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Short communication

A multiplex RT-PCR assay for simultaneous detection of four viruses from sweet cherry

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ABSTRACT

A multiplex reverse transcription polymerase chain reaction (mRT-PCR) assay was developed for simultaneous detection of four viruses: *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *Little cherry virus-2* (LChV-2) and *Cherry green ring mottle virus* (CGRMV). Random hexamer primer was used for cDNA synthesis. Detection primers were designed against the genomic sequences of four viruses. The reaction conditions were firstly optimized by selecting primers combinations and standardizing the individual primer proportion and PCR protocols. Then, sensitivity of multiplex RT-PCR was evaluated. The assay could detect all four viruses in diluted cDNA (10^{-6}, about 8.64×10^{-4} ng μL^{-1}) and RNA (10^{-1}, about 0.2 μg). The reliability of the assay was evaluated by cloning and sequence alignments. The developed multiplex RT-PCR method was then used to test virus infections from field samples of sweet cherry in this paper. It will be quite helpful for plant quarantine and certification programs. To our knowledge, it is the first report of the multiplex RT-PCR assay for simultaneous detection of PNRSV, PDV, LChV-2 and CGRMV.

1. Introduction

Virus and virus-like disease have caused serious problems in cherry production. It was reported that at least 29 viruses have ever been found to infect sweet cherry (Myrta and Savino, 2008), 9 of which have been identified in China, including *Prunus necrotic ring spot virus* (PNRSV), *Prune dwarf virus* (PDV), *Apple mosaic virus* (ApMV), *Apple chlorotic leafspot virus* (ACLSV), *Cherry green ring mottle virus* (CGRMV), *Cucumber mosaic virus* (CMV), *Cherry virus A* (CVA), *Little cherry virus-2* (LChV-2) and *Cherry necrotic rusty mottle virus* (CNRMV) (Zhou et al., 1996, 2011, 2013; Rao et al., 2009, 2011; Tan et al., 2010). At present, the most effective approach to manage the disease is to remove the virus-infected trees from the orchards. Multiplex RT-PCR is considered as one of the most effective methods to detect the pathogens simultaneously. It cost less time and is easier to operate than single RT-PCR. A similar multiplex RT-PCR assay of detecting virus infections from sweet cherry trees have ever been reported (Noorani et al., 2013). However, that method cannot be applied in the survey of virus infection in China because of the tested targets it chose.

We describe here a protocol of two-step multiplex RT-PCR system for detecting the most prevalent four virus species in Chinese sweet cherry orchards, PNRSV, PDV, LChV-2 and CGRMV. In this paper, sensitivity and reliability analysis of the assay was performed and a magnetic nanoparticles assisted method for total virus nucleic acid extraction was adopted, which enable the assay more convenient and simple.

2. Materials and methods

2.1. Plant materials and virus source

Leaf samples of sweet cherry (*Prunus avium* L. cv. Red lamp) were collected from the orchards in Shandong province in China. The plant samples which were confirmed to be infected with four viruses simultaneously by RT-PCR were used as the positive control during the field samples detection. It was also used to evaluate the optical reactions, the sensitivity and specificity of the multiplex RT-PCR assay. The virus-free plantlets and sterile ddH_2O were used as the negative controls.

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2.2. Primer design

The primers for the multiplex RT-PCR and simplex RT-PCR assays were designed separately according to the conserved region for each virus genome sequence. The sequences were listed in Table 1.

2.3. Total virus RNA extraction

Total virus RNA was extracted using a commercial kit (Wawasye, Wuhan, China) based on magnetic nanoparticles (MNPs) technique. Briefly, leaf samples was ground into a fine powder in liquid nitrogen, mixed with 500 μL lysis buffer and then centrifuged at 12,000 rpm for 5 min. 200 μL supernatant was mixed with 150 μL binding buffer and 20 μL MNPs, briefly vortexed and then incubated at 70 °C for 10 min. The contents were centrifuged and then adsorbed by a magnet. The contents adsorbed on MNP were cleaned by 200 μL washing buffer I, 200 μL buffer II and 200 μL buffer III successively, centrifuged briefly and adsorbed again by a magnet. Total virus RNA extracts were dissolved in 50 μL elution buffer by incubated at 70 °C for 5 min.

2.4. Reverse transcription and multiplex virus detection

For cDNA synthesis, reverse transcription (RT) was carried out using the random hexamer primer according to instructions of Fermentas RevertAidTM First Strand cDNA Synthesis kit (Fermentas, Thermo Scientific).

For simplex RT-PCR assay, a 25 μL reaction mixture contained 2.5 μL of 10× Taq Buffer (TIANGEN, Beijing), 2 μL of 2.5 mM dNTP mix, 1 μL of 5 μM forward primer, 1 μL of 5 μM reverse primer, 1 μL of cDNA, 0.5 μL of Taq DNA polymerase (TIANGEN, Beijing) and 17 μL ddH_2O. The reactions were performed in an Eppendorf Mastercycler Gradient 5331 (Hamburg, Germany) at 95 °C for 5 min for denaturation and 35 cycles of amplification (95 °C for 50 s, 55 °C for 50 s and 72 °C for 50 s), with a final step of 72 °C for 10 min.

