al.*, 2002). To this aim, the primer ADAS-36 (5'-GCCTTCGTCGACGACGACAG-3'), complementary to positions 83 to 102 of the ADFVd reference sequence (Di Serio *et al.*, 1996) and corresponding to the upper strand of the central domain conserved in ADFVd and ASSVd, the primer AS-37 (5'-CGGTGACAAAGGAGCTGCCAG-3'), homologous to positions 98 to 118 of the ASSVd reference sequence (Hashimoto and Koganezawa, 1987) and the primer AD-38 (5'-CCTTTGAGACTTGACCGGTTCCTC-3'), homologous to position 156-179 of the ADFVd reference sequence, were used in combination in a PCR reaction containing the product of reverse-transcriptions performed with the primer ADAS-36. As reported (Di Serio *et al.*, 2002), cDNA fragments corresponding to the full-length ASSVd cDNA (330bp) and to the partial ADFVd cDNA of 254 bp are generated in ASSVd and ADFVd-infected samples, respectively. The primers and the RT-PCR method reported by Malfitano *et al.* (2004) were used for amplifying the PBCVd full-length cDNA (315 bp) that was directly sequenced in both orientations using the same primers. PCR-amplified products were analysed by electrophoresis in 1.2% agarose gels and detected by ethidium bromide staining and irradiation with a UV lamp.

Molecular hybridizations. Tissue prints were done by pressing fresh cut ends of leaf petioles onto Hybond-N+ (Roche Diagnostics GmbH, Germany) membranes. In Bosnia and Herzegovina imprinting was done in autumn 2005 directly in the field (field TPH). Printed membranes were stored at 4°C and processed 2-4 weeks later. During sampling, one-year-old shoots were also collected from tested plants, stored for 8 weeks at 4°C in the dark, and forced to sprout in a greenhouse. A second TPH experiment, for confirming the results of the field survey, was done with petioles of leaves emerging from forced shoots of many plants that tested positive in the first assay. From the same leaves, TNAs were prepared as reported above and analysed by dot-blot and northern-blot hybridizations.

For dot-blot analyses, aliquots (5 µl) of undiluted and 1:10 dilutions of TNA were spotted onto membranes. Northern-blot hybridization was done following separation of TNAs by PAGE in 5% gels containing 8M urea or by two consecutive PAGEs under non-denaturing and denaturing conditions (Flores *et al.*, 1985), and transferring RNA to positively charged membranes by means of Trans-Blot SD Semi-Dry Transfer Cell (Bio Rad, Hercules, CA, USA). DIG-labeled riboprobes were synthesized with T3 or T7 RNA polymerase using linearized recombinant plasmids, which contained monomeric full-lengh cDNAs of ASSVd, ADFVd (kindly supplied by R. Flores, IBMCP-UPV, Spain) and PBCVd (kindly supplied by A. Ragozzino, University of Naples, Italy). Pre-hybridizations and hybridizations with specific RNA probes were performed at 68°C in

the DIG-Easy Hyb Granules solution according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). Membranes were washed for 10 min at room temperature in a buffer containing 0.1% SDS in 2x SSC (0.3M NaCl, 0.03M sodium citrate) and twice for 15 min at 65°C in a buffer containing 0.1% SDS in 0.1x SSC. The membranes were then treated with RNase A (1 µg/ml) in 2x SSC and were incubated with an anti-DIG antibody conjugated to alkaline phosphatase before adding the chemiluminescent substrate CDP-Star (Roche Diagnostics GmbH, Germany).

Cloning and sequencing. PBCVd full-length cDNAs from Bosnian infected pear plants were generated by RT-PCR using primers PB2RV (5'-AGGGGAGCAC-CACAGGAACCTCAGG-3') and PB2FW (5'-TGTG-GTGCTCCCCTGACCTGCGTTCC-3'), which are complementary and identical to positions 5 to 29 and 16 to 41 of PBCVd reference variant (Hernández *et al.*, 1992b), respectively. These primers are partially overlapping to cover a very short genome sequence (36 nt) highly conserved among known PBCVd variants. Amplified cDNAs were electrophoresed in agarose gels and the products of the expected size were eluted and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Inserts were sequenced automatically (MWG-Biotech, Germany).

