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Molecular detection of *Papaya meleira virus* in the latex of *Carica papaya* by RT-PCR

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Abstract

A RT-PCR assay was developed for early and accurate detection of *Papaya meleira virus* (PMeV) in the latex from infected papayas. The meleira disease is characterized by an excessive exudation of more fluidic latex from fruits, leaves and stems. This latex oxidises and gives the fruit a "sticky" texture. In the field, disease symptoms are seen almost exclusively on fruit. However, infected plants can be a source of virus for dissemination by insects. Primers specific for PMeV were designed based on nucleotide sequences of the viral dsRNA obtained using a RT-RAPD approach. When tested for RT-PCR amplification, one of these primers (C05-3′) amplified a 669-nucleotide fragment using dsRNA obtained from purified virus particles as a template. The translated sequence of this DNA fragment showed a certain degree of similarity to the amino acid sequence of RNA-dependent RNA polymerases from other dsRNA viruses. When used as the single primer in two RT-PCR kits available commercially, primer C05-3′ also amplified the DNA fragment from papaya latex of infected, but not from healthy plants. The RT-PCR-based method developed in this study could simplify early plant disease diagnosis, assist in monitoring the dissemination of the pathogen within and between fields, and assist in guiding plant disease management.

Keywords: Diagnosis; Papaya "sticky" disease; Papaya meleira disease; PMeV; Virus

1. Introduction

Papaya (*Carica papaya* L.) is the most cultivated species of the *Caricaceae* family. It is grown in lowland tropical and subtropical areas around the world, and it is consumed usually as fresh fruit. Viruses are the main group of pathogens limiting papaya cultivation in Brazil, which is the largest papaya producing country. Virus diseases result in considerable increase in production cost and losses in productivity. Three virus diseases infect papaya in Brazil: *Papaya ringspot virus* (PRSV); *Papaya lethal yellowing virus* (PLYV); and a virus causing the papaya "meleira" disease, also known as papaya "sticky" disease (Lima et al. 2001).

Papaya meleira disease was first detected in the second half of the 1980s (Lima et al., 2001), and since then it has become the most important disease to limit papaya yields in some of the major production regions in Brazil, where commercial orchards can have up to 100% of the plants affected. The first evidence that the causal agent of this disease is a virus was provided by Kitajima et al. (1993), who detected isometric particles of about 50 nm in diameter. These investigators also purified a 12 kb-long dsRNA from latex extracted from fruits and leaves of symptomatic plants. The viral origin of the causal agent of this disease was confirmed by Maciel-Zambolim et al. (2003) and the name *Papaya meleira virus* (PMeV) was proposed. No resistance to this disease is known. The only management strategy applied by growers is rouging, and infected plants are diagnosed based on symptoms in the field.

The diagnosis of papaya meleira disease is based on symptoms observed on the fruit. For this reason, an infected plant without fruit can remain unnoticed for months in the field, acting as source of inoculum before it is detected and eliminated

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(Rezende and Fancelli, 1997). Papaya plants affected by this disease show an intense and apparently spontaneous latex exudation from the fruit. The exuded latex has an aqueous appearance, being more watery than normal latex, which is typically milky. The external surface of the fruit becomes stained because the latex darkens by oxidation. When fruit are cut, small internal spots are seen in the flesh. The fruit shows a softer consistency of the flesh and is less tasty. Infected plants also show green and white spots on the fruit, and the leaves near the top may have rounded borders and can exhibit gum exudation and necrosis (Kitajima et al., 1993). According to Nascimento et al. (2000), the fruit of a papaya plant infected by the meleira virus is more attractive to fruit flies such as *Ceratitis capitata* (Wiedmann) and *Anastrepha obliqua* (Macq).

Diagnosis of this disease is also possible by dsRNA purification through a CF11 column, followed by visualization on acrylamide or agarose gels, according to the protocol developed by Doods et al. (1984). However, this is a laborious and time consuming assay which is not practical when handling a large number of samples. Tavares et al. (2004) developed two diagnostic assays for the disease based on the extraction of nucleic acids from the latex of papaya plants and visualization of the dsRNA on agarose gels. These assays are easy to perform, very reliable, and can be used when handling dozens of samples at once; however, they use phenol, chloroform, and/or isoamylic acid, which can be a health issue for laboratory personnel. These assays also require between 350 and 500 μL of papaya latex, which can be difficult to obtain when working with small plants.

