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Simultaneous detection and differentiation of three viruses in pear plants by a multiplex RT-PCR

Bingyu Yao[a,b], Guoping Wang[a,b,c], Xiaofang Ma[b], Wenbin Liu[b], Huihui Tang[b], Hui Zhu[b], Ni Hong[a,b,c,*]

[a] National Key Laboratory of Agromicrobiology, Huazhong Agricultural University, Wuhan, Hubei 430070, China
[b] The Key Laboratory of Plant Pathology of Hubei Province, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, China
[c] National Indoor Conservation Center of Virus-free Germplasm of Fruit Crops, Huazhong Agricultural University, Wuhan, Hubei 430070, China

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A B S T R A C T

A multiplex RT-PCR (mRT-PCR) assay was developed for detection and differentiation of the *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV), which are viruses frequently occurring in pear trees. Different combinations of mixed primer pairs were tested for their specificity and sensitivity for the simultaneous detection of the three viruses. Three primer pairs were used to amplify their fragments of 247 bp, 358 bp and 500 bp, respectively. The primer pair for ASPV was designed in this work, while the primer pairs for ACLSV and ASGV were from previous reports. The sensitivity and specificity of the mRT-PCR assay for the three viruses were comparable to that of each uniplex RT-PCR. The mRT-PCR was applied successfully for the detection of three viruses in leaves of pear and apple plants, but was unreliable in the detection of ASGV in dormant barks. In conclusion, this mRT-PCR provides a useful tool for the routine and rapid detection and the differentiation of three pear viruses.

1. Introduction

Pear is one of the most economically important and world-widely grown fruit crops. In commercially cultivated pear trees, *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV) are the most commonly occurring viral pathogens (Németh, 1986). These viruses are also important pathogens in apple. The single infection of these viruses in apple cultivars does not usually cause obvious symptoms. In contrast, these viruses cause diverse symptoms in pear. ACLSV infection may induce severe leaf malformation and chlorotic rings or line patterns (Németh, 1986; Desvignes and Boyé, 1988; Fridlund, 1989) in some pear varieties, whereas ASGV is found to be associated with pear black necrotic leaf spot (PBNLS) in Korea (Shim et al., 2004). ASPV infection causes vein yellowing or red mottling of pear leaves (Fridlund, 1989; Schwarz and Jelkmann, 1998). The mixed infections of two or more viruses are very common in cultivated pear and apple plants, and can cause top-working disease of pear and apple trees on susceptible rootstocks, resulting in significant reduction in fruit quality and yields (Campbell, 1963; Posnette et al., 1963; Schmidt, 1972; Yanase, 1983; Desvignes and Boyé, 1988; Yanase et al., 1989; Desvignes, 1999; Cembali et al., 2003). The severity of symptoms elicited by those viruses depends largely on the plant species and virus strains (Desvignes and Boyé, 1988; Fridlund, 1989; Rana et al., 2010). ACLSV, ASGV and ASPV are naturally disseminated by infected propagating materials. They belong to the same family *Betaflexiviridae* (Adams et al., 2012), and are the type species of the genera *Trichovirus*, *Capillovirus* and *Foveavirus*, respectively. Their flexible filamentous particles contain single-stranded positive-sense genomic RNAs. The genomic RNAs of these viruses consist of two to five open reading frames (ORFs) and a poly (A) tail at their 3′-terminus.

Until now, no insect vector for these viruses has been found. The use of healthy propagation materials and plants is a very effective way for preventing the transmission of these viruses and controlling the viral diseases. Therefore, the establishment of sensitive, reliable, fast and inexpensive detection methods for these viruses is of critical importance for the certification and quarantine programs of pear plants and germplasm. Previously, these viruses were mostly based on biological indexing with woody indicators (Fridlund, 1989) and serological assays (Clark and Adams, 1977; Gugerli and Ramel, 2004). Currently, RT-PCR assays have been developed and widely used for the detection and identification

* Corresponding author at: Huazhong Agricultural University, College of Plant Science and Technology, Shizishan Street No. 1, Wuhan 430070, China.
Tel.: +86 27 87283278; fax: +86 27 87384670.
E-mail address: whni@mail.hzau.edu.cn (N. Hong).