Sequence analyses. Multiple sequence alignments were generated by using the CLUSTAL W program (Thompson *et al.*, 1994) with minor manual adjustments. Secondary structures of lowest free energy were calculated with the circular version of the MFold program (Zuker *et al.*, 1999).

RESULTS AND DISCUSSION

Development of a tissue printing detection method for pome fruit viroids. In order to establish a fast and reliable method for large-scale indexing, we tested the efficiency of TPH for detecting ASSVd, ADFVd and PBCVd in trees of cv. Spy 227, which had been separately graft-inoculated with these viroids. At first, viroid infection of inoculated plants was confirmed by RT-PCR (Di Serio *et al.*, 2002), which yielded amplicons of the expected sizes from ADFVd- and ASSVd-inoculated plants. An amplification product of the expected size (315 bp) was also generated by RT-PCR as described by Malfitano *et al.* (2004) using the TNA preparation from Spy 227 inoculated with PBCVd. Correspondence of the amplicon to the PBCVd monomeric full length cDNA was confirmed by its direct sequencing (data not shown). These data showed that the analyzed apple plants were infected by the respective viroids and could be used as positive controls for further studies.

In a second step, DBH and TPH using non-radioactive labelled probes were used to detect ADFVd, ASSVd and PBCVd in experimentally inoculated apple plants. Fig. 1 shows that the three viroids can be detected effectively by both DBH and TPH. Each probe hybridized strongly and exclusively with the complementary viroid RNA (Fig. 1). Moreover, the best tissue printing hybridization signals were always obtained from petioles collected from the apical part of the shoots (Fig. 1), suggesting that the sensitivity of the method could be improved by using petioles of young leaves. Non-specific hybridizations of each probe with the other non-targeted pome fruit viroids were not observed (Fig. 1), thus showing that the stringent hybridization conditions adopted in our protocol and the treatment of the hybridized membranes with RNase A avoided one of the limits reported previously for pome fruit detection methods based on molecular hybridization (Di Serio *et al.*, 1996; 2002). Therefore, TPH seems to be a useful assay for specifically detecting and discriminating among ASSVd, ADFVd and PBCVd in pome fruit trees.

An additional outcome of this study is the finding that

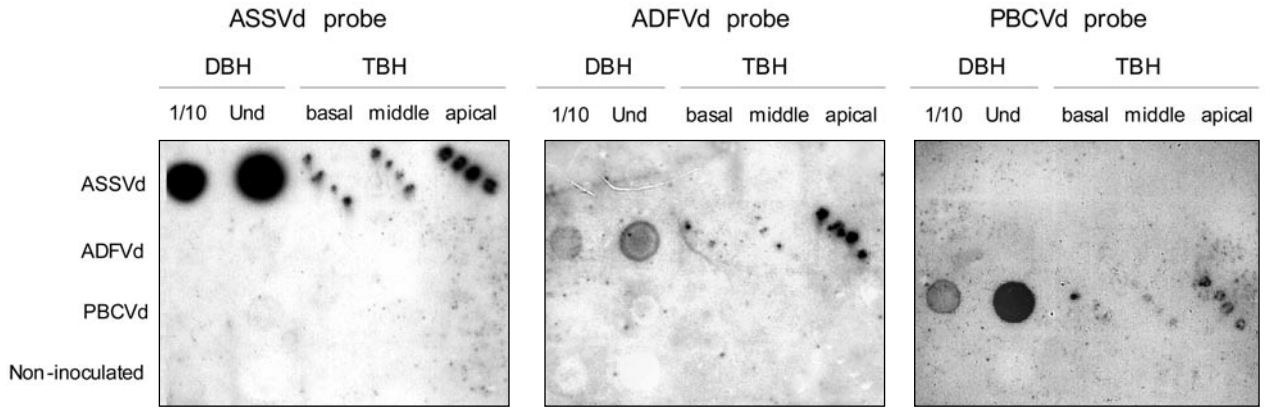

Fig. 1. Detection of ASSVd, ADFVd and PBCVd by dot-blot and tissue-printing hybridization in graft-inoculated apple plants cv. Spy 227. From each assayed plant, aliquots of TNA preparations, diluted 1:10 or undiluted (und), were spotted. Petioles from the basal, middle and top portion of 1-year-old shoots were printed on the membrane. The samples were applied in the same order onto three membranes that were separately hybridized with probes specific for ASSVd, ADFVd or PBCVd.