Polymerase chain reaction (PCR)-based techniques offer advantages over traditional methods of detection and diagnosis because they require normally only a few microliters of template nucleic acid. Also, PCR-based techniques are rapid, sensitive and can be applied to hundreds of samples at the same time. In this study, a RT-PCR assay was developed for the early and accurate detection of PMeV, the causal agent of meleira disease, in papaya latex in a one-step procedure.

2. Material and methods

2.1. Virus purification and isolation of viral dsRNA

Latex from papaya plants symptomatic for meleira disease was collected and used for virus purification according to Maciel-Zambolim et al. (2003). The dsRNA extraction was carried out according to Guo et al. (1998). This dsRNA was used as template for RT-PCR, as well as a positive control when validating the RT-PCR assays.

2.2. RT-RAPD and primer design

Viral dsRNA was used as a template in RT-RAPD (Reverse Transcription-Random Amplified Polymorphic DNA) to randomly produce DNA sequences from the genome of PMeV (Tavares et al., 2003). RT-RAPD fragments, amplified using a set of different 10-mers (Operon Biotechnology), were cloned into pCR2.1-TOPO vectors (Invitrogen) and sequenced using M13 forward and reverse primers in the DNA sequencing facility at Embrapa Genetic Resources and Biotechnology, using Dye Terminator chemistry and an automated sequencer. Base calling and quality assignment of individual bases of the RT-RAPD sequences were carried out using the program PHRED (Ewing et al., 1998). Removal of ribosomal, poly (A) tails, low quality sequences, and vector and adapter regions was conducted as described by Telles and da Silva (2001). The resulting sets of high quality sequences were assembled into clusters of overlapping sequences using the CAP3 assembler (Huang and Madan, 1999), with individual base quality and default parameters. The consensus sequences were used to design specific primers from the genome of PMeV, which were then applied for further characterization of the viral genome (de Araújo et al., 2005) and to develop the RT-PCR diagnosis assay. The software Gene Runner Version 3.05 (Hastings Software, Inc.) was used for primer design.

2.3. Amplification, cloning and sequencing of RT-PCR fragments

Prior to RT-PCR, an aliquot of 50 μL of viral dsRNA was treated with RNase-free DNase I at 37 °C for 30 min, and then heated at 60 °C for 5 min, in order to eliminate possible DNA contaminants. An aliquot of 1.5 μg of DNase I-treated dsRNA was combined with 200 ng of antisense primer and DEPC water, in a final volume of 15 μL, and then incubated at 99 °C for 6 min. This denaturing solution was then kept on ice until the RT reaction.

Reverse transcription (RT) was undertaken in a 50 μL solution containing 15 μL of denaturing solution, 5 μL of DTT (0.1 M), 0.8 μL of dNTP mix (25 mM each), 2 μL of Rnasin (40 U/μL), 2 μL of Moloney murine leukemia virus (M-MLV) reverse transcriptase (200 U/μL), 10 μL of 5× M-MLV RT buffer (Invitrogen), and 15.2 μL of DEPC-water. Incubation was at 37 °C for 60 min. The RT solution was then kept at −20 °C until the PCR reaction.

The PCR reaction was also carried out in a 50 μL solution, which contained 5 μL of 10× PCR buffer (Invitrogen), 0.8 μL of dNTP mix (25 mM each), 1.5 μL of MgCl2 (50 mM), 1.0 μL of sense primer (100 ng/μL), 1.0 μL of antisense primer (100 ng/μL), 5 μL of the RT solution, and 0.5 μL of Taq DNA polymerase (5 U/μL) (Invitrogen). The PCR reaction was carried out in a PTC 100 thermal cycler (MJ Research) programmed for one cycle at 94 °C for 1 min followed by 30 cycles at 94 °C for 1 min, 57 °C for 2 min, and 72 °C for 2 min. A 7 min extension at 72 °C completed the program.

The RT-PCR-amplified fragments were cloned into the pGEM-T Easy vector (Promega), and sequenced using M13 forward and reverse primers. Base calling and quality assignment of individual bases of the RT-PCR sequences were carried out as described for RT-RAPD. The resulting sets of high quality sequences, eight per fragment, were assembled into clusters of overlapping sequences using the CAP3 assembler (Huang and Madan, 1999), with individual base quality and default parameters. The consensus sequences were analyzed by BLASTx (Altschul et al., 1997) against viruses in the nr database at NCBI (Benson et al., 2007).