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of viruses of pear, apple and other fruit trees (Candresse et al., 1995; Rowhani et al., 1995; Kinard et al., 1996; Jelkmann and Keim-Konrad, 1997; MacKenzie et al., 1997; Kummert et al., 1998; Malinowski et al., 1998; Nemchinov et al., 1998; Singh, 1998; James, 1999; Klerks et al., 2001; Menzel et al., 2002; Mathioudakis et al., 2008; Komorowska et al., 2010). The high specificities and sequence complementarities of primers are very important for the effective detection of viruses by different RT-PCR protocols. The molecular characteristics of three viruses ACLSV, ASGV and ASPV have been extensively studied, and it is found that these viruses may have high genetic diversity (Magome et al., 1997; Komorowska et al., 2010, 2011; Mathioudakis et al., 2010; Hong et al., 2012), which may cause differential serological and biological properties. Among these viruses, ASPV is the mostly variable. Our previous studies also showed that ACLSV isolates from sandy pear grown in China were molecularly and serologically divergent (Song et al., 2011). The relatively high divergence of viral genomes often results in the failure of RT-PCR and leads to false negative results (Nemchinov et al., 1995, 1998; Malinowski, 2005; Komorowska et al., 2010). Therefore, the primers used in RT-PCR should be designed to match the most conserved sequences in the genome of each virus. On the other hand, although the RT-PCR assays are used most widely for the detection of plant viruses due to their high sensitivity, the application of individual protocol adapted for each virus to the same sample is time, labour and cost expensive for routine diagnosis. The development of the polyvalent PCR-based assays using primers targeting conserved regions of viral genomes has allowed the simultaneous detection of a number of different viruses in a single PCR assay, such as the detections of potyviruses (Langeveld et al., 1991), viruses of three viral genera in the family *Flexiviridae* (Foissac et al., 2005), and clostero- and viti-viruses of the grapevine (Saldarelli et al., 1998). Multiplex RT-PCR (mRT-PCR) using several pairs of primers specific for the genomes of targeted pathogens in one reaction allows rapid and sensitive detection and differentiation of a variety of pathogens simultaneously in a single assay (Nassuth et al., 2000; Saade et al., 2000; Ito et al., 2002; Menzel et al., 2002; Roy et al., 2005; Sánchez-Navarro et al., 2005; Hassan et al., 2006; Li et al., 2012), which greatly reduces cost and increases efficiency of viral surveys. Previously, Menzel et al. (2002) reported that four apple viruses ACLSV, ASGV, APSV and ApMV (*Apple mosaic virus*) from one extract could be detected by two multiplex RT-PCR assays, of which each was used for two viruses. Hassan et al. (2006) developed a pentaplex reverse-transcription polymerase chain reaction (Pentaplex RT-PCR) for the simultaneous detection of four pome fruit viruses ASPV, ASGV, ACLSV and ApMV. However, those assays were mainly optimized by using virus-infected apple materials, and the developed assays frequently failed in the detection of those viruses in pear trees. The high divergence of viruses, together with the inference of high contents of polysaccharide and polyphenol components in pear tissues, has impeded to apply these RT-PCR-based methods for efficient detection of viruses infecting pear trees.

This study was undertaken to develop an mRT-PCR assay for the simultaneous and efficient detection of three economically important viruses ACLSV, ASPV and ASGV infecting pear plants. The optimized mRT-PCR was evaluated by detecting those viruses in different tissues of pear and apple plants, and proved to be reliable and sensitive.

2. Materials and methods

2.1. Plant materials

In vitro cultured pear (*Pyrus communis*) plants infected with ASGV, ACLSV and ASPV were used for the optimization of all RT-PCR assays.

Plasmids containing cloned cDNAs of a 582 bp CP gene (Accession no. AY728180) of an ACLSV isolate from peach, a 500 bp fragment covering 62.7% of the CP gene of a ASGV isolate from pear (Zheng et al., 2005), and a fragment of the CP gene of a ASPV isolate from pear, respectively, were used as templates to optimize the PCR conditions and evaluate its sensitivity for the detection of those three viruses.

Samples of field-grown sandy pear (*Pyrus pyrifolia* cv. Cuiguan and Yuanhuang), of which the infections with ASGV, ACLSV and ASPV were confirmed by uniplex RT-PCR (uRT-PCR), together with in vitro cultured plants of *P. communis*, were used for the validation of the efficiency and specificity of mRT-PCR. Some other pear and apple samples randomly collected from different fields were included in the validation of the efficiency of optimized mRT-PCR applied for the detection of the three viruses in field-grown plants. In vitro virus-free plants of *Pyrus betulifolia* were used as negative controls for all assays.

2.2. Design of virus-specific primers

Our previous studies indicated that ASGV-specific primer set ASGV-U/ASGV-2 (James, 1999) could give reliable detections of ASGV in pear plants. Preliminary experiments performed in our laboratory revealed that ASPV isolates were highly variable and there were problems with RT-PCR detection of some ASPV isolates by using primers reported previously (MacKenzie et al., 1997; Malinowski et al., 1998; Schwarz and Jelkmann, 1998; Komorowska et al., 2010). Thereby, in this study, three primer pairs specific for ACLSV and four primer pairs specific for ASPV (Table S1) were designed based on the sequences specific for each virus available in GenBank and tested in our laboratory (unpublished), and those primer sets were designed to have similar annealing temperatures (50–58 °C). The primer sets ACLSV-52/ACLSV-53 specific for ACLSV (German et al., 1990) and ASPV370-F/ASPV370-R specific for ASPV reported previously (Menzel et al., 2002) were also included in the assays. The specificity of each primer set was analyzed by comparisons and Blastn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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2.3. Evaluation of the specificity and compatibility of primer pairs

Each primer pair was tested separately in uniplex RT-PCR using the RNA extracts from in vitro plants as templates to determine their amplification efficiency and specificity against targeting viruses. The products amplified using each set of primers were cloned into the vector pMD18-T (TaKaRa, Dalian, China) and sequenced by a commercial sequencing service (Genscript Biological Science, Nanjing, China) to ensure that the products were from targeted viruses. First-strand cDNA synthesis was performed by using 0.5 μM of the reverse random primer and M-MLV reverse transcriptase (Promega, Madison, USA) at 37 °C for 1 h. PCRs were taken in a 25 μl volume with reaction mixtures containing 2.5 μl of 10× PCR buffer, 0.1 mM of each dNTP, 0.2 μM of each primer, one unit of *Taq* DNA polymerase (TaKaRa, Dalian, China), and 3 μl of originally synthesized cDNA templates. The thermal cycling condition was as follows: 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 45 s at 72 °C, followed by a final incubation of 5 min at 72 °C. The PCR products were electrophoresed on 1.5% agarose gels in TAE buffer and visualized by staining with ethidium bromide (0.5 μg/ml) under UV illumination.

2.4. Optimization of the reaction conditions for mRT-PCR

The virus-specific primer sets with high amplification efficiencies were selected for further evaluation in multiplex RT-PCR