PBCVd can infect apple Spy 227, in which it accumulates sufficiently to allow detection by both RT-PCR and nucleic acid hybridization. To our knowledge, this is the first report of PBCVd in *M. pumila*, suggesting that cultivated apples are potential natural hosts of PBCVd.

Survey of pome fruit viroid infections in Bosnia and Herzegovina by tissue printing. No fruit or bark symptoms comparable to those reported for ASSVd and ADFVd and for PBCVd were observed in the surveyed field plants.

Out of the 310 tested samples, 178 were from apples assayed for ASSVd and ADFVd, and 130 were from pears tested for PBCVd. Two quince trees were assayed for infection by each of the three viroids. TPH with a probe specific for PBCVd showed infection of 24 of the 130 tested pear trees, corresponding to 15 cultivars (Table 1) grown in both the central and the northern regions of Bosnia and Herzegovina.

On the contrary, no apple tree tested by TPH with probes specific for ASSVd or ADFVd generated positive hybridization signals (data not shown). Samples from quince did not hybridize with any viroid probe (data not shown).

Confirmation of TPH results. In February 2006, shoots collected in autumn 2005 from pear trees in the field and forced to sprout in greenhouse, were tested again by TPH using the probe specific for PBCVd. These analyses were performed on the shoots collected from 28 field-grown pear trees, including, for each positive cultivar, at least one representative positive plant identified by the previous field TPH with the exclusion of samples from cvs Abate Fetel, Trevuska, and Jeribasma, which were not available. Shoots from several pear cultivars that were negative in the previous TPH assay (cvs Bella di Giugno, Conference, Bonita, and Sampionka) were also incorporated in these analyses as additional controls. This second test largely confirmed the results of the first TPH, with the exception of two pear trees (cvs Red William and Santa Maria), that gave slight positive signals in the first assay (Table 1) and were negative in the second one, thus indicating that some occasional false positives can be obtained with TPH.

To further confirm the results of TPH, the emerging leaves of shoots forced to sprout in the greenhouse and tested by TPH were also tested by dot-blot hybridization (DBH). Most PBCVd-infected samples identified by TPH also gave positive hybridization signals in dot-blot assays using the PBCVd-specific probe (Table 1). However, samples from cvs Red William and Santa Maria, which gave contradictory results with TPH, were negative also when assayed by DBH, again indicating that TPH can occasionally yield false positives. Interestingly, all samples that tested negative with TPH were confirmed as non-infected by PBCVd when assayed by DBH. This can be taken as an indication that PBCVd detection by TPH is unlikely to yield false negatives. To verify possible mixed infections in the same host by more than one pome fruit viroid, the same samples were also hybridized with probes specific for ADFVd and ASSVd. In this case, no signal was obtained (data not shown), suggesting the absence of natural infections by these two viroids in the assayed pears.

A similar approach was followed to confirm results of TPH with apple trees. In this case, shoots of 18 trees, corresponding to 6 different cultivars were forced to sprout and analysed by both TPH and DBH. No hybridization signal was obtained with any of the three specific viroid probes, confirming the absence of single and mixed infections in the analyzed apple samples. These results are consistent with the lack of symptomatic apple trees in the surveyed fields and also suggest

Table 1. Confirmation of results of field tissue printing hybridization assays (field TPH) by additional TPH and dot-blot hybridization (DBH) tests using shoots forced to sprout in greenhouse.

Cultivar[1]	Field TPH[2]	TPH and DBH using forced shoots[3]
William	3 / 8[4]	1 / 6
Starkrimson	2 / 2	1 / 1
Kaludjerka	2 / 6	1 / 2
Butirra Hardy	2 / 8	1 / 2
Klapovka	2 / 2	1 / 1
Karamut	2 / 4	1 / 1
Rancica	1 / 2	1 / 1
Bijela Kaiserica	1 / 2	1 / 1
Kanjiska	1 / 2	1 / 1
Sjerkovaca	1 / 2	1 / 1
Red William	1 / 2	0 / 1
Santa Maria	1 / 4	0 / 2
Bella di Giugno	0 / 2	0 / 2
Sampionka	0 / 2	0 / 2
Conference	0 / 2	0 / 2
Bonita	0 / 2	0 / 2
Abate Fetel	2 / 4	NT[5]
Trevuska	1 / 4	NT
Jeribasma	2 / 2	NT
Total positive/tested	24 / 62	10 / 28

