Detection by tissue printing hybridization of Pome fruit viroids in the mediterranean basin

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Abstract

Available data on the incidence and biodiversity of pome fruit viroids in the Mediterranean basin are limited. Before starting a research survey to fill this gap, a tissue-printing hydridization (TPH) method to detect *Apple scar skin viroid* (ASSVd), *Pear blister canker viroid* (PBCVd) and *Apple dimple fruit* viroid (ADFVd) has been developed and validated. Afterward, TPH was used in large-scale indexing of pome fruit viroids in Bosnia and Herzegovina, Malta, Lebanon and Turkey. A total of about 1,000 trees was randomly collected and tested. Positive results obtained by TPH were confirmed by at least one additional detection method (RT-PCR and/or Northern-blot hybridization) and viroids were finally identified by sequencing full-length cDNA clones. PBCVd was detected in 13%, 12.4% and 5.4% of the tested pear trees in Bosnia and Herzegovina, Malta and Turkey, respectively, showing a wider diffusion of this viroid than expected. In contrast, ASSVd was never detected and ADFVd was only found in symptomatic trees (cv. Starking Delicious) in Lebanon, confirming a restricted presence of these viroids in the Mediterranean basin. Altogether, these data support the use of TPH as an easy and valuable tool for exploring pome fruit viroid spread.

Keywords: Viroid disease, viroid spread, Pome fruit trees, detection methods, molecular hybridization

Introduction

Pear blister canker viroid (PBCVd) (Hernández et al., 1992) is the causal agent of bark alteration in the pear indicator "A20" (Ambrós et al., 1995). Commercial pear cultivars infected by PBCVd generally do not develop bark symptoms. *Apple dimple fruit viroid* (ADFVd) (Di Serio et al., 1996) may cause a severe fruit disorder in apple trees, characterized by malformed fruits with green scattered depressed spots of 3 to 4 mm in diameter, predominantly around the calyx (Di Serio et al., 2001). Fruits showing similar symptoms or with scar patches on the skin accompanied by a corky texture can also be observed in plants infected *by Apple scar skin viroid* (ASSVd) (Hashimoto and Koganezawa, 1987).

These viroids belong to the genus *Apscaviroid*, family *Pospiviroidae* (Flores et al., 2005). Available data on the incidence and biodiversity of pome fruit viroids in the Mediterranean basin are limited (Flores et al., 2003; Di Serio et al., 2003; Koganezawa et al., 2003). Several efficient methods for detecting pome fruit viroids have been reported previously (Ambros *et al*., 1995; Di Serio et al., 2001; 2002; Ragozzino et al., 2004).

These technologies, based on RT-PCR or on molecular hybridization of labeled probes with plant extracts, need technical expertise for nucleic acid preparations, which is time consuming and relatively expensive. In contrast, tissue printing hybridization (TPH), an alternative detection method based on molecular hybridization, does not require nucleic acid preparation because nucleic acids are applied to the membrane by directly imprinting the fresh plant tissues to be tested. Although TPH has been successfully applied for detecting several viroids infecting fruit trees, such as *Hop stunt viroid* (Astruc et al., 1996; Amari et al., 2001) and *Peach latent mosaic viroid* (Loreti et al., 1999; Torres et al., 2004), the sensitivity of this method may depend on several factors, including the viroid-host combination and the seasonal fluctuation in the viroid titer in the infected plant, as shown by Duran-Vila et al. (1993) in the case of viroids infecting citrus. In the case of pome fruit viroids, detection by TPH of ASSVd in apple plants graft-inoculated with infected material (Podleckis et al., 1993; Hurtt et al., 1996) suggested that this method could be also applied for large-scale indexing of this and other pome fruit viroids, but no data in this respect were available at the beginning of our study. To fill this gap, we developed and validated a tissue-printing hybridization (TPH) method to detect ASSVd, PBCVd and ADFVd (Lolic et al., 2007).

Here we summarize the results obtained in the last few years applying this detection method in large-scale indexing of pome fruit viroids in Bosnia and Herzegovina (Lolic et al., 2007), Malta (Attard et al. 2007), Lebanon (Choueiri et al., 2007), Morocco and Turkey, allowing the identification of several new pome fruit viroid isolates. Our studies show a wider spread of PBCVd and ADFVd in the Mediterranean basin than thought previously and supply additional data on the sequence variability of these viroids**.**

Material and methods

Field surveys and sample collection: Surveys for symptoms and sample collections were made in apple (*Malus pumila* Mill.), pear (*Pyrus communis* L.) and quince (*Cydonia oblonga* Mill.) varietal collections, commercial orchards and nurseries located in Bosnia and Herzegovina (northern and central areas), Malta, Lebanon (northern areas), Morocco (northern areas) and Turkey (western areas). In total, about 1,000 trees were tested for viroid infection (310 samples from Bosnia Herzegovina, 113 from Malta, 264 from Lebanon, 249 from Morocco and 89 from Turkey). 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 assayed for the respective viroid infection and then used as positive controls in the RT-PCR and molecular hybridization tests.

