DEVELOPMENT OF MOLECULAR TESTS FOR THE DETECTION OF ILAR AND LATENT VIRUSES IN FRUIT TREES

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ABSTRACT

The detection throughout the year of latent and ILAR viruses in fruit tress by classical serological tests appear to be unreliable. We have developed RT-PCR tests for a reliable detection of latent and ILAR viruses in fruit trees. These assays were then simplified to allow the direct use of crude plant extracts instead of total RNA preparations, and the analyses of pooled samples. In this way, such RT-PCR protocols are suitable for a routine diagnosis of latent and ILAR viruses in fruit tree certification.

INTRODUCTION

Latent and Isometric Labile Ringspot (ILAR) viruses are important pathogens of fruit trees in temperate climates. The most common latent viruses of pome fruits include *Apple Chlorotic Leafspot virus* (ACLSV), *Apple Stem Pitting Virus* (ASPV) and *Apple Stem Grooving Virus* (ASGV). The main ILAR virus affecting stone fruits are *Prune Dwarf Virus* (PDV), *Prunus Necrotic Ringspot Virus* (PNRSV) and *Apple Mosaic Virus* (ApMV). This latter virus can also infect apple trees. These viruses can cause fruit yield losses and affect fruit maturity or tree growth of many commercial *Malus* and *Prunus* species.
The infections caused by latent and ILAR viruses are frequently symptomless. They pose a special risk for the movement of germplasm, by accidentally introducing the virus along with the host plant. The use of virus-free vegetatively propagated planting material is essential (1) for the distribution of germplasm for breeding and evaluation programs and (2) for disease control and management. Therefore a systematic detection of infected plants from basic stocks is required. Thus, routine tests have to be suitable for a reliable and quick detection of these viruses and compatible with analysis of a large number of samples.
ILAR and latent viruses are usually found in low concentrations in infected plants. ELISA tests lack sensitivity for detecting low virus concentrations in infected materials. Molecular methods, based on the detection of specific sequences of nucleic acids, are highly sensitive and could provide a mean of reliable pathogen detection in routine testing. However, the classical protocols generally rely on labour intensive extraction of the nucleic acids. Molecular tests developed in this study were thus simplified to allow the direct use of crude plant extracts more suitable for routine tests.

MATERIALS AND METHODS

Plant material

Orchard trees (apple, pear, plum and cherry) from the collections of the Plant Pathology Unit of Gembloux Agricultural University (FUSAGx), Belgium, and the Gembloux Center of Agronomical Research (CRAGx), Belgium, have been tested. Two one-year-old branches per tree were collected in winter. Bark or leaves tissues were removed on different parts of these branches to minimise the effects of potential uneven virus distribution.
Twigs of infected trees (GF 305 peach seedlings and orchard trees) were kindly provided by B. Pradier (SQL, Lempdes, France), R. Guillem (LNPV, Villenave d'Ornon, France), T. Candresse (INRA, Villenave d'Ornon, France), T. Malinowski (RIPF, Poland), K. Petrzik (IPMB, Czech Republic) and J.C. Desvignes (CTIFL, Lanxade, France).

Total nucleic acid extraction

Total RNA were extracted from 0.4 g of bark or leave tissues of fruit trees, according to the method described by Spiegel *et al.* (1996) with slight modifications.

Preparation of crude plant extracts

0.2 g of leaf or bark tissues of fruit trees were ground with 2 ml of a buffer developed by Spiegel *et al.* (1996) (1.5% SDS, 300mM LiCl, 10mM EDTA, 1% Na déoxycholate, 1% Nonidet P40). Leaf extracts were centrifugated at 10000 *g* during 5 min. Different dilutions were prepared : 10x, and 100x. The clarified diluted extracts were kept at 4°C. Two µl of each extract was used for the RT-PCR test.

Primer design

Specific primers for PDV, PNRSV, ApMV, ACLSV, ASGV and ASPV were identified by computer analysis using the PILEUP, FASTA and PRIME programs. These programs were applied to sequence data available in EMBL and GenBank databases as well as partial sequences obtained in the laboratory. The specificity of primers was previously demonstrated by RT-PCR reactions applied to total RNA preparations from trees or herbaceous hosts infected with different reference isolates (Kummert *et al.*, 2001).

