Development of Routine Duplex RT-PCR Tests for Certification of Fruit Tree Multiplication Material

S. Massart, S. Roussel, J. Kummert, O. Dutrecq and M.H. Jijakli
Plant Pathology Unit
Agricultural University of Gembloux (FUSAGx)
Passage des Déportés 2
B-5030 Gembloux
Belgium

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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 of fruit trees throughout the year. The simultaneous detection of several viruses in a single PCR tube is a promising but challenging way which can reduce the cost of the analysis. So, we have developed three duplex RT-PCR protocols for the simultaneous detection of two viruses: ASPV-ASGV, ACLSV-ApMV and PDV-PNRSV. The development of a multiplex detection protocol requires numerous steps: adequate virus combination, optimisation of the PCR mix, limit of detection in comparison with the single detection protocol, adequate integration in the certification process, intra-laboratory validation. We described this step-by-step development of duplex protocols with special emphasis on *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV). The three duplex protocols were successfully validated in our laboratory. There are currently validated through a European ring-test including 10 partners.

INTRODUCTION

Latent viruses and Isometric labile Ringspot (ILAR) viruses are important pathogens causing yield losses and affecting tree growth and fruit maturity of fruit trees in temperate climates. The detection of these viruses is currently an important component of many phytosanitary certification programs for fruit trees.

Molecular tools, such as RT-PCR, have a great potential to detect viruses in a phytosanitary certification procedure. They may be rapid, sensitive, reliable and user-friendly. RT-PCR protocols for the individual detection of three ILAR viruses (ApMV, PDV and PNRSV) and three latent viruses (ASGV, ASPV and ACLSV) were previously developed (Kummert et al., 2000; Roussel et al., 2004).

Nevertheless, further efforts have to be undertaken to reduce the cost of RT-PCR for routine testing in a certification procedure. In this context, the simultaneous identification of several viruses in a single PCR tube (multiplexing) requires less time, labour and cost but represents a challenging way. The development of multiplex RT-PCR relies on numerous steps. First of all, the appropriate combinations of viruses for simultaneous detection have to be selected on the basis of four criterias: spatial distribution in the canopy, size of the amplicon, primer compatibility and occurrence in different hosts. In a second step, the PCR conditions have to be optimised and the duplex protocol compared to the individual detection of the viruses. Finally, the compatibility of the duplex protocol with a simplified method for sample preparation from leave or bark and with the sample pooling has to be studied.

Using the primer pairs previously published for the individual detection of viruses (Kummert et al., 2000; Roussel et al., 2004), we describe here the step-by-step development of duplex protocols for the simultaneous detection of fruit tree viruses with special emphasis on *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV).

MATERIAL AND METHODS

Plant Material

Orchard trees (apple, pear, plum and cherry) from the collection of the Gembloux agricultural University (FUSAGx) and the Walloon Center of Agronomical Research (CRA-W) were used. Twigs of infected trees were kindly provided by B. Pradier (SQL, Lempdes, France), R. Guillem (LNPV, Villenave d'Ornon, France), T. Candresse (INRA, Villenave d'Ornon, France), J.-C. Desvignes (CTIFL, Lanxade, France), T. Malinowski (RIPF, Poland) and K. Petrzik (IPMB, Czech Republic).

Sample Preparation

Total RNA was extracted from 0.4 g of leaf or bark tissue, according to the technique of Spiegel et al. (1996). Crude extracts were prepared as previously described using the Pollähne press (Kummert et al., 2003) and the Kaji buffer (DNAlis, Gembloux, Belgium). The crude extracts were diluted 100 times in water and 5 µl aliquots were added directly to the PCR mix. A healthy control sample was included in each sample preparation serie to control the absence of cross-contamination between samples during grinding with the Pollähne press.

RT-PCR Amplification

The One-Step RT-PCR kit (Qiagen, Hiden, Germany) was used for RT-PCR amplification. The primer pairs described by Kummert et al. (2000) and Roussel et al. (2004) were used for latent viruses detection and ilarviruses detection, respectively.

Detection of the Amplification Products

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

Bio-Informatic

The DNAMAN software version 5.2.2 (Lynnon Biosoft, Quebec, Canada) was used to evaluate the minimal free energy of complementarity between two primers specific to different viruses.