2.4. RT-PCR procedures

'Titan™ One Tube RT-PCR System' (Roche Applied Sciences) and 'Ready-To-Go RTPCR Beads' (Amersham Biosciences), two commercially available RT-PCR kits, were used in this study.

When using the 'Titan™ One Tube RT-PCR System', 0.4 μL of dNTP mix (25 mM each), 200 ng of the primer, 2.5 μL of DTT (0.1 M), 10 μL of 5× Titan RT-PCR buffer (1.5 mM MgCl2), 0.6 μL of Titan™ enzyme mix, and 2.5 μL of latex diluted (1:1) in 0.1 M ammonium citrate (pH 6.5) were mixed in a 0.5 ml microfuge tube. When using the 'Ready-To-Go RT-PCR Beads', 200 ng of primer and 2.5 μL of diluted latex (1:1) in 0.1 M ammonium citrate (pH 6.5) were placed in the microfuge tube containing the beads. In both cases, RT-PCR was carried out in a 50 μL final volume. The RT-PCR were performed in a PTC 100 thermal cycler (MJ Research) programmed for one cycle at 60 °C for 30 min and 94 °C for 2 min, followed by nine cycles at 94 °C for 30 s, 57 °C for 30 s and 68 °C for 1 min; then four cycles at 94 °C for 30 s, 57 °C for 30 s and 68 °C for 1.5 min; then four cycles at 94 °C for 30 s, 57 °C for 30 s and 68 °C for 2 min; then four cycles at 94 °C for 30 s, 57 °C for 30 s and 68 °C for 2.5 min; then 4 cycles at 94 °C for 30 s, 57 °C for 30 s and 68 °C for 3 min; then four cycles at 94 °C for 30 s, 57 °C for 30 s and 68 °C for 3.5 min; and finally four cycles at 94 °C for 30 s, 57 °C for 30 s and 68 °C for 4 min. A 7 min extension at 68 °C completed the program.

3. Results and discussion

3.1. Primers specific for RT-PCR amplification of PMeV dsRNA fragments

Isometric PMeV particles (Fig. 1a) were obtained from latex extracted from papaya plants symptomatic for meleira disease. The nucleic acid isolated from these particles was shown to resist treatment with RNase-free DNase I but not to RNAse A (Fig. 1b). The RNA purified from the particles were exposed to temperatures ranging from 65 to 95 °C for a period of 3 min, and the RNA was degraded at temperatures of 80 °C or higher (data not shown). These results corroborate those of Maciel-Zambolim et al. (2003), who demonstrated that the nucleic acid purified from virus particles has physicochemical properties of a dsRNA molecule: "melting" when heated to 96 °C for 3 min, resistant to S1 nuclease and DNase digestion, and susceptible to RNase A digestion.

As pointed out by Maciel-Zambolim et al. (2003), PMeV is distinct from any other virus described to date from bacteria, fungi, plants, invertebrates and vertebrates. PMeV seems to be restricted to the lactiferous vessels of papaya plants, an environment that seems especially antagonistic to plant viruses. Its genome is a dsRNA molecule of approximately 12 kb, which is unique among plant viruses. Therefore, the molecular characterization of this dsRNA needed to be carried out without a reference viral genome. In order to generate random sequence data from this dsRNA, a RT-RAPD approach was used in this study.

Random amplified polymorphic DNA (RAPD) analysis is a polymerase chain reaction (PCR)-based assay that requires only small amounts of template DNA. It does not require prior DNA sequence information, is simple and quick to perform, using single, short, random primers, usually 10-mers (Williams et al., 1990). Combined with reverse transcription (RT), RAPD can be applied to generate random DNA sequence data from an uncharacterized virus RNA genome, such as that from PMeV.

