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Simultaneous detection of the three characterized ilarviruses affecting stone fruit trees by non-isotopic molecular hybridization and multiplex RT-PCR.

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ABSTRACT

The three most economically damaging ilarviruses affecting stone fruit trees on a worldwide scale are *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple mosaic virus* (ApMV), all of which are closely related phylogenetically. Non-isotopic molecular hybridization and multiplex RT-PCR methodologies were developed that simultaneously could detect all these viruses and the latter technique had the advantage of being discriminatory. For RT-PCR a degenerate antisense primer was designed which, in conjunction with three virus-specific sense primers, allowed the identification of the three ilarviruses in a single test. The amplification efficiencies for the detection of the three viruses in the multiplex RT-PCR reaction were identical to those obtained in the single RT-PCR reactions for individual viruses. This cocktail of primers was able to amplify sequences from all of the PNRSV, ApMV and PDV isolates tested in five different *Prunus spp*. hosts (almond, apricot, cherry, peach and plum) occurring in nature in a single, double or triple infections. For ApMV isolates, differences in the electrophoretic mobilities of the PCR products were observed. The nucleotide sequence of the amplified products of two representative ApMV isolates was determined and comparative analysis revealed the existence of a 28 nt deletion in the sequence of isolates showing the faster electrophoretic mobility. To our knowledge, this is the first report on the simultaneous detection of three plant viruses by multiplex RT-PCR in woody hosts. This multiplex RT-PCR could be a very useful method that would save time and costs in indexing these three ilarviruses which damage stone fruit tree yields and for helping in the analysis of mother plants in certification programs.

INTRODUCTION

Stone fruit trees are affected by a large number of diseases of viral etiology that can cause substantial economic losses (6,15,30). The most important ilarviruses affecting stone fruits are *Apple mosaic virus* (ApMV), *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV). These viruses, alone or in combination, can affect fruit yield, fruit maturity and tree growth (30). In addition, PNRSV and PDV have been shown to be pollen and seed-borne transmitted (2,14) which contributes to their rapid spread. These three ilarviruses are phylogenetically closely related (26) and they show at least 65% of sequence similarity at the coat protein level.

To date the only effective way to prevent virus spread in woody crops is through the use of healthy material. Therefore, great efforts have been made during recent years to improve the sensitivity and speed of molecular diagnostic methods. Both molecular hybridization-based methods (5,17,19,23,27) and PCR-based methods (8,19,20,21,23,29) detect these viruses even in low concentration limits. As a next step towards development of faster and more time-saving methods, attempts have been made to simultaneously detect at least two viruses. This has been easily achieved by using non-isotopic molecular hybridization, for instance in the detection of the primary viruses affecting tomato (22) or carnation (24) plants. As regards PCR-based techniques, there are still few examples of simultaneous detection of more than two plant viruses in a single assay (e.g. 3, 7). Concerning the stone fruit viruses, so far PNRSV *and Plum pox virus* (PPV) (11) or *Apple chlorotic leaf spot virus* (ACLSV) and PPV (16) were detected in a single assay by multiplex RT-PCR with the same sensitivity as that observed during individual RT-PCR analyses.

Additionally, a PCR-ELISA procedure has been described for the simultaneous detection of ApMV and PNRSV (4) but, in this case, an extra probe-capture step was required to differentiate between the two viruses.

In this work we describe the simultaneous detection of the three characterized ilarviruses affecting stone fruit trees (PNRSV, PDV and ApMV) by non-isotopic molecular hybridization and a modified multiplex RT-PCR. For the latter technique, we took advantage of the high sequence similarity at the 3'-untranslated region (3'-UTR) of the RNA 3 of these three viruses to design a degenerate antisense primer, which in conjunction with sense primers that were virus-specific, allowed the simultaneous detection and identification of the three viruses in a single assay. To our knowledge, this is the first report on detecting three plant viruses simultaneously in a multiplex RT-PCR in woody hosts.

MATERIALS AND METHODS

Plant material and RNA extraction.

