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BRIEF REPORT

A multiplex RT-PCR method to detect papaya meleira virus complex in adult pre-flowering plants

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Abstract

Papaya sticky disease (PSD), which can destroy orchards, was first attributed to papaya meleira virus (PMeV). However, the discovery of papaya meleira virus 2 (PMeV2) associated with PSD plants impose the need to detect this viral complex. We developed a multiplex RT-PCR (mPCR) technique capable of detecting two viruses in a single assay from pre-flowering plant samples, which is a useful tool for early diagnosis of PSD. We also determined the limit of detection (LOD) using asymmetric plasmid dilutions of both PMeV and PMeV2, which revealed that a higher titer of one virus prevents detection of the other. Thus, this technique is an alternative method for detecting PMeV and PMeV2 in a single reaction.

Abbreviations

PSD	papaya sticky disease
PMeV	papaya meleira virus
PMeV2	papaya meleira virus 2
mPCR	multiplex PCR
LOD	limit of detection
RdRp	RNA-dependent RNA polymerase
ORF	open reading frame

Officially reported in Brazil and Mexico, papaya sticky disease (PSD) is a severe disease that can devastate papaya orchards. Initially, the causal agent of PSD was identified as papaya meleira virus (PMeV), a virus with a double-stranded RNA genome similar to those of members of the family *Totiviridae* enclosed in a 42-nm-diameter isometric particle [1, 2]. Later, papaya meleira virus 2 (PMeV2), a single-stranded RNA virus closely related to members of the genus *Umbravirus*, was also discovered in association with PSD plants. These viruses have an interesting relationship in mixed infections, because the PMeV and PMeV2 genomes are separately encapsidated in particles formed by the PMeV capsid protein [3].

No papaya cultivars have been found that are resistant to PMeV and PMeV2 (PMeV complex) [4]. Visual identification of diseased plants and their eradication (roguing) is the only available control method [5]. However, symptoms of PSD appear only after flowering. Thus, an infected symptomless plant in a field may remain unnoticed for an extended period, acting as a virus inoculum source [5, 6]. Therefore, development of diagnostic procedures for early detection is imperative.

Previous reports have described alternative diagnostic methods for PMeV: (i) viewing in an agarose gel the viral dsRNA band purified from latex [7], (ii) conventional reverse transcription PCR (RT-PCR) from nucleic acids obtained from latex diluted in ammonium or sodium citrate [8], and (iii) conventional RT-PCR and quantitative RT-PCR (qRT-PCR) from small quantities of leaf-purified RNA [9]. Despite these advances, the discovery of PMeV2 associated with PSD plants [3] requires new diagnostic methodologies. A method modified from conventional RT-PCR was described by Antunes et al. [3], who used primers based on sequenced genomes. However, the methodology requires synthesis of two cDNAs and two PCR reactions, one for each virus, making it laborious and time-consuming, especially when screening a large number of samples.

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In contrast, the multiplex PCR (mPCR) method is based on a single PCR that can simultaneously detect different viruses [10]. The method has been used to simultaneously detect papaya ringspot virus (PRSV-P), papaya leaf distortion mosaic virus (PLDMV), and papaya mosaic virus (PapMV). These viruses are difficult to distinguish visually since they cause similar symptoms [11].

The sensitivity or limit of detection (LOD) of a PCR method is an important parameter used to evaluate the minimum amount of amplicon DNA that can be detected and quantified [12, 13]. It is commonly determined using total nucleic acids [14, 15], nucleic acids extracted from viral particles purified from infected plants [16], or plasmids containing the target [17–19]. These templates are quantified, mixed in equimolar amounts, serially diluted and used as a template for mPCR. However, an equimolar mix may not be a proper template to determine the LOD. This can lead to misleading results, as the different viruses in mixed infection do not usually have the same titer in a host [20–22]. Here, we report a mPCR method for simultaneous identification of PMeV and PMeV2 in pre-flowering papaya plants. Moreover, we propose that an asymmetric mixture of PMeV and PMeV2 templates is the most appropriate target for determining the sensitivity of the mPCR method.

A survey was conducted on four groups of plants at different stages on several papaya production farms in the north of Espírito Santo state, Brazil. For the first group, (i) papaya seedlings ($n = 10$) were kept under greenhouse conditions for two months before leaves were collected. For the other groups, the papaya leaves in the field were collected from trees (ii) that were in the adult pre-flowering stage ($n = 10$), (iii) that were asymptomatic in the post-flowering stage ($n = 16$), and (iv) that were symptomatic in the post-flowering stage ($n = 6$). Leaf samples were taken on the same day from different papaya plants.