Screening was carried out for 10-mers primers capable of directing the amplification of DNA fragments when using PMeV dsRNA as template (Fig. 1c). The same primer was used for both RT and RAPD reactions. RT-RAPD fragments amplified when using nine different primers, out of 20 tested, were cloned and sequenced, generating 146 forward and reverse sequence reads. The assembling of the high quality DNA sequences from these results generated nine contigs, which together were 7.828 nucleotides long, approximately 65% of the 12 kb dsRNA

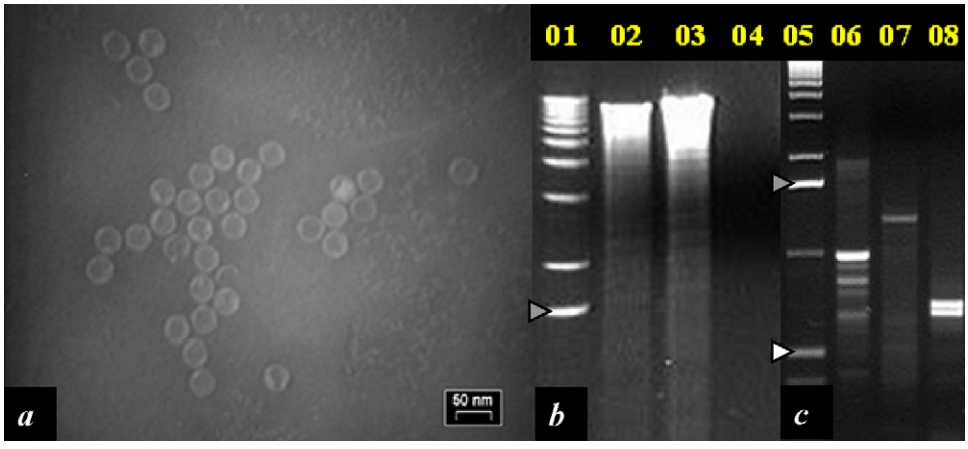

Fig. 1. (a) Electron micrograph of purified PMeV virions. (b) dsRNA isolated from purified PMeV virions. (c) RT-RAPD amplification of PMeV dsRNA fragments. (1 and 5) 1 kb DNA ladder (Invitrogen); (2) non-treated dsRNA; (3) RNase free DNase I-treated dsRNA; (4) RNase A-treated dsRNA; (6–8) RT-RAPD amplification with three different 10-mers from the OPK kit; respectively, primers 01, 04 and 12. Size of DNA fragments indicated by arrows points of different colors: gray – 1636 bp and white – 506/517 bp.

Table 1
Primer name, primer sequence, RT-PCR fragment length, and best BLASTx hit against viruses (ORGN) in the nr database in the NCBI for all three primers which successfully amplified DNA fragments when used alone in the RT-PCR assay for *Papaya meleira virus*

Primer name	Primer sequence	Size (bp) of the RT-PCR fragment	Best BLASTx hit against viruses (ORGN) in the nr database in the NCBI
C04-5′	5′-TTACCACCCAGACGTCC-3′	1482	ORF FPV081 Metalloprotease, Accession: NP_039044, Fowlpox virus, Viruses; dsDNA viruses, no RNA stage; Poxviridae; Chordopoxvirinae; Avipoxvirus, Expect = 0.68
C05-3′	5′-ACCACAATGGGTATTTAAAG-3′	669	dsRNA-dependent RNA polymerase, Accession: ABC86751, Trichomonas vaginalis virus, Viruses; dsRNA viruses; Totiviridae; Giardiavirus, Expect = 0.17
C06-3′	5′-AAGCTACGGTTTAATGAGG-3′	522	RNA-dependent RNA polymerase, Accession: NP_619714, Groundnut rosette virus, Viruses; ssRNA positive-strand viruses, no DNA stage; Umbravirus, Expect = 0.078

(Tavares et al., 2003). The consensus sequences of the nine contigs were used subsequently for the design of specific primers for PMeV (Souza Jr. et al., in preparation).

Thirty different combinations of primers specific for PMeV were tested for RT-PCR amplification using dsRNA isolated from virion particles as a template. These primers were used in pairs, as sense and antisense, but also alone. Most of the combinations examined allowed amplification of RT-PCR fragments. However, three of these primers also allowed amplification when used alone (Table 1), and these were therefore chosen for further analysis. All three RT-PCR fragments displayed BLASTx hits against viruses in the NCBI nr database, but only the fragment generated with the C05-3′ primer displayed a hit to a sequence from a dsRNA virus (Table 1). Three putative ORFs were identified by ORF Finder (Rombel et al., 2002) in this RT-PCR fragment. A putative conserved domain of a totivirus RNA-dependent RNA polymerase was observed by CD Search (Marchler-Bauer and Bryant, 2004) on the largest ORF, 190 amino acid-long (Fig. 2).

