Development of RT-PCR Tests for the Routine Detection of Latent and ILAR Viruses in Fruit Trees

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Abstract

The detection throughout the year of latent and ILAR viruses in fruit trees by classical serological tests appears to be unreliable. Recently, these problems have smoothed themselves out by the use of molecular methods. We have developed RT-PCR protocols which are simple and reliable for routine detection of these viruses throughout the year.

Keywords: ACLSV; ASPV; ASGV; PDV; PNRSV; ApMV; fruit trees; RT-PCR; RT-PCR-ELOSA; Minor Groove Binder-DNA probe

INTRODUCTION

Latent and Isometric LAbile Ringspot viruses (ILAR) viruses are important pathogens of fruit trees in temperate climates. The most common latent viruses of pome fruits include *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV). The main ILAR viruses affecting stone fruit consist in *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV). The detection of these six viruses is currently an important component of many phytosanitary certification programs of fruit trees. Biological detection methods for these viruses were time-consuming and labor intensive. ELISA tests were much more rapid but appeared reliable only during a short period of the year because they lacked sensitivity for detecting low virus concentrations in woody material. Molecular techniques, such as Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR), have been successfully developed for a more sensitive detection of these viruses. However, the classical RT-PCR protocols relied on labor intensive extractions of nucleic acids and ethidium bromide-stained agarose gels (KUMMERT *et al*. 1995). These steps have hampered the routine use of RT-PCR techniques.

The simplification of the (RT)-PCR protocols for routine detection has thus to meet different requirements: (1) the reduction of the risk of contaminations linked to the manipulations of a large number of samples, (2) the simplification of samples preparation, and (3) the use of alternative detection protocols of the amplified products.

RESULTS

The samples processing protocols

Template preparation represents a critical step for routine use of PCR technology. PCR assay was simplified using crude plant extracts (MARINHO *et al*. 1998; KUMMERT *et al*. 1998, 2000, 2001). Samples (bark or leaves) 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 dilutions in the buffer. The detection step was based on agarose gel electrophoresis. This rapid

and easy test validated for ASGV (MARINHO *et al.* 1998) permits a very reliable detection of this virus directly in leaves and in bark tissues throughout the year.

A colorimetric detection of amplification products

We focused on an other type of detection of amplified products, based on a convenient microplate colorimetric assay. We have successfully developed RT-PCR-ELOSA detection protocols using sandwich hybridization of specific PCR-amplified products to a phosphorylated capture probe and a biotin-labeled detection probe. This process was followed by a colorimetric reaction on microplates. This test performed with crude extracts was validated for ACLSV, ASGV, ASPV, PDV and PNRSV (KUMMERT *et al.* 2000, 2001). For ASGV, the ELOSA detection was more sensitive than electrophoresis (MARINHO *et al.* 1998).

Real-time RT-PCR assay

To eliminate the post-amplification manipulations, we have developed a real-time fluorescent RT-PCR system. For viruses with high genome variability such as latent and ILAR viruses, it was necessary to target a small conserved cluster of nucleotides among the different isolates. For this, fluorogenic hydrolysis probes (of ca. 12–17 nucleotides) conjugated to a minor groove binder (MGB) were used. The covalent attachment of the minor groove binder moiety at the 3' end of the probe increased the probe/target duplex stability and raised the melting temperature to a range suitable for real-time analysis. For each virus studied (ASPV, ACLSV, PDV, PNRSV, ApMV), MGB probes were defined within conserved regions of the genome based on available information and on sequencing of field isolates. The five MGB probes were tested on a broad spectrum of isolates (reference isolates and field isolates) of various geographic origin and host plant. For ACLSV (SALMON *et al.* 2002a) and PNRSV (MARBOT *et al.* unpublished data), the real-time assay amplified, from total RNA, all the isolates tested (56 isolates for ACLSV and 61 for PNRSV). For these two viruses, this method was demonstrated to be at least as sensitive as gel analysis. For PDV, ASPV (SALMON *et al.* 2002b) and ApMV, we can not detect all isolates with our MGB probes. For PDV, the comparison of the sequence of the MGB probe tested so far with the corresponding sequence of these isolates revealed a mismatch in the middle of the nucleotidic sequence.

DISCUSSION

As shown by the two first tests, the ability to detect latent and ILAR viruses during both active growth and the dormant season from crude extracts will provide a valuable tool for certification programs. However the post-detection step based on agarose gel electrophoresis is cumbersome and time-consuming. The discrimination of bands in gel electrophoresis can be particularly difficult especially at the limits of detection. Therefore, until now, running of gels can not be automated. Precast agarose gels overcome these drawbacks and provide an alternative tool for fast, convenient and automated DNA electrophoresis. When combined to a detection with precast gels, RT-PCR test will be a means to detect all six viruses in routine in fruit trees. The use of colorimetric detection of labeled PCR products on microtitre plates obviate the need to run gels. Colorimetric PCR uses similar reading equipment as ELISA, which is an advantage. This technique which allows high throughput of samples using a plate format can be also used in testing laboratories for detection of latent and ILAR viruses.

However all these post-PCR manipulations increase the risk of carry-over contaminations and false positive results. The use of in-tube fluorescent detection eliminates the need for detection step. The closed-tube format used by TaqMan also removes the major source of PCR contamination, namely PCR products aerosols created during post-PCR manipulations. This reduces the risk of false positive results. Interpretation of the data in real-time is therefore easier than the detection on agarose gel. This assay is considerably less subjective, leaving the fluorescence detector to denote a significant increase in fluorescence over background, rather than visual comparison of DNA bands on a gel. Therefore, the RT-PCR protocol is simple and rapid, allowing analysis of up to 96 samples in a standard format. This test provides a means to apply PCR technology to a large number of samples for detection of viruses. We recommend then the utilization of MGB probes for early and opportune detection of PNRSV and ACLSV in fruit trees, throughout the year.

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