Total RNA was extracted from 100 mg of papaya leaves using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). RNA purity (A_{260}/A_{280}) was assessed using a NanoDrop® ND2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The templates used for RT-PCR reactions were obtained from 1 µg of purified RNA that had been treated with DNAse I (Invitrogen, Carlsbad, CA, USA). For the uniplex PCR reaction, the RNA was incubated at 96 °C for 3 min and 70 °C for 10 min to denature the dsRNA (PMeV) and ssRNA (PMeV2). For the mPCR reaction, the RNA was denatured at 96 °C for 3 min. First-strand cDNA synthesis was performed using random hexamers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Two primer pairs were utilized for both uniplex PCR and mPCR diagnosis. The PMeV-specific primer pair targets the predicted PMeV ORF1 at nucleotide position 2446-2816 (PMeVC1F, 5´CTTGGTTAGGCATAACTG TAGGT3´; PMeVC1R, 5´CACGGACTCTTAGAAACG TCTATC3´) [3]. The PMeV2-specific primer pair targets ORF2 at nucleotide position 1430-2244 (PMeV2F, 5´CGC CAAGTGGGATAAGTTTAGA3´; PMeV2R, 5´CGATTT GAGCACAAGGGTTAATG3´) based on an available genomic sequence (NCBI GenBank no. KT921785). The primers were designed using the PrimerQuest Tool (https://www.idtdna.com/PrimerQuest/Home/Index), and their specificity was verified using BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers for PMeV amplify a 370-bp fragment, and those for PMeV2 amplify an 814-bp fragment.

Uniplex and mPCR reactions were performed in a Mastercycler Thermocycler (Eppendorf, Hamburg, Germany) using Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). To determine the optimal PCR conditions, different annealing temperatures (52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C) and concentrations of each specific primer set (0.5:0.5 µM or 1.5:0.5 µM) were tested.

Following optimization, PCR amplification was performed in a 10-µl volume containing 1.54 µl of PCR mix (1 µl of 10X PCR Buffer -Mg^{2+}, 0.3 µl of 50 mM $MgCl_2$, 0.2 µl of 10 mM dNTP mixture and 0.04 µl of recombinant Taq DNA polymerase [5 U/ µl]), and deionized water. The uniplex PCR reaction for detection of PMeV or PMeV2 contained 1 µl of PMeV or PMeV2 primers (10 µM), while multiplex PCR reactions were performed with half the amount of both primers. Both uniplex and mPCR reaction were performed with 55 ng of cDNA. The PCR mix and primers were manufactured by Invitrogen, Carlsbad, CA, USA.

The PCR protocol consisted of the following: 94 °C for 3 min followed by 35 cycles of amplification (94 °C for 45 s, 58 °C for 30 s, 72 °C for 1 min) and a final extension at 72 °C for 10 min. PMeV2 conditions were the same as for PMeV, but the extension time during the cycles was increased to 1.2 min. The mPCR program was the same as for the PMeV2 uniplex reaction. PCR amplicons were analyzed by electrophoresis on 1% (w/v) agarose gels stained with ethidium bromide and visualized under UV light.

To assess the LOD of the uniplex and mPCR assays, we generated recombinant plasmids by ligating the RT-PCR products into the plasmid pGEM®-T Easy Vector (Promega, Fitchburg, WI, USA). The specificity of PCR was validated by Sanger sequencing. The plasmid copy number was determined [18], and serial tenfold dilutions (10^8-10^1 copies/µl) were used as a template in 10-µL uniplex PCR mixtures. To determine the sensitivity of the mPCR, two different assays were performed. In the first one, equal volumes of each plasmid dilution were used as a template in different PCR reactions. In the second, different ratios of the PMeV and PMeV2 plasmids were used (10^8:10^3, 10^8:10^2, and 10^8:10^1) to mimic situations in which different viral titers are present

in field samples. All reactions were performed according to the program described above for mPCR.