3.2. Using papaya latex as a template in a RT-PCR assay for PMeV diagnosis

The RT-PCR assay developed proved to be effective for the amplification of DNA fragments from PMeV dsRNA. However, when considering its use as a diagnostic assay, the need for virion purification and dsRNA isolation would be a serious disadvantage. No amplification was achieved using the

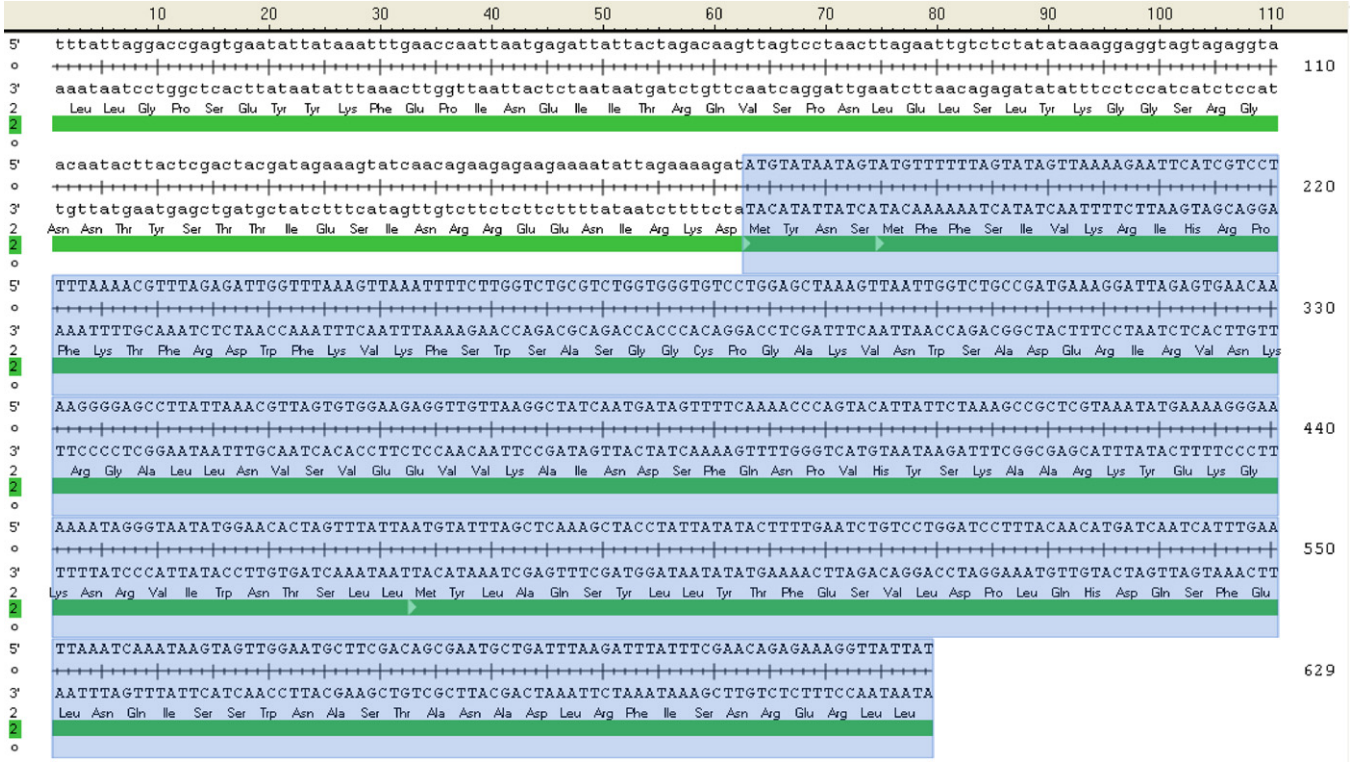

Fig. 2. Nucleotide and amino acid sequences of the RT-PCR fragment routinely amplified from plants symptomatic for meleira disease, but not from healthy plants, when using primer C05-3′ as the single primer. Sequence of the primer was removed from both sides, and only the core sequence of the RT-PCR fragment is shown. Sequence of the largest putative ORF is highlighted in blue.