[1] From each cultivar possibly infected by PBCVd on the bases of the field TPH assays, at least one positive plant was selected and some of its shoots were forced to sprout in greenhouse and used as source material for additional TPH and DBH assays. Only cvs with samples tested positive in the field TPH and/or assayed by additional TPH and DBH using forced shoots are reported. The cvs with samples whose PBCVd infection has been confirmed are shown in bold.
[2] Reported results refer to the field TPH assay performed in autumn 2005.
[3] TPH and DBH assays performed on forced shoots in 2006 gave coincident results.
[4] Number of positive plants /number of tested plants for each cultivar.
[5] NT, not tested.

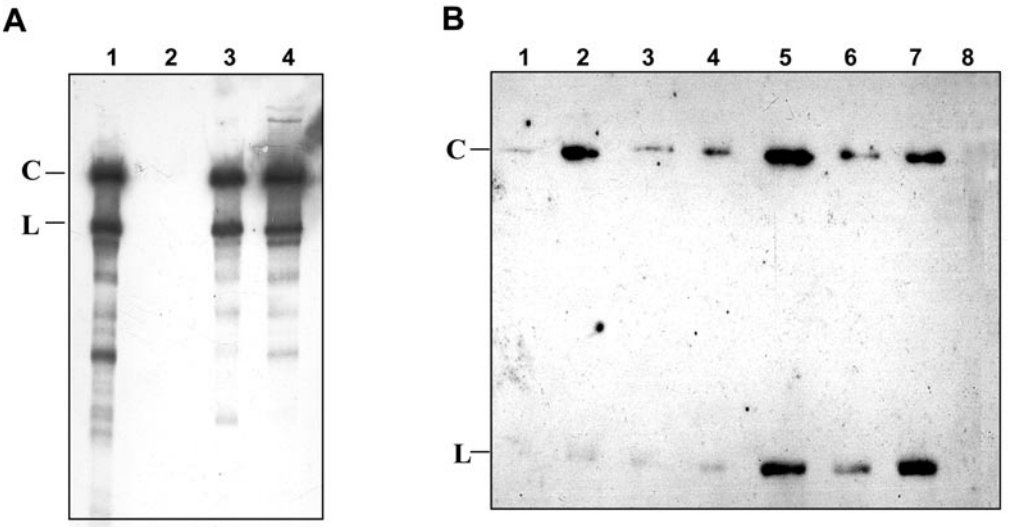

Fig. 2. Analysis by northern-blot hybridization with PBCVd-specific digoxigenin-labeled riboprobes. A. TNAs separated by denaturing PAGE: 1: positive controls (apple cv Spy 277); 2: healthy; 3: cv Bijela Kaiserica; 4: cv Rancica; B. TNAs separated by two consecutive PAGEs (a non- denaturing followed by a denaturing PAGE): 1: cv. Karamut; 2 : cv Starkrimson; 3: cv Sjerkovaca; 4: cv Klapovka; 5: positive control (apple cv Spy 277); 6: cv Kanjiska; 7: cv Rancica; 8: healthy; positions of circular (C) and linear (L) PBCVd RNAs are indicated on the left.

that both ASSVd and ADFVd are not prevalent in Bosnia and Herzegovina. This tallies with the alleged absence, or limited presence, of these viroids in other European countries (Loreti *et al.*, 1998; Desvignes *et al.*, 1999a; Di Serio *et al.*, 2003).

PBCVd infections of pear trees were also confirmed by northern-blot hybridisation assays. To this aim, TNA preparations from cvs Rancica and Bijela Kaiserica known to be positive by DBH were separated by denaturing PAGE, transferred to nylon membranes and hybridised with a PBCVd-specific probe. Two bands, comigrating with the linear and circular forms of PBCVd infecting the positive control (Spy 227) were obtained from both samples (Fig. 2A), indicating that the plants were infected by a viroid RNA very similar to PBCVd. In order to further characterize the electrophoretic mobility of these RNAs, northern-blot hybridisation analyses was also done after separating TNA preparations by double PAGE, an electrophoretic technique specifically proposed for studying small circular RNAs (Flores *et al.*, 1985). This assay was carried out with TNA preparations already tested by DBH, corresponding to TPH-positive trees of cvs. Starkrimson, Klapovka, Karamut, Kanjiska, and Sjerkovaca. The TNA preparation from the apple tree cv. Rancica, analyzed previously, was used as an additional control. As shown in Fig. 2B, this assay confirmed that trees grown in Bosnia and Herzegovina and positive to TPH were infected by an RNA very similar to and comigrating with circular and linear forms of PBCVd.