Single infections of ApMV and PDV in dormant cuttings of almond (*Prunus amygdalus*) (isolates AL015 and Al016, respectively) and PNRSV-infected cuttings of plum (*Prunus salicina*) (isolate PL44) were obtained from a virus collection kept at the Istituto Agronomico Mediterraneo (Bari, Italy). The infected cuttings were forced to grow at room temperature and leaves from these cuttings were used for molecular hybridization and preliminary RT-PCR studies. In addition, leaf samples from 42 trees of *Prunus ssp*. (almond, apricot, cherry, peach and plum) which were grown in the field and naturally infected by one, two or three ilarviruses were used to evaluate the effectiveness of the non-isotopic molecular hybridization and multiplex RT-PCR techniques. These

samples were originating mainly from southern Italy (thirty-two), the USA (three) or the Czech Republic (seven).

Total RNA was extracted from 0.4 g fresh leaf tissue as described previously (23) except for samples from cherry trees where a treatment to eliminate polysaccharides was incorporated (18). Alternatively, 0.2 g of tissue per sample was homogenized in a plastic bag with 10 volumes of sodium citrate buffer (50 mM sodium citrate, 5 mM EDTA, pH 8.5) (23). The homogenates were clarified by centrifugation at 5000 g for 1 min and the supernatants were applied to nylon membranes (Boehringer Mannheim, Germany) and analyzed by non-isotopic molecular hybridization (17).

In vitro transcription of digoxigenin- labeled riboprobes

An ApMV clone containing the CP gene was kindly provided by Dr. P. H. Berger and P. J. Shield (University of Idaho, USA) (28). A PDV clone containing the CP gene and 3' non coding region was kindly provided by Dr. S. W. Scott (Clemson University, USA) (27) and a PNRSV clone containing the RNA 4 sequence (25,26) were used to synthesize riboprobes. One μg of plasmid was linearized with Bam HI, Hind III or Pst I for the ApMV, PDV or PNRSV clones, respectively. Linearized plasmids were made blunt-ended with T4 DNA polymerase. After linearization and blunt-ending, the plasmids were purified with phenol-chloroform, ethanol-precipitated and resuspended in sterile water. The synthesis of the digoxigenin-labeled RNA probes was conducted as described previously (13,17).

Dot-blot hybridization

Total RNA or clarified sap preparations were diluted fivefold with healthy extracts and 3.5 μl of each dilution were directly applied to nylon membranes (Boehringer Mannheim). Membranes were air dried and the nucleic acids were crosslinked to the membrane by exposure to UV irradiation from a transilluminator for 3 min. Prehybridizations and hybridizations with single or mixed probes were carried out as previously described (17,24), except that the hybridization temperature was raised to 70ºC for the clarified sap extracts. Chemiluminescent detection using CSPD reagent as substrate was performed as recommended by the manufacturer (Boehringer Mannheim). Films were exposed for 10-60 min.

Primers and multiplex RT-PCR.

The primers used for single or multiplex RT-PCR are shown in Table 1. A degenerate antisense primer (VP77) was used for the reverse transcription reaction. First strand cDNAs were synthesized in a 20 μl reaction containing 2 μl of total RNA (3-5 μg approx.), 50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 1 mM of each dNTP, 40 U ribonuclease inhibitor (Amersham International, Cleveland, OH), 8 U AMV-RT (Promega Corp., Madison, WI) and 100 pmol of VP77 antisense primer. To perform specific PCR amplifications for each virus, the universal antisense VP77 and the virus-specific sense primers (VP79 for ApMV, VP80 for PDV and VP78 for PNRSV), identical to a region in the corresponding CP genes (see Table 1) were used. Multiplex PCR was carried out using a mixture of four primers in a single tube. PCR cocktail included: five μl of the reverse