For multiplex RT-PCR, the reaction was carried out according to the optimization conditions assay. The final optimized 30 μL of reaction mixture contained 3 μL of 10× Taq Buffer (TIANGEN, Beijing), 2 μL of 2.5 mM dNTP mix, 5 μL of 5 μM forward primers mixture (containing 2 μL, 1.5 μL, 0.5 μL and 1 μL for PNRSV, PDV, LChV-2 and CGRMV), 5 μL of 5 μM reverse primers mixture (the same as above), 1 μL of cDNA, 0.5 μL of Taq DNA polymerase (TIANGEN, Beijing) and 13.5 μL ddH_2O. And the reaction was carried out using the procedure of 95 °C 5 min, 95 °C 50 s, 50 °C 50 s, 72 °C 50 s for 35cycles, followed by 72 °C 10 min. The PCR products were analyzed on a 1.5% agarose gel.

2.5. Cloning and sequencing

To verify the reliability of the multiplex RT-PCR assay, each fragment amplified by the multiplex RT-PCR was purified from the agarose gel (TIANgel Midi Purification Kit, TIANGEN, China) and cloned into the plasmids pMD18-t vector (TAKARA, Dalian, China) for sequencing. Sequence alignment was carried out using DNAMAN 6.0 Software (Lynnon Biosoft, USA).

3. Results

3.1. Optimization of multiplex RT-PCR

For the optimization of the reaction, the concentration of primers for each virus was evaluated. A mixture containing 2 μL of forward primer and 2 μL of backward primer for each virus could not achieve stable amplification for all viruses (data not shown). As a result, a mixture of 5 μL of 5 μM forward primers containing 2 μL, 1.5 μL, 0.5 μL and 1 μL for PNRSV, PDV, LChV-2 and CGRMV, and 5 μL of 5 μM backward primers (the same as above) could amplify four fragments with the expected sizes (733 bp for PNRSV, 467 bp for PDV, 337 bp for LChV-2 and 192 bp for CGRMV). In addition, the annealing temperature of the reaction was also optimized in this study using a procedure of 95 °C for 5 min followed by 95 °C 50 s, 47–67 °C 50 s, 72 °C 50 s for 35 cycles and 72 °C for 10 min. As shown in Fig. 1, when the reaction was performed with the annealing temperature between 47 °C and 50 °C for 35 cycles, the amplification products exhibited four distinct fragments with the expected size of 733 bp, 467 bp, 337 bp and 192 bp and no amplification was observed in the negative controls (Fig. 1). In the following assay, we used 50 °C as the optical annealing temperature for all viruses.

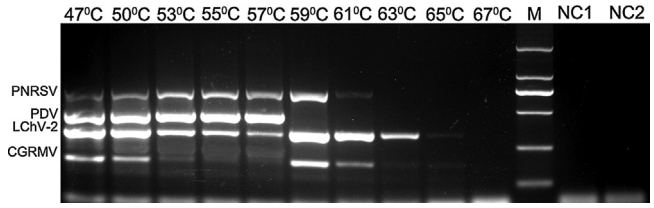

Fig. 1. Effects of annealing temperature on detection of four viruses by multiplex RT-PCR. M: DL2000 DNA Marker.

3.2. Sequencing and alignment analysis

To validate the multiplex RT-PCR assay, each fragment of the amplicon was purified from the gel and cloned for sequencing. After obtaining the sequence data it was subjected to BLAST and aligned with the earlier reported virus sequences. Sequencing analysis showed that all the amplification products were highly homologous to the genome sequence of the corresponding virus (data not shown). This result further confirmed the identity and reliability of the amplification.

3.3. Sensitivity analysis of multiplex RT-PCR

To determine the sensitivity of the multiplex RT-PCR assay, firstly, a series of 10-fold dilutions of cDNA templates transcribed from 2 μg total RNA were tested. The results indicated in Fig. 2A that PNRSV (733 bp), PDV (467 bp), LChV-2 (337 bp) and CGRMV (192 bp) could be detected when the cDNA templates were diluted

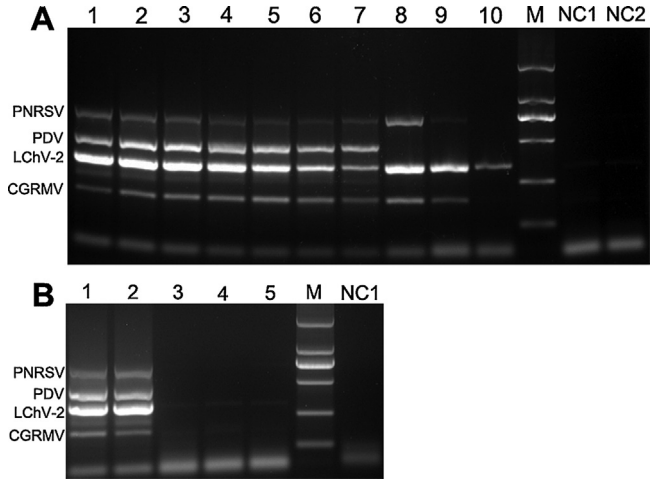

Fig. 2. Sensitivity analysis of multiplex RT-PCR for detecting four cherry viruses. (A) Sensitivity analysis using 10-fold serial dilutions of cDNA. Lanes 1–10: 10^0–10^{-9} serial dilutions. M: DL2000 DNA Marker. (B) Sensitivity analysis using 10-fold serial dilutions of total virus RNA. Lanes 1–5: 10^0–10^{-4} serial dilutions. M: DL2000 DNA Marker.