Infection of pear trees by PBCVd was also confirmed by transmission assays. Chip buds from two field trees that were positive in molecular hybridization assays (cvs. Klapovka and Rancica) were grafted onto LA62 pear seedlings. Although these indicator plants did not show any symptom, they proved positive when TPH and DBH were made four months post-inoculation (data not shown).

Sequencing and genetic diversity of a Bosnian PBCVd isolate. For a further molecular characterization, we selected the PBCVd isolate from a pear tree of the native cv. Rancica (PB-BR isolate) and performed RT-PCR with primers PB2RV and PB2FW. Cloning of the amplification product and sequencing of six independent clones showed inserts corresponding to full-length monomeric PBCVd cDNA, ranging in size from 315 to 316 nt. Multiple alignments revealed that the Rancica PBCVd variants share high sequence similarity with the reference variant (NC001830, Hernández *et al.*, 1992a) and with those previously deposited in the databank. Five new polymorphic positions (G125⇒A; C234⇒G; G235⇒C; deleted U242; G⇒267A with respect to the reference variant) were identified, with those located at position 234 and 235 shared by all the six variants from the PB-BR isolate. All the mutations in the new polymorphic positions do not result in major modifications to the secondary structure of lowest free energy proposed for this viroid (Hernández *et al.*, 1992a) (data not shown). Further analyses of the multiple alignment showed that three variants from cv. Rancica are identical, thus corresponding to the possible master sequence (accession number EF530209) in the PBCVd population infecting this field isolate. The other three variants differed from the master sequence because of a single point mutation or deletion. Therefore, from this study a total of four new PBCVd variants dif-

fering slightly from those previously reported were identified and deposited in the GenBank database with the accession numbers from EF530209 to EF530212.

Altogether, these results confirm the presence of PBCVd in pear trees grown in Bosnia and Herzegovina, and represent its first report in the country. This study also validated the TPH method for large scale surveys of pome fruit viroid infections. Moreover, it showed that both TPH and DBH can be successfully used for detecting PBCVd in shoots forced to sprout in a greenhouse. This finding is interesting from an applied point of view because it offers the possibility of extending to several months the time between sampling and assay. Although ADFVd and ASSVd were not found in Bosnia and Herzegovia, the TPH method developed here has been recently successfully applied in other Mediterranean countries, allowing the identification of new ADFVd isolates (A. Myrta and F. Di Serio, unpublished information). The identification of several native and ancient pear cultivars infected by PBCVd and the presence of infected pear trees in both the northern and central part of Bosnia and Herzegovina should encourage surveys in other Balkan countries.

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REFERENCES

Amari K., Cañizares M.C., Myrta A., Sabanadzovic S., Di Terlizzi B., Pallás V., 2001. Tracking Hop stunt viroid infection in apricot trees during a whole year by non-isotopic tissue printing hybridisation. *Acta Horticulturae* **550**: 315-320.

Ambrós S., Desvignes J.C., Llácer G., Flores R., 1995a. Pear blister canker viroid: sequence variability and causal role in pear blister canker disease. *Journal of General Virology* **76**: 2625-2629.

Ambrós S., Desvignes J.C., Llácer G., Flores R., 1995b. Peach latent mosaic and pear blister canker viroids: detection by molecular hybridization and relationships with specific maladies affecting peach and pear trees. *Acta Horticulturae* **386**: 515-521.

Astruc N., Marcos J.F., Macquaire G., Candresse T. and Pallás V., 1996. Studies on the diagnosis of hop stunt viroid in fruit trees: identification of new hosts and application of a nucleic acid extraction procedure based on non-organic solvents. *European Journal of Plant Pathology* **102**: 837-846

Choueiri E., Zammar E., Jreijiri F., Hobeika C., Myrta A., Di Serio F., 2007. First report of *Apple dimple fruit viroid* in Lebanon. *Journal of Plant Pathology* **89**:294.

