Identification of Ilarviruses in almond and cherry fruit trees using nested PCR assays

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Abstract

In this study nested PCR assays have been developed for the detection of *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV) modifying a previously reported assay for the generic detection of ilarviruses. In all cases one generic upstream primer was used along with a virus-specific downstream primer in respective nested PCR assays. The application of the same thermocycling profile allowed all amplifications to run in parallel. *Ilarvirus* isolates from different hosts were used for the evaluation of the detection range of the assays, which were afterwards applied for screening almond and cherry plant material. In almond trees the incidence of PNRSV and PDV was 41% and 21.5%, respectively. In cherry orchards the opposite was observed with PDV (56.6%) being the prevalent virus followed by PNRSV (19.4%). Mixed infections with both viruses were also encountered in approximately 10 and 17% of cherry and almond trees, respectively. ApMV was not detected in any of the samples tested. This is the first extensive survey conducted in Greece in order to monitor the distribution of these viruses using molecular assays.

Keywords: *Prune dwarf virus*, *Prunus necrotic ringspot virus*, *Apple mosaic virus*, cherry, almond, nested PCR

Introduction

Stone fruits are susceptible to many virus associated diseases (Nemeth, 1986). Some of the most important viruses of cherry and almond belong to the genus *Ilarvirus and* exist either in single or in mixed infections within the trees (Nemeth, 1986). Vegetative propagation and grafting are mainly responsible for their wide dissemination and the production of certified propagating material is the most effective way for their control. However, an important step in the process of controlling fruit tree viruses is the application of sensitive and reliable molecular diagnostic techniques. A nested PCR assay has been recently developed for the generic detection of ilarviruses amplifying a 371 bp RdRp fragment (Maliogka et al., 2007). Using this method a survey was conducted on a number of almond and cherry trees in Greece and revealed high rates of *Ilarvirus*-related infections. In order to further identify the viral agents involved in these infections the nested PCR step of the generic assay was modified so as to specifically detect three of the most widespread ilarviruses of stone fruits namely, *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV). The developed assays were applied for screening almond and cherry orchards from different geographic districts so as to provide further insight on the distribution and relative importance of PDV, PNRSV and ApMV on these plant species in Greece.

Materials and methods

Virus isolates: PDV, PNRSV and ApMV isolates from different hosts (cherry, almond, apple, rose, plum, and apricot) were used for the evaluation of the detection range of the herein developed assays.

RT-PCR: The first generic RT-PCR took place as reported earlier (Maliogka et al., 2007), using 2 µl of total RNA (Rott & Jelkmann, 2001) as template.

Virus-specific nested PCR assays: For the detection of each virus, specific downstream primers (Fig. 1) were designed from conserved RdRp regions, after a proper alignment of homologous nucleotide sequences available in the genebank and/or determined herein, and used along with the generic upstream "Ilarpolsequp"

5'-TCGAMRTTYGAYAARTCGCA-3' (Maliogka et al., 2007)

in respective nested PCR assays. The reaction mixture contained in all cases 1µl of the first RT-PCR, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 3% DMSO, 0.2 mM of each dNTP, 1 unit Dynazyme II™ DNA polymerase (Finnzymes), 1 µM of "Ilarpolsequp" and depending on the specificity of the detection 0.2 µM from each of the PDV, PNRSV or ApMV downstream primers. The same thermal profile was applied for all reactions which consisted of a 3 min incubation at 94 °C followed by 40 cycles at 94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 20 sec and a final extension step of 72 °C for 2 min.

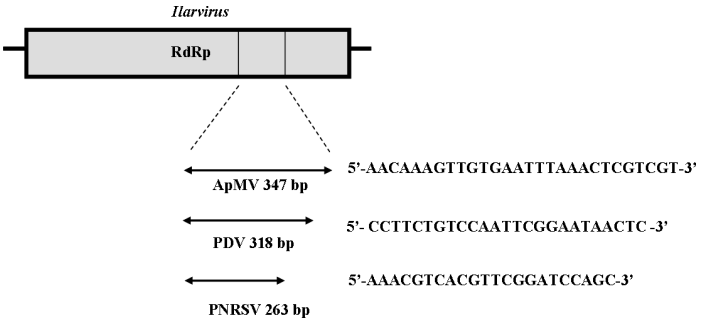

Fig. 1 Position of the PDV, PNRSV and ApMV specific downstream primers on the RdRp gene and size of the nested PCR products.

Plant material: A total of 265 almond and 196 cherry samples were collected in a random way from different districts of Greece (Table 1) during 2005-2007 and tested using the herein developed nested PCR assays.