RT-PCR amplification

RT-PCR amplifications were performed using the One Step RT-PCR Kit from Qiagen (Hilden, Germany). This kit allows reverse transcription and amplification to be performed in a single tube.
Twenty five µl of RT-PCR reaction mixture containing total RNA, 0.6 µM of both primers and the reagents from the kit (buffer, dNTP mix, Enzyme Mix) were submitted to cDNA synthesis (30 min at 50°C), activation of the Hot-

Start Taq polymerase (15 min at 95°C) and 40 amplification cycles (15 s at 95°C, 45 s at 55°C and 1 min at 60°C) and final extension (10 min at 72°C). To ensure the absence of contamination, each RT-PCR run included a water control. Total RNA preparations from a virus infected plant were included as positive controls in each RT-PCR run. Total RNA preparations of a virus-free cherry tree and a virus-free apple tree, which were tested periodically by RT-PCR using the same primers during the preceding two years, were included as healthy controls in each RT-PCR run. All RT-PCR experiments were repeated three times giving reproducible results.

Agarose gel electrophoretic analysis

Aliquots (8 µl) of end amplification products were submitted to electrophoresis in ethidium bromide stained agarose gel (2%), in 1 x TAE buffer (40 mM/l tris-acetate, 1mM/l EDTA, pH 8.0).

RESULTS

Detection of isolates from total RNA preparations

For PDV, PNRSV, ApMV, ACLSV, ASGV and ASPV, the RT-PCR assay allowed the discrimination of virus-free and virus-infected trees. Figure 1 shows an example of the results obtained with total nucleic acid preparations from a set of isolates of PNRSV, two PDV isolates, and one ApMV isolate.

Detection of isolates from crude plant extracts

The test using crude extracts from the same samples gave results comparable to those obtained by the test using total RNA (Figure 2). For each virus, the specific signal was observed for the dilutions used (10x, 100x, 1000x). For the latent viruses, the higher intensity of the signal was observed for sap diluted 1000x. For the sap diluted 10x and 100x, the signal was rather weak thus suggesting the activity of potential inhibitors of the RT-PCR reaction when the sap is not diluted sufficiently (Figure 2). For the ILAR viruses, the higher intensity of the signal was observed for sap diluted 100x (unpresented results).

Analysis of pooled samples

Bark or leaves were taken from both infected and healthy fruit trees. The extract was constituted in such a way that one volume of extract from an infected sample was mixed with 19, 39 and 79 volumes of extracts from healthy trees. For each virus, the infected sample was detected even its extract was diluted 80 times with extracts of healthy plants. Figure 3 illustrated these results in the case of crude extracts of leaves of an apple tree infected by ASPV.

DISCUSSION

Over recent years, the use of molecular methods for the detection of plant pathogens has been extensively investigated. Despite these efforts, the routine use of these methods in diagnostic laboratories has been limited. In particular where mass-scale testing is required, the problems of labour-intensive preparation of nucleic acid extracts have made molecular methods difficult to implement.

RT-PCR tests have been developed for the detection of latent and ILAR viruses in fruit trees. These tests were initially validated by using total RNA preparations. With such assays, all the isolates of various geographical origins and host plants were detected. These tests were then simplified for direct use of diluted crude plant extracts. Samples were ground in an extraction buffer, diluted and added directly into the PCR mix. The inhibitory effects of plant polysaccharides or phenolic compounds of crude plant extracts on PCR amplification were avoided by their dilutions in the buffer. The results obtained from crude barks or leave tissues are similar to those obtained from total RNA preparations. These rapid and easy tests allow a very reliable detection of these viruses directly from leaves or bark tissues throughout the year.

For each studied virus, the infected sample can be detected even if its crude extract was diluted 80 times in extracts from healthy plants. These tests are thus compatible with the processing of pooled samples. The use of pooled samples allows to test an increased number of samples without dramatically increasing the cost of the tests. It can thus also bring a positive solution to sanitary control procedures by increasing the statistical reliability of the obtained results.

The assays developed here can be performed within 24 hours and can be particularly useful for large-scale applications where sensitivity, reliability, and speed are required. They are suitable for a routine detection of ILAR viruses and latent viruses in fruit trees. Such assays could be developed for the detection of other plant viruses present in latent infections and involved in the sanitary programs.

