Development of RT-PCR Assays Using Fluorogenic 3' Minor Groove Binder DNA Probes for Detection of Fruit Tree Viruses

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Abstract

In phytosanitary certification, there is currently a need for the development of reliable, sensitive and rapid tests for the routine detection of ilarviruses and latent viruses from fruit trees during the dormant season. We have here developed real-time RT-PCR assays that allow the reliable detection of *Prunus necrotic ringspot virus* (PNRSV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV) in bark tissues of dormant wood. These assays are well adapted for the routine detection of these three viruses because they eliminate one risk of contamination by performing the whole test in a single closed tube.

INTRODUCTION

Latent viruses and Isometric Labile Ringspot (ILAR) viruses are important pathogens of fruit trees in temperate climates. These viruses cause fruit yield losses and affect fruit maturity or tree growth of many commercial *Malus* and *Prunus* species. Consequently, detection of these viruses is currently an important component of many phytosanitary certification programs for fruit trees.

Serological methods such as ELISA appear reliable only during a short period of the year because they lack sensitivity for detecting low virus concentrations in dormant woody material sampled in winter. Molecular technique such as reverse transcriptase-polymerase chain reaction (RT-PCR) can surpass ELISA in sensitivity for detection of *Prunus necrotic ringspot virus* (PNRSV) in dormant fruit trees. However, this method generally requires agarose gel electrophoresis analysis for its interpretation. The post-amplification manipulations increase the risk of carry-over between samples and contamination that can subsequently lead to misdiagnosis. This post-detection step has hampered the routine use of the RT-PCR technique.

The real-time detection of specific amplification products using hydrolysis probes is particularly useful because eliminates the need for post-PCR processing steps. The fluorogenic 5'-nuclease assays are based on the real-time measurement of fluorescence resulting from the hydrolysis of a fluorescent probe (TaqMan® probe) that hybridizes specifically to the target PCR product during amplification. For viruses with high genome variability, such as latent and ILAR viruses, it is necessary to target a small cluster of conserved nucleotides shared among the different isolates. For this, the covalent attachment of a minor groove binder (MGB) moiety at the 3' end of the fluorogenic hydrolysis probe increased the probe/target duplex stability and raised the melting temperature to a range suitable for real-time analysis (Kutyavin et al., 2000).

We describe here the development of real-time RT-PCR assays using MGB probes for detection of PNRSV, *Apple Chlorotic Leaf Spot Virus* (ACLSV), *Apple Stem Pitting Virus* (ASPV), *Prune Dwarf Virus* (PDV) and *Apple Mosaic Virus* (ApMV).

MATERIALS AND METHODS

Plant Material

Orchard trees (apple, pear plum and cherry) from the collections of the 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 tissues were removed on different parts of these branches to minimize 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) and K. Petrzik (IPMB, Czech Republic). Twigs of dormant wood collected from trees containing ASPV, ACLSV and ASGV in single or mixed infections were kindly provided by J.C. Desvignes (CTIFL, Lanxade, France).

Total Nucleic Acid Extraction

Total RNAs were extracted from 0.4 g of bark tissue, according to the method described by Spiegel et al. (1996).

Primer Design

Specific primers for PDV, PNRSV, ApMV, ACLSV 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 demonstrated by RT-PCR on total RNA preparations from trees or herbaceous hosts infected with different reference isolates (Kummert et al., 2001).

3' Minor Groove Binder Probe Design

The 3' minor groove binder (MGB) DNA probes were selected as described by Livak et al. (1996), on the basis of sequences aligned using the PILEUP program. The probes were designed within a conserved region of the genome based on available information and on sequencing of field isolates. They contained VIC, FAM or TET as reporter dye, covalently attached at the 5' end, and a quencher and the MGB moiety at the 3' end.

RT-PCR Amplification

RT-PCR amplifications were performed on the total RNA preparations using the OneStep RT-PCR Kit from Qiagen (Hilden, Germany). The kit allows reverse transcription and amplification to be performed in a single tube. The GeneAmp 5700 Sequence Detection System (Applied Biosystems, Forster City, USA) was used for thermal cycling. A 25 µl RT-PCR reaction mixture containing total RNA, 0.6µM of both primers, 0.2µM of the MGB probe and the reagents from the kit (buffer, dNTPs and enzyme) was submitted to cDNA synthesis (30 min at 50°C), activation of the HotStart *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).