Tab. 1 Sampling of Plant Material and incidence of PDV and PNRSV

Area surveyed	No. of samples collected	PDV positive samples	PNRSV positive samples
Almond			
Larissa	75	18	40
Thessaloniki	22	0	0
Magnesia	42	15	19
Ioannina	10	1	1
Pieria	38	6	8
Kavala	19	3	19
Evros	19	0	2
Ilia	3	0	0
Serres	37	14	20
Total	265	57 (21.5%)	109 (41%)
Cherry			
Imathia	37	11	5
Pella	19	12	4
Pieria	52	26	16
Kavala	3	2	2
Thessaloniki	22	18	4
Evros	19	17	4
Ioannina	19	3	0
Grevena	3	3	3
Komotini	22	19	0
Total	196	111 (56.6%)	38 (19.4%)

Sequence analysis: PDV and PNRSV amplicons were directly sequenced using "Ilarpolsequp" and the virus specific downstream primers. The obtained sequences were compared after clipping the primer-binding regions with homologous sequences of other PDV and PNRSV isolates already deposited in the genebank.

Results

Evaluation of the assays: All PDV, PNRSV and ApMV isolates tested, originating from different hosts, were successfully amplified using the developed assays (Fig. 2). No amplicon was obtained in each reaction from the isolates of the other two viruses or the healthy controls.

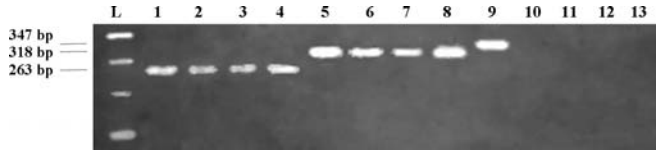

Fig. 2 Agarose gel electrophoretic analysis of the nested PCR products for the detection of PDV, PNRSV and ApMV. Lane 1: PNRSV infected cherry, lane 2: PNRSV infected almond, lane 3: PNRSV infected rose, lane 4: PNRSV infected apricot, lanes 5-6: PDV infected cherries, lane 7: PDV infected almond, lane 8: PDV infected plum, lane 9: ApMV infected apple, lanes 10-13: healthy cherry, almond, plum and apricot, respectively, lane L: 100 bp DNA ladder.

Incidence of PDV, PNRSV and ApMV: A high incidence of PDV and PNRSV was encountered on both plant species tested (Table 1). In almond trees PNRSV was identified in 41% of the analysed samples and it was prevalent in the areas of Larissa, Magnesia and Serres. PDV was identified at lower rates (21.5%) mainly in the areas of Larissa, Magnesia, Kavala and Serres. Both viruses were detected, though at lower rates (10%), in wild almonds (region of Ioannina, Table 1). In cherry orchards the opposite was observed with PDV (56.6%) being the prevalent virus followed by PNRSV (19.4%). PDV exhibited a higher infection rate in the regions of Komotini, Evros and Thessaloniki, whereas PNRSV was mainly found in Pieria. Mixed infections with both viruses were also encountered in approximately 10 and 17% of cherry and almond trees, respectively. ApMV was not detected in any of the samples tested.

Sequence analysis: Sequencing of an almond isolate from PNRSV and an almond and cherry isolate from PDV confirmed the specificity of the assays. The almond partial RdRp sequence of PNRSV was 99% identical in nucleotides with that of an apricot isolate (Acc. No. AM412232). The homologous sequences from the almond and cherry isolates of PDV showed 91 and 99% nucleotide identities, respectively with that of an already published Greek cherry isolate (Acc. No. AM412231). The sequences determined herein were deposited in the EMBL-EBI database under the accession no. FN556183, FN556184 and FN556185.

Discussion

In this study nested PCR assays were developed for the specific detection of three major stone fruit ilarviruses, namely PDV, PNRSV and ApMV. Various isolates of the three viruses originating from different host plants were used for the evaluation of the specificity and detection range of the assays while the application of the same thermocycling profile enabled all amplifications to run in parallel. The assays were successfully applied for screening almond and cherry plant material. The results indicated high infection rates with PDV and PNRSV while ApMV was not detected. The low sanitary status of cherry and almond trees calls for the implementation of certification programs in the production of their propagating material in Greece in order to prevent further spread of the viruses. To our knowledge, this is the first extensive survey conducted in Greece for monitoring the distribution of PDV, PNRSV and ApMV in almond and cherry trees using molecular assays. The sequence information that exists so far in the databases for PDV, PNRSV and ApMV comes mainly from the coat protein gene of the viruses. Thus, the herein developed assays can be applied for the enrichment of sequence data on the RdRp gene from different isolates of the three viruses, which would in turn contribute to future molecular variability studies. Finally, these methods could be used in combination with the generic PCR previously reported for ilarviruses (Maliogka et al., 2007) for the identification of genetically distant PDV, PNRSV and ApMV variants or even putatively new *ilarvirus* species.

Literature

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