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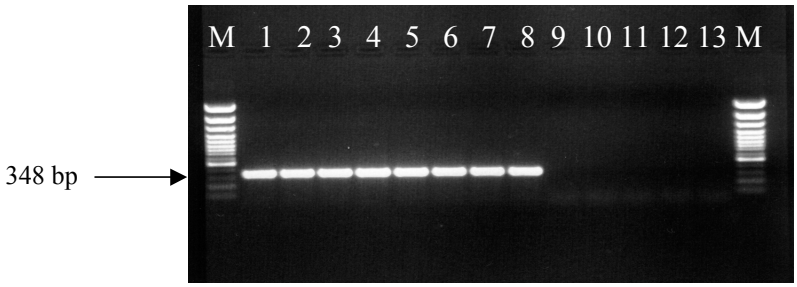

Figure 1. Agarose gel analysis of RT-PCR assays using the PNRSV primers

Lane 1-8 : RT-PCR products of total nucleic acid extracts of identified PNRSV isolates (1, 2, 3, 4, 5, 6, 7, 8) grafted on peach twigs.
Lanes 9-10 : RT-PCR products of total nucleic acid extracts of standard PDV isolates.
Lane 11 : RT-PCR products of total nucleic acid extracts of ApMV standard isolate.
Lane 12 : RT-PCR products of total nucleic acid extracts of healthy tree.
Lane 13 : water control.
M : 100 bp DNA ladder (GibcoBRL).

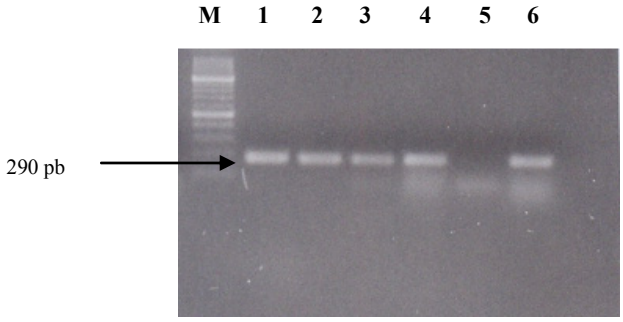

Figure 2. Agarose gel analysis of RT-PCR assays using the ACLSV primers

Lane 1 : RT-PCR products of total nucleic acid extracts of identified ACLSV isolate grafted on peach twigs.
Lane 2 : RT-PCR products of crude plant extracts diluted 10x. Lane 2: RT-PCR products of crude plant extracts diluted 10x.
Lane 3 : RT-PCR products of crude plant extracts diluted 100x.
Lane 4 : RT-PCR products of crude plant extracts diluted 1000x.
Lane 5 : water control.
Lane 6 : positive control
M : 100 bp DNA ladder (GibcoBRL).

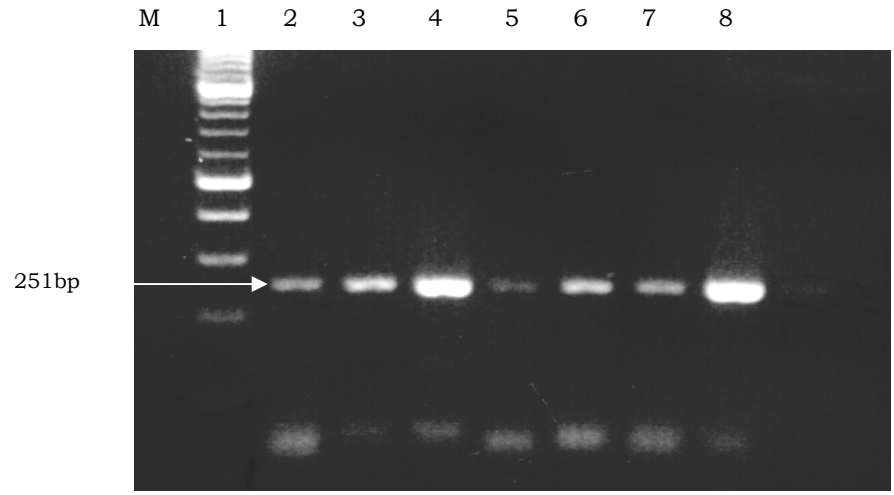

Figure 3. Detection of RT-PCR products from crude plant extracts from leaves of two ASPV- infected trees (Tree n°1 and Tree n°2).

Tree n°1
Lane 1 : extract diluted 80 times with extracts from healthy tree
Lane 2 : extract diluted 40 times with extracts from healthy tree
Lane 3 : extract diluted 20 times with extracts from healthy tree

Tree n°2
Lane 1 : extract diluted 80 times with extracts from healthy tree
Lane 2 : extract diluted 40 times with extracts from healthy tree
Lane 3 : extract diluted 20 times with extracts from healthy tree
Lane 7 : undiluted extracts from ASPV-infected leaves (tree 1)
Lane 8 : water control
M : 100 bp DNA ladder (GibcoBRL).