transcription products, 10 µl of 10X buffer (166mM $(NH_4)_2SO_4$, 670 mM, Tris-HCl pH 8.8, 0.1% Tween 20), 3 mM $MgCl_2$, 0.2 mM of each dNTP and 60 pmol of each primer in a total volume of 50 µl. Prior to thermal cycling, 1 U Ecotaq DNA polymerase (Ecogen SRL, London) was added to the cocktail and overlaid with mineral oil (Sigma Chemical Co., St. Louis, MO). PCR was carried out in a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer Corp., Norwalk, CT) programmed for one cycle at 94 ºC for 2 min followed by 30 cycles of 94 ºC for 35 sec, 52 ºC for 35 sec and 72 ºC for 35 sec, and finally with an extension temperature at 72 ºC for 5 min. For multiplex PCR on naturally-infected cherry samples collected in the field, the same RT-PCR conditions were used, except that 1% PVP was added to the PCR reaction mixture to avoid inhibitory effects of phenolic compounds (12). Amplified products (5 µl each) were electrophoresed in 2% agarose or 5% polyacrylamide gels in 40 mM Tris-acetate and 1 mM EDTA, pH 8.0 (TAE) and stained with ethidium bromide. For southern-blot analysis, 2 µl of the PCR products were electrophoresed in triplicated 5% polyacrylamide gels with TAE buffer. Prior to transfer, gels were washed 10 min in 0,5 M NaOH/ 1 M NaCl and 10 min in 0,5 M Tris-HCl pH 7.5/ 1,5M NaCl and then electrotransferred in TAE buffer onto nylon membranes. Hybridizations were performed as described by dot-blot analysis except that the hybridization temperature was 60 °C. For sequencing of the amplified fragments of some ApMV isolates, the PCR products were extracted from 2% agarose gels and directly sequenced in both orientations by using VP77 and VP79 primers with and automated DNA sequencer (ABI PRISM 377, Perkin Elmer).

RESULTS

Multiple non isotopic molecular hybridization detection.

Total RNA prepared from singly infected *Prunus* hosts with ApMV, PDV or PNRSV and their five-fold serial dilutions were applied onto four nylon membranes (Figure 1A). The first three membranes were hybridized separately with specific digoxigenin-labeled riboprobes, while the remaining membrane was treated with a mixture of the three riboprobes (multiple hybridization).

In single hybridizations, specific signals were clearly observed trough dilutions of 5^{-6} for PDV and 5^{-5} for ApMV and PNRSV, corresponding to the equivalent of 0.5 μg and 0.1 μg, respectively, of infected leaf tissue. No signal was detected in the non-related infected extracts (Figure 1A). The simultaneous detection of the three ilarviruses when the riboprobes were mixed within the hybridization solution showed the same sensitivity limit as observed in the single hybridizations (Figure 1A). Therefore, based on these results, multiple hybridization procedure could be used for the detection of any of these three viruses in a *Prunus* host.

In order to simplify the extraction procedure, tissue extracts from the same infected samples used in Figure 1A were prepared by a very fast method as previously described for PNRSV and other plant viruses (23). Although the specificity of the procedure was as good as total nucleic acids extraction method, the sensitivity was decreased up to 125 times (Figure 1B). However, regardless of the sample preparation method used, the end point dilutions obtained in the multiple hybridizations were similar to those obtained in the single hybridizations, indicating the suitability of this easy procedure for the routine simultaneous detection of the three viruses.

Multiplex RT-PCR.

With the aim of setting up a procedure that would allow the simultaneous detection of the three ilarviruses affecting stone fruit trees by a PCR-based method, an antisense degenerate primer was designed to the 3' end of their RNAs 3 and 4 (Table 1). This degenerate primer was designed to avoid the need to include three different primers in the reverse transcription reaction. Virus specific sense primers were then made to be used for the unequivocal identification of each of the three viruses (Table1).

As a first step in determining the versatility of the degenerate primer, the same total RNA extracts used in the molecular hybridization experiments obtained from *Prunus* hosts individually infected with ApMV, PDV or PNRSV were analyzed by RT-PCR. PCR products of the expected size were obtained for PNRSV (356 bp) and PDV (517 bp), whereas for ApMV a PCR product of a slightly smaller size than the expected one (390 bp vs 417 bp) was obtained (Figure 2). Both molecular hybridization experiments and southern blot analysis confirmed the validity of the amplified segment to be of an isolate of ApMV (see below). No interfering bands were obtained indicating that the degenerate antisense primer was able to recognize the genome of all three ilarviruses tested. No PCR products were observed in the healthy control sample (Figure 2, lane 4).