Dalmay T., Rubino L., Burgyán J., Kollár Á., Russo M., 1993. Functional analysis of cymbidium ringspot virus genome. *Virology* **194**: 697-704.

Desvignes J.C., 1970. Les maladies à virus du poirier et leur détection. *CTIFL Docum.* **26**:1-12.

Desvignes J.C., Grasseau N., Boyé R., Cornaggia D., Aparicio F., Di Serio F., Flores R., 1999a. Biological properties of apple scar skin viroid: isolates, host range, different sensitivity of apple cultivars, elimination, and natural transmission. *Plant Disease* **83**: 768-772.

Desvignes J.C., Cornaggia D., Grasseau N., Ambrós S., Flores R., 1999b. Pear blister canker viroid: Host range and improved bioassay with two new pear indicators, Fieud 37 and Fieud 110. *Plant Disease* **83**: 419-422.

Di Serio F., Aparicio F., Alioto D., Ragozzino A., Flores R., 1996. Identification and molecular properties of a 306 nucleotide viroid associated with apple dimple fruit disease. *Journal of General Virology* **77**: 2833-2837.

Di Serio F., Malfitano M., Ragozzino A., Desvignes J.C., Flores R., 2001. Apple dimple fruit viroid: fulfillment of Koch postulates and symptom characteristics. *Plant Disease* **85**: 179-182.

Di Serio F., Malfitano M., Alioto D., Ragozzino A., Flores R., 2002. Apple dimple fruit viroid: sequence variability and its specific detection by multiplex fluorescent RT-PCR in the presence of Apple scar skin viroid. *Journal of Plant Pathology* **84**: 27-34.

Di Serio F., Malfitano M., Alioto D., Ragozzino A., Flores R., 2003. Apple dimple fruit viroid. In: Hadidi A., Flores R., Randles J.W., Semancik J.S. (eds). Viroids, pp. 146-149. CSIRO Publishing, Collingwood, Australia.

Duran-Vila N., Pina J.A., Navarro L., 1993. Improved indexing of citrus viroids. In: Moreno P., da Graça J.V., Timmer L.W. (eds). *Proceeding of the 12th Conference of the International Organization of Citrus Virologists*, pp. 202-211, IOCV, Riverside, CA, USA.

Faggioli F., Ragozzino E., Barba M., 2001. Simultaneous detection of stone and pome fruit viroids by single tube-RT-PCR. *Acta Horticulturae* **550**: 59-63.

Flores R., Duran-Vila N., Pallás V., Semancik J. S., 1985. Detection of viroid and viroid-like RNAs from grapevine. *Journal of General Virology* **66:**2095–2102.

Flores R., Ambròs S, Llácer G., Hernández C., 2003. Pear blister canker viroid. In: Hadidi A., Flores R., Randles J.W., Semancik J.S. (eds). Viroids, pp. 153-155. CSIRO Publishing, Collingwood, Australia.

Flores R., Hernández C., Martínez de Alba E., Daròs J.A., Di Serio F., 2005a. Viroids and viroid-host interactions. *Annual Review of Phytopathology*, **43**: 117-139.

Flores R., Randles J.W., Owens R.A., Bar-Joseph M., Diener T.O., 2005b. Viroids. In: Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball A.L. (eds). Virus Taxonomy, Eight Report of the International Committee on Taxonomy of Viruses, pp. 1145-1159 Elsevier/Academic Press, London, UK.

Foissac X., Svanella-Dumas L., Dulucq M.J., Gentit P., Can-

dresse T., 2001. Polyvalent detection of fruit tree Tricho, Capillo and Foveaviruses by nested RT-PCR using degenerated and inosine containing primers (PDO RT-PCR). *Acta Horticulturae* **550**: 37-43.

Hashimoto J., Koganezawa H., 1987. Nucleotide sequence and secondary structure of apple scar skin viroid. *Nucleic Acids Research* **15**: 7045-7052.

Hassen I.F., Roussel S., Kummert J., Fakhfakh H., Marrakchi M., Jijakli M.H., 2006. Development of a rapid RT-PCR test for the detection of peach latent mosaic viroid, pear blister canker viroid, hop stunt viroid and apple scar skin viroid in fruit trees from Tunisia. *Journal of Phytopathology* **154**: 217-223.