To ensure the absence of contamination, each RT-PCR run included a water control. Total RNA preparations of a virus-free prune tree and a virus-free apple tree were included as healthy controls in each RT-PCR run. All RT-PCR experiments were repeated three times with reproducible results.

Analysis of Amplified RT-PCR Products

The real-time detection (real-time RT-PCR) was compared with the analysis of end amplification products by agarose gel electrophoresis (conventional RT-PCR) in order to evaluate the efficiency of real-time RT-PCR assays.

1. Real-time Detection. Fluorescence was recorded at the extension step of each amplification cycle using the GeneAmp 5700 Sequence Detection System. The threshold cycle (Ct) is the cycle at which a significant increase in fluorescence occurs. The Ct values were calculated using the 5700 SDS software.

2. Agarose Gel Electrophoresis. Aliquots (8 µl) of end amplification products were electrophoresed in an ethidium bromide stained agarose gel (2%), in 1X TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0).

RESULTS

Detection of Isolates by Conventional RT-PCR

For PDV, PNRSV, ApMV, ACLSV and ASPV, the conventional RT-PCR assay discriminated between virus-free and virus-infected trees. Either no band or a band corresponding to the expected size of the amplification product was observed on the agarose gel. For each virus, the number of different isolates tested positive is presented in Table 1. Figure 1 shows an example of the expected (348 bp) amplification product obtained by RT-PCR on total RNA preparations from bark tissues of 8 different trees infected by PNRSV.

Detection of Isolates by Real-time RT-PCR

For ACLSV (Salmon et al., 2002a) and PNRSV (Marbot et al., 2002), all the isolates detected by conventional RT-PCR were also detected by real-time RT-PCR (Table 1). An example of normalized fluorescence measurements obtained during the PCR amplification with total RNA from bark tissues of 6 trees infected with PNRSV is shown in Figure 2.

For ASPV, among 60 isolates tested, only 3 were not detected in the real-time RT-PCR assay compared to the conventional RT-PCR assay (Table 1). For two of them, sequence data analysis revealed the presence of mismatches between the probe and the corresponding target sequence (Salmon et al., 2002b).

For PDV and ApMV, the specific amplification products of more isolates were not detected with the selected MGB probes (Table 1). For PDV, amplification products from 9 infected trees that were not detected in the real-time assay were cloned and sequenced. The comparison of the sequence of the MGB probe tested so far with the corresponding sequence of these isolates revealed the presence of a mismatch in the middle of the nucleotidic sequence.

For ACLSV, ASPV and PNRSV, we used serial ten-fold dilutions of total RNA preparations from infected trees. The real time RT-PCR assay was at least as sensitive as conventional RT-PCR, with a threshold between 20 and 2 pg of total RNA (data not shown).

DISCUSSION

For PNRSV, ACLSV, ASPV, the real-time RT-PCR assay allowed detection of the isolates tested. The assays correlated well with agarose gel electrophoresis, which was used as the reference technique for the detection of amplification products. The real-time RT-PCR assays are thus particularly suitable for the detection of these viruses in dormant woody material.

To our knowledge, this paper is the first report of the use of fluorogenic probes for the detection of latent and ilarviruses in fruit trees. To overcome the effect of the potential variability of the viral genome, a short fluorogenic hydrolysis probe (of ca. 12-17 nucleotides) conjugated to a minor groove binder (MGB) was preferred to the usual TaqMan probes (24-40 nucleotides). The covalent attachment of the MGB moiety stabilized the probe/target duplex and raised the melting temperature to a range suitable for 5'-nuclease assays.

For PDV and ApMV, our real-time RT-PCR assay fails to detect all virus isolates with a single specific probe. For PDV, we demonstrated that only one nucleotide difference between the probe and the corresponding target sequence prevents the fluorogenic reaction to proceed and generates false-negative results. For ASPV, PDV, and especially for ApMV, very few published sequences are available. We recommend then to sequence RT-PCR products from many different infected trees. The alignment of these sequences with those already published may allow to identify other potential MGB probes in an highly conserved genomic region.