We then addressed the question of using a cocktail of all four primers to check the method's efficiency confronted with the three possible single infections, three simulated double infections and a simulated triple infection. Virus-specific bands, which did not vary in the concentration or in the expected

size with respect to the products obtained in the case of single infections (Figure 3, lanes 1 to 3), were clearly detected in all combinations. A large molecular weight product was obtained from healthy sample (Figure 3, lane 8) which was absent in the infected samples, but this did not interfere with the interpretation of the results.

Detection limits for the cocktail of four primers in both single and simulated triple infections were determined by making five-fold serial dilutions of the corresponding total RNA extracts. In single infections, ApMV and PNRSV specific bands were detected up to 5^{-8} dilution whereas the PDV specific product was detected up to 5^{-10} dilution, corresponding to 20 ng and 0.8 ng of infected leaf tissue, respectively (data not shown). The same detection limits were obtained when the three viruses were amplified in the multiplex RT-PCR reaction (Figure 4). According to these results the technique was at least 125 times more sensitive than multiplex non-isotopic molecular hybridization.

To further evaluate the efficiency of this multiplex RT-PCR technique, leaf samples were collected from the trees grown in the field, which were suspected to be naturally infected with one or combinations of these three viruses. Total RNAs were extracted from leaves of 42 trees including 5 different *Prunus spp* (almond, apricot, cherry, peach and plum) and subjected to the multiplex RT-PCR procedure. Some of the results are shown in figure 5, representing most of the potential combinations. Single infections are shown in eight lanes. Interestingly two PCR bands of different size account for the detection of ApMV, the one described to represent the reference virus isolate in this work (Fig. 5A; lanes 1 and 2) and the one expected from the previously

characterized isolate (417 bp; Fig. 5A; lane 3) (28). To verify that the PCR products obtained were from the expected viruses, representative samples of the different combinations, including the two ApMV PCR bands differing in size (Fig 5A; lanes 2 and 11 vs 6 and 17) were analyzed by southern-blot hybridization by using the corresponding virus-specific riboprobes (Fig. 5B). Hybridization signals were obtained in the expected lanes, including those of the ApMV isolates that gave remarkable size heterogeneity. To study the origin of this size heterogeneity, these two PCR products were gel purified and their primary structures determined. As can be observed in figure 5C, a 28 nt deletion was present in the sequence of isolates that gave a band with faster electrophoretic mobility. Double infections of the type PDV-PNRSV (Fig. 5A; lanes 4, 5, 7, 8, 9 and 13) and ApMV-PNRSV (Fig. 5A; lanes 11 and 17) were clearly discriminated. A natural triple infection was also detected (lane 6). These results indicated that the technique described here is quite reliable and could be used for testing field samples. The detection limit for the triple infected tree sample was 5^{-3} for PNRSV and ApMV and 5^{-2} for PDV (data not shown). These limits were clearly lower than those observed in the simulated triple infection (Fig. 4). More triple infections need to be analyzed to know the real detection limits of this technique in these exceptional cases. It is important to note that when total RNA from cherry samples were used, no PCR bands were detected unless a specific treatment for eliminating polysaccharides (18) was carried out and PVP was incorporated in the PCR buffer (12) (not shown).

DISCUSSION

The present work demonstrates the success obtained in simultaneously

detecting three ilarviruses (PNRSV, PDV and ApMV) in tissue from fruit trees by non-isotopic molecular hybridization and a modified multiplex RT-PCR procedure, the latter having the additional advantage of allowing the identification of each virus in a single test.

When total nucleic acid extracts from simulated triple infections were analyzed by non-isotopic molecular hybridization, it was observed that the sensitivity limits were identical to those obtained in the corresponding single analyses. This result was consistent with previous reports in which this strategy has been successfully applied: to the phytosanitary certification of tomato plants (22) and to the large scale evaluation of the sanitary status of carnation mother plants (24). Although the use of sap extracts in the simultaneous detection of the three ilarviruses resulted in a 5-25 times reduction in sensitivity limits, this easier extraction procedure could facilitate handling of a significantly larger number of samples and should be considered in routine diagnostic procedures.