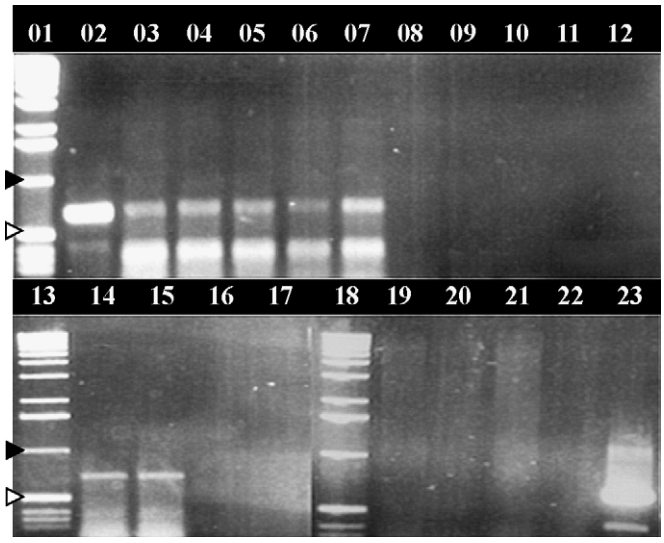

Fig. 3. RT-PCR assay for diagnosis of PMeV using primer C05-3′. (1, 13 and 18) 1 kb DNA ladder (Invitrogen); (2 and 23) RT-PCR amplification using dsRNA isolated from purified PMeV virion as template; (3–22) RT-PCR amplification using the 'Titan^{TM} One Tube RT-PCR System' kit; (3–7) latex from plants symptomatic for meleira disease; (8–12) latex from healthy plants, and (19–22) leaf sap from plants symptomatic for "meleira" disease); (14–17) RT-PCR amplification using the 'Ready-To-Go RT-PCR Beads' kit (14 and 15) latex from plants symptomatic for "meleira" disease, and (16 and 17) latex from healthy plants. Size of DNA fragments indicated by arrow points of different colors: black – 1018 bp and white – 506/517 bp.

above RT-PCR when using latex from infected plants diluted in ammonium citrate as template and any of three primers (C04-5′, C05-3′, and C06-3′) as the single primer in the reaction (data not shown).

When using C05-3′ as the single primer in the 'Titan^{TM} One Tube RT-PCR System' or the 'Ready-To-Go RT-PCR Beads' kits with 2.5 μL of latex diluted in ammonium citrate, the 669 nt fragment was routinely amplified from plants symptomatic for meleira disease, but not from healthy plants (Fig. 3). However, a second, shorter fragment was also amplified when using the 'Titan^{TM} One Tube RT-PCR System' but not the 'Ready-To-Go RT-PCR Beads' kit (Fig. 3).

All papaya samples used in this study were first analyzed using a protocol described by Tavares et al. (2004). In this protocol approximately 350 μL of latex is used, and the extraction of nucleic acids is based in treatment with proteinase K, phenol and chloroform:isoamylic acid. Both protocols, that developed in this study and that described in Tavares et al. (2005), gave the same results for all samples tested. The latex used as a negative control in this study (Fig. 3) came from plants grown in an area where PMeV has not yet been reported.

A dilution series for the latex from an infected plant in 0.1 M ammonium citrate (pH 6.5), from 1:1 to 1:1000, was carried out, and 2.5 μL from each dilution were used as a template for RT-PCR using the commercially available kits. The 669 nt fragment was amplified in all dilutions tested. However, the fragment was not amplified when 2.5 μL of papaya leaf sap, diluted (1:1) in the same buffer, was used as a template (Fig. 3). These results may help to explain the lack of sap transmissibility for this virus (Maciel-Zambolim et al., 2003).

The availability of an early diagnostic assay for PMeV, as described in this study, can benefit the papaya industry in several ways. First, it makes it possible to study and monitor the dissemination of the virus within and between fields from the moment when the plants are transplanted from the nursery. Diagnostic methods described previously for this disease could not be applied to very young plants, because of the need of a large amount of latex (Kitajima et al., 1993; Tavares et al., 2004). In order to use the assay described in this study, one needs only 2.5 μL of papaya latex, which can be obtained from very young plants. Second, this procedure can be incorporated into testing protocols when post-entry quarantines are required. Finally, it can be used in other countries to determine whether this disease is present.

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