It should be noted that the real-time RT-PCR assay requires expensive, special equipment and additional reagents compared with conventional RT-PCR. On the other

hand, the real-time RT-PCR assay can be performed within 3 h, occurs in a single closed tube, does not require any post-PCR detection step and therefore reduces the risk of carry-over contamination and misdiagnosis in routine use. The real-time RT-PCR assays can be particularly useful for large-scale applications where sensitivity, reliability, speed and quantitative data are required, such as seed indexing, field surveys, identification of virus reservoirs, screening of germplasm for sources of resistance, disease forecasting, gene expression profiling and allele discrimination.

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Tables

Table 1. Comparison of the number of isolates detected by conventional and by real-time RT-PCR

	ACLSV	PNRSV	ASPV	PDV	ApMV
Number of isolates detected by conventional RT-PCR	43	79	60	52	32
Number of isolates detected by real-time RT-PCR	43	79	57	30	23

Figures

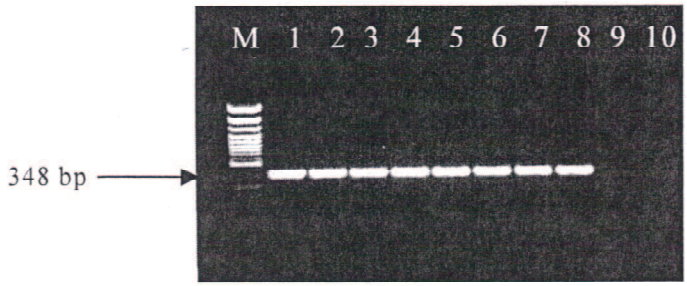

Fig. 1. Detection of 8 PNRSV isolates by conventional RT-PCR. Amplification of total RNA preparations from bark tissue followed by agarose gel electrophoresis (lanes 1-8). Lane 9, RT-PCR products of total nucleic acid extracts of a healthy prune tree. Lane 10, water control. Lane M, 100 bp DNA ladder (GibcoBRL).

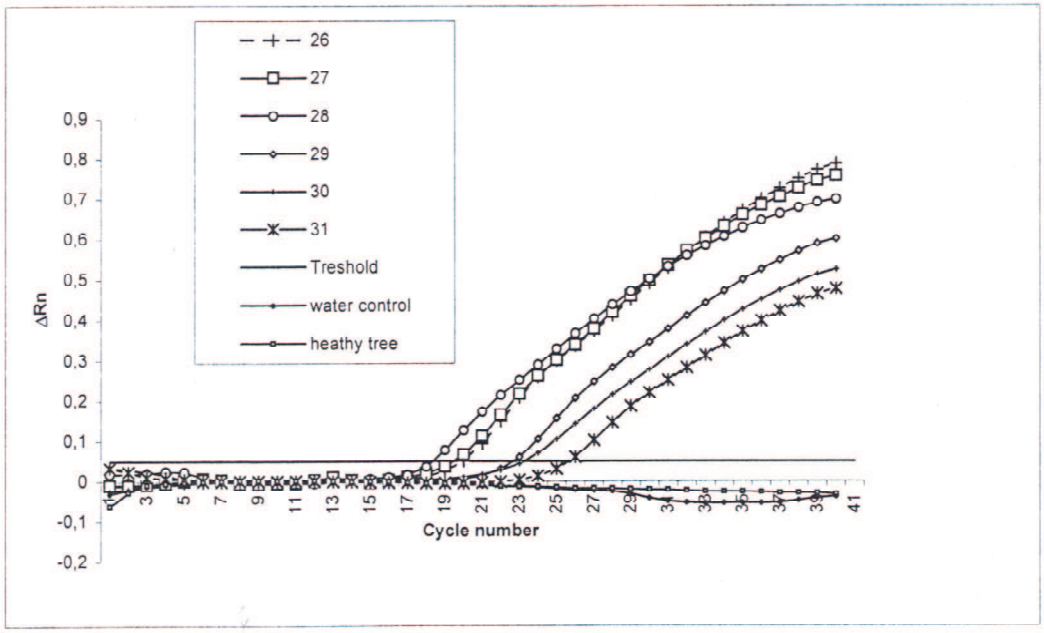

Fig. 2. Detection of 6 PNRSV isolates by real-time RT-PCR. Amplification of total RNA preparations from bark tissue of 6 PNRSV-infected trees (trees 26-31). Amplification plot showing cycle number versus normalized fluorescence (ΔRn). The horizontal line indicates the threshold.