RESULTS

Virus Combination

Four criterias were used to select the appropriate virus combinations:

- Spatial distribution in the canopy. Previous experiments (Kummert et al., unpublished datas) suggested that ACLSV and ApMV have an uneven distribution on the canopy while ASPV, ASGV, PDV and PNRSV present an homogenous distribution.
- Size of the amplicon. After electrophoresis on agarose gel, the PDV amplicon (303 bp) was not distinguishable from the ApMV (312 bp) or ACLSV (290 bp) amplicons. Furthermore, the PNRSV and the ASGV amplicons had too close sizes, 344 bp and 348 bp, respectively, to be distinguishable on agarose gel.
- Free energy of the primer complementarity. The most appropriate duplex combinations, corresponding to the highest minimal free energy of primer complementarity, were: PDV with ASPV, ApMV or ACLSV; PNRSV with ASPV or ACLSV; ASGV with ASPV; and ApMV with ACLSV.
- Host range and certification schemes. Among the studied viruses, the certification schemes PM 4/27(1), PM 4/29(1) and PM 4/30(1) of the EPPO recommend to test the presence of: (i) ApMV, ACLSV, ASPV and ASGV on *Malus* sp.; (ii) ACLSV, ASGV, ASPV on *Pyrus* sp.; and (iii) ApMV, ACLSV, PDV and PNRSV on *Prunus* sp. (plum and cherry).

According to all these criterias, three duplex combinations were selected for simultaneous detection of viruses: ASPV-ASGV, PDV-PNRSV and ApMV-ACLSV.

Optimisation of the RT-PCR Mix

During most but not all the duplex PCR optimisation, the PCR mix has to be optimised. In our experiments, the concentration of two components of the mix, the $MgCl_2$ concentration and the PCR buffer concentration, was tested. The modification of these two parameters did not improve the PCR efficiency. Consequently, the PCR mix was not modified.

Compatibility with Simplified Protocol and Sample Pooling

A simplified protocol for sample preparation, corresponding to crude extract preparation, was tested with our duplex protocol. The use of 100X diluted crude extract instead of purified total RNA in the PCR mix allowed the detection of the virus by the duplex RT-PCR protocol starting from leaves or from bark tissue.

Limit of Detection of Duplex and Uniplex Protocols

The limit of detection of the duplex protocol was compared to those of the uniplex protocol of each virus. A ten-fold serial dilution (10^{-1} to 10^{-6}) of a 100X diluted crude extract preparation, contaminated by both viruses, was submitted to RT-PCR. The viruses were still detected with duplex and uniplex protocols after a 10^{-4} dilution of the 100X diluted crude extract (Fig. 1).

Intra-Laboratory Validation

The ASPV-ASGV duplex protocol was tested on 46 apple and pear trees infected by both viruses (13 samples), by one of the virus (22 samples) or by none of the viruses (13 samples). Some results are displayed in Figure 2. The results obtained by the duplex protocol had a 98% of correlation with those obtained previously with the uniplex protocol. The only discrepancies corresponded to two samples ASPV positive with the uniplex protocol and ASPV negative with the duplex protocol.

DISCUSSION

In this paper, we described the development and the intra-laboratory validation of RT-PCR duplex protocols for the simultaneous detection of fruit tree viruses. The general guidelines for developing a multiplex PCR assay (Henegariu et al., 1997) were followed. Moreover, some criteria's, specific to the detection of fruit tree viruses in a certification process, were added.

The objective of developing a duplex PCR protocol has to be considered from the beginning of the research. So, during previous experiments, the primer pairs were selected according to the general guidelines (GC content, length, melting temperature, self-complementarity, etc.), but also taking into account the possibility of simultaneous detection by multiplex PCR. So, three additional criteria's were used: (i) close melting temperature for all the primers, (ii) absence of unacceptable primer-primer complementarily between different primer pairs and (iii) similar, but discernable, length of the amplicons. An identical PCR program was designed for the individual detection of the tested viruses. The polyvalence and the specificity of the individual detection were further validated for the tested viruses. The development of a multiplex protocol may start only after this validation of the individual detection tests.

The first step in the development of a multiplex PCR protocol is the selection of virus combination. In this paper, we showed that this selection has to follow biological (distribution in the canopy, host range), legal (certification requirements) and technical (amplicon length, primer compatibility) criteria.

The second step corresponds to the optimisation of the PCR protocol. To facilitate this step, it is very important to have previously designed identical thermal cycles for individual detection. Moreover, the co-amplification of DNA fragments of similar but

discernable lenghts should be recommended as fragments of very different sizes, can have different amplification efficiencies. It is generally recommended to adjust the annealing temperature or the $MgCl_2$ concentration (Henegariu et al., 1997) to have optimal efficiency in multiplex amplification. For virus detection, these adjustments have to be carried out carefully as the modification of these parameters might influence the polyvalence or the specificity of the primers. During our experiments, neither the $MgCl_2$ concentration nor the PCR buffer concentration had a positive effect on PCR efficiency and virus detection.