PCR-based technology has been shown to be several-fold more sensitive than ELISA and nucleic acid hybridization (10,19). In addition, it has recently been shown that this technology allows the simultaneous amplification of more than one locus in the same reaction (9). There are still few examples showing its application to simultaneously detect more than two viruses. Some of them include the detection of five seedborne legume viruses (3) and of three viruses infecting globe artichoke (7). In regard to stone fruit viruses a maximum of two different viruses have been detected by different variations of the multiplex RT-PCR. Examples include the simultaneous detection of ACLSV and PPV (16), PNRSV and PPV (11) or PNRSV and ApMV (4). This strategy has also been applied to differentiate two biologically distinct groups of PNRSV

isolates from cherry (8). In our study, we have shown that an antisense degenerate primer and three virus-specific sense primers can be used to simultaneously detect (and discriminate between) the three characterized ilarviruses affecting stone fruit trees. To design the PCR-primers we took advantage of the high degree of sequence identity at the 3' end of RNA 3 in the three viruses (26). It is well known in primer design that the 3' end is more important than the 5' end, and although the antisense primer has five degeneracies, the last eight bases at the 3' end were identical in all three viruses. When using a multiplex PCR, there are several critical parameters that significantly influence the yield and quality of the different loci (9). In general, raising the buffer concentration to 2X improves the efficiency of the multiplex reaction. In the case of PDV, however, this modification led to the almost total disappearance of the product (data not shown), probably due to the fact that longer products are harder to denature. In addition, in contrast to previous recommendation (9), lowering the annealing temperature by 4-6ºC in the multiplex mixtures was not necessary for amplification of the three different targets. It was assumed that, in our case, the established conditions for single RT-PCR worked well enough for the multiplex RT-PCR. This can be explained by: (i) only one antisense degenerate primer was required, (ii) the virus specific sense primers were designed in such a way that they have identical melting temperature and, (iii) the size differences of the amplified products were not so significant as to require any modification of the critical parameters.

Another important feature of the multiplex RT-PCR procedure described here is that it works well with all the isolates tested in this work. In the case of PNRSV, the sense primer was designed within a region that was strictly

conserved in the fifteen PNRSV isolates previously characterized (1). Concerning PDV and ApMV no sequence variability data were available to use in designing sense primers but the primers were able to amplify all the field isolates tested and verified their efficiency. In this respect, from the results obtained in the testing of field samples, it can be concluded that ApMV isolates can be present in significant size variability at the last 400 nt of their RNAs 3 and 4. Two main different PCR products were observed among all the ApMV isolates analyzed in this work. Sequence analysis allowed us to determine that the isolates that gave a faster migrating PCR product presented a 28 nt deletion in their 3'-UTRs. Interestingly, secondary structure analysis of the deleted sequence revealed that the deleted sequence could be folded in a very stable stem-loop structure.

In summary, the multiplex RT-PCR procedure described here could be a very useful method for saving time and costs during indexing of the three characterized ilarviruses that affect stone fruit trees. It may also be used in the testing of mother plants for these viruses in certification programs and particularly for testing CAC (Conformitas Agraria Communitatis) propagating material, which is already compulsory for EU countries.

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Figure 1. Detection of PNRSV, ApMV and PDV in infected tissue by non-isotopic molecular hybridization in single (top six membranes) or simultaneous (bottom, mixed probes) assays. Total RNA (A) and clarified sap extracts (B) from stone fruit leaves individually infected with ApMV, PDV or PNRSV and their five-fold serial dilutions were applied onto nylon membranes in the rows indicated at the left of the figure. The membranes were hybridized using the individual or a mixture of the three different RNA probes (mixed-riboprobes) in the hybridization solution. Numbers at the bottom of figure represent the dilutions performed on the original undiluted sample.