To determine the optimal annealing temperature for the PCR reactions, a gradient test was performed in uniplex and mPCR reactions. No differences in the efficiency of the reaction were found when different temperatures were tested; therefore, the annealing temperature was chosen to be 58 °C. This temperature was also used to test different PMeV and PMeV2 primer ratios in mPCR using cDNA from symptomatic post-flowering plants. Based on the intensity of amplicons, the 0.5:0.5 µM primer ratio was used in further reactions. Moreover, reliable diagnosis of the PMeV complex using current techniques requires synthesis of a cDNA with two different denaturation temperatures (one for each virus) [3], and this consumes double the materials and reagents for PCR detection. We tested these two cDNA samples in the mPCR assay, but only the PMeV dsRNA denaturation protocol (96 °C for 3 min) gave results that were consistent with those obtained with the uniplex RT-PCR (data not shown).

The sensitivity test showed that the uniplex RT-PCR assay could detect 10 copies of PMeV, whereas the LOD for PMeV2 was 100 copies (Online Resource 1). The sensitivity of the uniplex PCR was compared with that of the mPCR, and they were found to have equal sensitivity, although the band intensity was weaker at all dilutions in the mPCR.

To validate the mPCR assay for use in field surveys, samples collected from papaya plants at stages i, ii, iii, and iv were tested. Forty-two papaya plants were tested, and the results are summarized in Table 1. In seedlings, all samples were positive for PMeV but negative for PMeV2. All adult pre-flowering plants tested positive for both PMeV and PMeV2, although some differences were found between the post-flowering groups. PMeV2 was detected in 12 out of 16 samples from group iii and in all samples of group iv, while fewer samples (three in the asymptomatic group and two in the symptomatic group) tested negative for PMeV. To rule out a false-negative diagnosis, we perform the uniplex RT-PCR assay with samples that tested negative for one of the viruses (data not shown). The results for the four samples from group iii that tested negative for PMeV2 and all seedling samples agreed with the mPCR results. On the other hand, uniplex RT-PCR confirmed the infection in the remaining samples, revealing a discrepancy between the results of this experiment and those of the LOD experiment.

Table 1 Results of a uniplex and multiplex PCR survey testing 42 plants from a greenhouse and papaya orchards in Espírito Santo, Brazil

Development stage	Uniplex*		Multiplex*	
	PMeV	PMeV2	PMeV	PMeV2
Seedlings	10/10	10/0	10/10	10/0
Adult pre-flowering	10/10	10/10	10/10	10/10
Post-flowering asymptomatic	16/16	16/12	16/13	16/12
Post-flowering symptomatic	6/6	6/6	6/4	6/6

*No. of total plants/no. positive

The sensitivity test using an equimolar plasmid ratio demonstrated that PMeV and PMeV2 detection in the mPCR was not altered when compared to the uniplex PCR (Online Resource 1). Therefore, it was not clear why some field samples were positive for PMeV in the uniplex PCR but not in the mPCR assay. One possible reason could be related to differences in viral titer. The use of equal amounts of PMeV and PMeV2 recombinant plasmids in sensitivity assay may not reflect the actual amounts of these viruses in the papaya plants. To test this hypothesis, we performed assays with different PMeV:PMeV2 plasmid copy number ratios (10^8:10^3, 10^8:10^2, and 10^8:10^1) in the mPCR. The results showed that when one virus was present at a high titer, the band intensity for the other virus in the agarose gel was lower (Online Resource 1).

The discovery of a second virus associated with sticky disease in plants indicated the need for the development of a new diagnostic tool. In this study, an already available primer pair [3] and a new one were used to develop an mPCR assay to detect PMeV and PMeV2 in a single reaction and its sensitivity and applicability for use in field surveys were evaluated.

It is a common practice to use equimolar amounts of PCR templates to determine the detection limit of an mPCR assay [14, 17–19]. Here, we determined the detection limit when different ratios of templates were used and found that altering the relative amount of the templates indeed affected the results (Online Resource 1). Although PMeV was detected more frequently than PMeV2 in pre-flowering papaya plants [23], this difference was more pronounced at later stages of infection (post-flowering symptomatic), as both viruses were successfully detected by mPCR in all adult pre-flowering plants. Amplicons produced by mPCR in the sensitivity test showed lower band intensity in an agarose gel. The presence of two primer pairs forces competition between the amplicons by the PCR reagents and thus reduces the yield of either the amplicons [24]. When templates were used in equal amounts, the reduced yield did not affect the LOD for both PMeV or PMeV2 amplicon.

In this study, we developed a multiplex PCR method for simultaneous detection of PMeV and PMeV2 in papaya pre-flowering plants. This method is very useful for early diagnosis because it can be used to screen simultaneously for both viruses in a large number of samples. Therefore, this procedure will contribute to a better understanding of PSD epidemiology and to the development of disease management strategies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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