Detection by molecular hybridization and RT-PCR: Total nucleic acid (TNA) extracts were prepared from 100-200 mg of leaf tissues as reported by Dalmay et al. (1993) and directly used for dotblot (DBH) and Northern blot hybridization experiments or were further purified by a modified silica-gel capture system (Foissac *et al.*, 2001) before performing RT-PCR reactions. Tissue prints were done by pressing fresh cut ends of leaf petioles onto Hybond-N+ (Roche Diagnostics GmbH, Germany) membranes. Labelling of riboprobes, DBH and Northern-blot hybridization experiments were carried out as previously described (Lolic et al., 2007). Detection of ASSVd and ADFVd by RT-PCR was performed as previously reported (Di Serio et al., 2002), whereas PBCVd was detected by RT-PCR following the protocol of Malfitano et al. (2004). PCR-amplified products were analyzed by electrophoresis in 1.2% agarose gels and detected by ethidium bromide staining and irradiation with a UV lamp.

Cloning and sequencing: PBCVd and ADFVd amplified cDNAs of expected sizes from infected pear and apple plants were directly sequenced in both orientations or were eluted from agarose gels and cloned into the pGEM-T-Easy vector (Promega, Madison, WI, USA). Inserts were sequenced automatically (MWG-Biotech, Germany).

Results and discussion

No bark symptom comparable to that reported for PBCVd in the pear indicator “A20” (Ambrós et al., 1995) was observed in the surveyed fields. Although no PBCVd infected pear was identified in Morocco, unexpected high infection rates of 13%, 12.4 and 5.4% were found in Bosnia and Herzegovina, Malta and Turkey, respectively (Table 1). Indeed, out of the 398 pear trees assayed for PBCVd infection by TPH, 35 tested positive. These findings were further confirmed by Northern blot hybridization assays and/or RT-PCR and, for some isolates, by cloning and sequencing of the amplified cDNA products (data not shown). These confirmation tests also showed that some additional pear trees testing positive to TPH were actually not infected, indicating that TPH may occasionally generate a false positive signal.

A careful testing of more than 800 trees for ASSVd and ADFVd was done by TPH in the surveyed regions. ASSVd and ADFVd were insistently searched in the surveyed regions testing by TPH more than 800 trees. ASSVd was not detected in any tested apple tree in Morocco, Lebanon and Bosnia and Herzegovina, suggesting the absence or limited spread of this viroid in such countries and supporting previous indications of the relatively rare incidence of ASSVd in Europe (Koganezawa et al., 2003). Interestingly, ASSVd was not detected in any of the 194 tested pear trees in Morocco and Lebanon. This result partially contrasts with the previous widespread and highly frequent occurrence of this viroid reported in wild and cultivated pear trees in Greece (Kyriakopoulou et al., 2001), suggesting that the incidence of this viroid in pear trees may largely differ among European countries.

Tab. 1 Large scale indexing by TPH of pome fruit viroids in Morocco, Lebanon, Malta, Bosnia and Herzegovina and Turkey.

Viroid / Location	PEAR ASSVd ADFVd	PBCVd	APPLE ADFVd	ASSVd	PBCVd	QUINCE ASSVd ADFVd	PBCVd
Morocco	0/81*	0/81	0/168	0/168	0/168	/	/
Lebanon	/**	/	17/264	0/264	0/264	/	/
Malta	0/113	14/113	/	/	/	/	/
Bosnia & Herz.	/	17/130	0/178	0/178	/	0/2	0/2
Turkey	/	4/74	/	/	/	0/15	1/15
TOTAL	0/194	35/398	17/610	0/610	0/432	0/17	1/17

*Number of positive plants/number of tested plants; ** /, not tested

The identification of ADFVd in cv Starking Delicious in Lebanon is of particular interest because this is the second country in which this viroid has been identified so far. Similar to a previous report from Italy (Di Serio et al., 2001), fruits from the Lebanese infected plants showed typical symptoms of ADFVd infection. It is known that, after experimental inoculation, some apple cultivars (i.e. cv. Golden) may tolerate ADFVd infections without eliciting symptoms (Di Serio et al., 2001). However, this viroid was not found in any of the almost 600 symptomless apple plants assayed in this study, suggesting that the natural diffusion of ADFVd is likely limited at present to symptomatic cultivars and to restricted areas.

Finally, identification of one quince infected by PBCVd is in line with previous data on natural hosts of this viroid (Flores et al., 2003). Altogether these data, show that the spread of PBCVd in the Mediterranean basin is wider than thought before, whereas ADFVd and ASSVd are still rare in this area. Our studies also strongly support the implementation of control measures based on importing viroid-free pome fruit germplasm into European countries and on the use of viroid-indexed mother trees for producing propagation material.

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