We also demonstrated that the limit of detection of the duplex protocol was identical to those of the uniplex protocols and that 98% of the results were identical between both protocols. Furthermore, the duplex protocol was also compatible with a simplified method of sample preparation and with the sample pooling. The compatibility with these two criteria's is very important in order to reduce the cost of the certification process.

The following step will correspond to a European ring-test including 10 laboratories. This step will allow the inter-laboratory validation of these duplex protocols for the detection of the 6 studied viruses.

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Figures

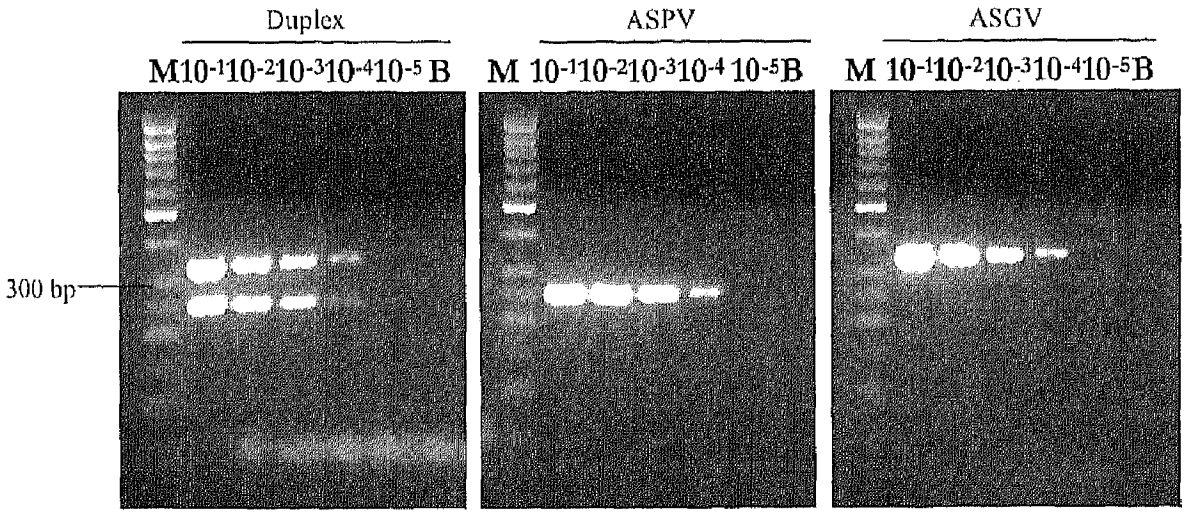

Fig. 1. Comparison of the limit of detection of the duplex and the uniplex protocol for ASPV and ASGV detection. Gel electrophoresis of PCR product amplified from a 100-fold serially diluted sample. M: molecular marker; B: blanc, 10^{-1} to 10^{-5}: dilution of the curde extract. Expected size for ASPV: 251 bp and for ASGV: 344 bp.

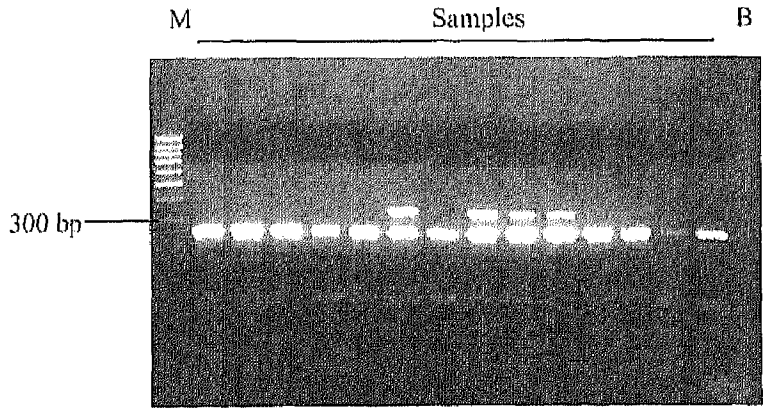

Fig. 2. Simultaneous detection of ASGV/ASPV viruses by gel electrophoresis of PCR products amplified from 14 trees through the duplex protocol for ASPV/ASGV. M: molecular marker; B: blanc. Expected size for ASPV: 251 bp and for ASGV: 344 bp.