Figure 2. RT-PCR products obtained from total RNA extracted from stone fruit leaves individually infected with PNRSV (lane1), ApMV (lane 2) or PDV (lane 3). The RT-PCR reactions were performed with the common antisense degenerate primer and the specific sense primer for each ilarvirus and electrophoresed in 2% agarose gel. Arrows indicate the virus-specific amplified bands corresponding to 358 bp for PNRSV, 417 bp for ApMV and 517 for PDV. Lane M: Ladder 1 Kb marker; Lane 4: RT-PCR products obtained from total RNA extracted from healthy sample.

Figure 3. Evaluation of multiplex RT-PCR technique carried out with a cocktail of the four primers (one degenerated antisense primer and three virus-specific sense primers). Total RNA extracted from the individually infected samples and mixtures of these extracts simulating the three double infections and the triple infection were amplified with the four primers cocktail and the products obtained were analyzed in 5% PAGE. Arrows indicate the virus-specific bands. Lane M: Ladder 1 Kb marker; Lane 1: PNRSV infected sample; Lane 2: ApMV infected sample; Lane 3: PDV infected sample; Lane 4: simulated PNRSV-ApMV infected sample; Lane 5: simulated PNRSV-PDV infected sample; Lane 6: simulated ApMV-PDV infected sample; Lane 7: simulated PNRSV-ApMV-PDV infected sample; Lane 8: healthy sample; Lane 9: water control;

Figure 4. Sensitivity limits of multiplex RT-PCR technique in simulated triple

ilarviruses infection. Five-fold serial dilutions of a mixture of total RNA from individually infected samples with ApMV, PDV or PNRSV were made with healthy extract and amplified by single reactions with the four primers cocktail. The products obtained were analyzed in 5% PAGE. The specific bands were detected up to 5^{-8} dilution for ApMV and PNRSV and 5^{-10} dilution for PDV. Numbers at the top of figure represent the dilution performed on the original undiluted sample. Lane M: Ladder 1 Kb marker; lane H: healthy sample.

Figure 5. Representative results obtained by multiplex RT-PCR technique for the ilarviruses detection in field tree samples. Total RNA from 42 stone fruit *Prunus* ssp which were thought to be naturally infected with one or more of the three ilarviruses were analyzed with the four primers cocktail and electrophoresed in 5% PAGE (A). M: Ladder 1 Kb marker; C: simulated triple infection used as positive control; H: healthy sample; Lanes 1 to 17: representative results obtained from 42 *Prunus ssp* samples analyzed. Some of the samples analyzed in (A) covering the triple (lane 6), double (lanes 7 and 11) and single (lanes 2, 14 and 15) infections were electrophoresed in triplicate and analyzed by southern-blot hybridization with virus-specific riboprobes (B). In the case of ApMV, samples that gave different PCR bands with different electrophoretic mobilities (see text) were included in this analysis. In (C) the nucleotide sequence of the PCR products corresponding to ApMV isolates of lanes 2 and 17 was determined and compared to the reference isolate (ApMV-A; 28). The region causing the differences in the electrophoretic mobilities of ApMV isolates is boxed. Nucleotide residues that can be folded in a very stable stem-loop structure are in italic. The stop codon for the coat protein cistron is in bold. Dots indicate identical residues. Dashes denote gaps in the sequence.

TABLE 1. Sequence of degenerate antisense and virus-specific sense primers used in the multiplex RT-PCR reaction.

Degenerate antisense primer	Specific sense primers				Size of amplified product
	Sequence	Virus	Code	Location within the RNA 3	
VP77[a] T TT AG 5'-GCCTCC T GGGGCATC-3' A CA T T	5'-CGTCGAGGAAGTTTAGGTTG-3'	ApMV	VP79	from 1638 to 1656 nt	417 bp
	5'-CAACGTAGGAAGTTCACAG-3'	PDV	VP80	from 1610 to 1628 nt	517 bp
	5'-GAACCTCCTTCCGATTTAG-3'	PNRSV	VP78	from 1586 to 1599 nt	356 bp

[a]VP77 primer corresponds to the complementary sequence of the last 19 nucleotides of PNRSV, ApMV and PDV.