Hernández C., Elena S. F., Moya A., Flores R., 1992a. Pear blister canker viroid is a member of the apple scar skin viroid subgroup (apscaviroids) and also has sequence homologies with viroids from other subgroups. *Journal of General Virology* **73**: 2503-2507.

Hernández C., Llácer G., Desvignes J.C., Flores R., 1992b. Evidence supporting a viroid etiology for pear blister canker viroid. *Acta Horticulturae* **309**: 319-324.

Hurtt S.S., Podleckis E.V., Howell W.E., 1996. Integrated molecular and biological assays for rapid detection of Apple scar skin viroid in pear. *Plant Disease* **80:** 458-462.

Joyce P.A., Constable F.E., Crosslin J., Eastwell K., Howell W.E., Rodoni B.C., 2006. Characterisation of Pear blister canker viroid isolates from Australian pome fruit orchards. *Australasian Plant Pathology* **35**: 465-471.

Koganezawa H., 1986. Further evidence for viroid etiology of apple scar skin and dapple diseases. *Acta Horticulturae* **193**: 29-33.

Koganezawa H., Yang X., Zhu S.F., Hashimoto J., Hadidi A., 2003. Apple scar skin in apple. In: Hadidi A., Flores R., Randles J.W., Semancik J.S. (eds). Viroids, pp. 137-141. CSIRO Publishing, Collingwood, Australia.

Kyriakopoulou P.E., Giunchedi L., Hadidi A., 2001. Peach latent mosaic and pome fruit viroids in naturally infected cultivated pear *Pyrus communis* and wild pear *P. amygdaliformis*: Implications on possible origin of these viroids in the Mediterranean region. *Journal of Plant Pathology* **83**: 51-62.

Kyriakopoulou P.E., Osaki H., Zhu S.F., Hadidi A., 2003. Apple scar skin viroid in pear. In: Hadidi A., Flores R., Randles J.W., Semancik J.S. (eds). Viroids, pp. 142-145. CSIRO Publishing, Collingwood, Australia.

Loreti S., Faggioli F., Barba M., 1997. Identification and characterization of an Italian isolate of pear blister canker viroid. *Journal of Phytopathology* **145**: 541-544.

Loreti S., Faggioli F., Barrale R., Barba M., 1998. Occurrence of viroids in temperate fruit trees in Italy. *Acta Horticulturae* **472**: 555-559.

Loreti S., Faggioli F., Cardoni M., Mordenti G., Babini A.R., Poggi Pollini C., Barba M, 1999. Comparison of different diagnostic methods for detection of peach latent mosaic viroid. *Bulletin OEPP/EPPO Bulletin* **29**: 433-438.

Malfitano M., Barone M., Ragozzino E., Alioto D., Flores R., 2004. Identification and molecular characterization of Pear blister canker viroid isolates in Campania (Southern Italy). *Acta Horticulturae* **657**: 367-371.

Podleckis E.V., Hammond R.W., Hurtt S.S., Hadidi A., 1993. Chemiluminescent detection of potato and pome fruit viroids by digoxigenin-labelled dot-blot and tissue blot hybridization. *Journal of Virological Methods*, **43**: 147-158.

Puchta H., Luc Yang, X., Hadidi A., Hammond R.W., 1990. Nucleotide sequence of apple scar skin viroid reverse transcribed in host extracts and amplified by the polymerase chain reaction. *Acta Horticulturae* **309**: 305-309.

Thompson J. D., Higgins D. G., T. J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Torres H., Gómez G., Pallás V., Stamo B., Shalaby A., Aouane B., Gavriel I., Kominek P., Caglayan K., Sipahioglu M., Michelutti R., Myrta A., Fiore, N., 2004. Detection by tissue printing of stone fruit viroids, from Europe, the Mediterranean and North and South America. *Acta Horticulturae* **657**: 379-383.

Yang X., Hadidi A., Sänger H.L., 1992. Nucleotide sequence and secondary structure of apple scar skin viroid (ASSVd) from China. *Plant Molecular Biolology* **14**: 1065-1067.

Zuker M., Mathews D.H., Turner D.H., 1999. Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. In:. Barciszewski J., Clark B.F.C. (eds). RNA Biochemistry and Biotechnology, NATO ASI Series, pp. 11-43. Kluwer Academic Publishers, Boston, USA.

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