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Detection of Papaya Ring Spot Virus Infection by RT-PCR together with an Amplification of Reference Gene

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ABSTRACT

Papaya ring spot virus (PRSV) is a major devastating viral disease in papaya cultivation in Sri Lanka. It has been a primary requirement to identify a PRSV disease resistant source to use in breeding programs. Previously it was reported that mountain papaya grown in high elevation in Sri Lanka exhibits resistance to PRSV. Therefore this study was conducted to evaluate the potential resistance to papaya ring spot virus in mountain papaya as there are no resistant sources found among *Carica papaya*. In this regard *Carica papaya* and mountain papaya were inoculated with PRSV by mechanical inoculation technique and the symptoms were evaluated visually. Simultaneously, in order to detect, the latent infection if any, total RNA was isolated and PRSV RNA genome segments were amplified using three sets of primers by RT-PCR. In this experiment, functional ability of the cDNA was examined using SAND family protein gene (*sand*) as the reference gene used in papaya expression studies. According to the results, visual symptoms were not detected in mountain papaya from PRSV inoculation while standard symptoms appeared in *Carica papaya*. RNA was successfully extracted and *sand* gene amplification was observed proving the correct functionality of the cDNA. Three sets of primers were able to amplify respective corresponding amplicons in inoculated *Carica papaya* while negative amplification was observed in mountain papaya. These findings give some insight into the understanding of the resistance of mountain papaya for PRSV infection.

KEYWORDS: Molecular detection, Mountain papaya, Papaya ring spot virus, *Sand* reference gene

INTRODUCTION

Ring spot disease caused by papaya ring spot virus (PRSV) is considered as the major disease of papaya (*Carica papaya*) in the world (Purcifull *et al.*, 1984).

PRSV is a member of the family Potyviridae and it has two strains (Purcifull *et al.*, 1984), PRSV type P which infects both papaya and some cucurbits and PRSV type W which infects only cucurbits. The virus is transmitted by aphids (Tennant *et al.*, 2007). In PRSV–P infection, papaya exhibits yellowing leaf distortion and severe mosaic, oily or water soaked spots on the trunk and petioles.

PRSV can be controlled by different methods such as destroying infected plants, using barrier crops and growing transgenic resistant plants (Dillon *et al.*, 2005). Resistance against the PRSV is the most effective methods of controlling the disease (Gonsalves *et al.*, 2006). In many countries including Sri Lanka, genetically modified transgenic plants are not permitted. Therefore, the best strategy would be to develop resistant plants via classical breeding using naturally resistant sources. However, among *Carica papaya*, there is no natural resistant varieties found against PRSV. Several wild papaya species such as *Vasconcellea cundinamarcensis, V. cauliflora, V. quercifolia* and *V. stipulata* are reported to exhibit heritable resistance to the papaya ring spot virus type P which is the most severe disease of papaya (Drew *et al.*, 1998). Hence, these genetic resources may able to be used to develop resistance in *C. papaya* against PRSV *via* breeding techniques.

Mountain papaya existed in the Montane zone of Sri Lanka, has been recognized as *Carica pubescence* in the Revised Flora of Ceylon (Dissanayake *et al.*, 1995). However, according to the latest classification (Badillo, 2000), genus *Carica* of Caricaceae contains only one species, *Carica papaya*, and genus called *Vasconcellea*, comprised of 20 wild papaya species including *Vasconcellea cundinamarcensis,* referred as mountain papaya.

Both *Carica* and *Vasconcellea* are genetically diploids and share the same chromosome number, 2n=18 (Storey, 1976). Possible hybridization also have been reported between *Carica* spp. and *Vasconcellea* spp. Therefore, development of a resistant variety *via* hybridization of *Carica* and *Vasconcellea* might be a useful approach. In this regards, it is necessary to verify whether this mountain papaya present in Nuwara Eliya is resistant to the PRSV disease. Therefore, present study was

planned to detect the possible PRSV infection in mountain papaya by artificial inoculation. Due to the fact, that visual observation of PRSV symptoms is not reliable, molecular detection of the disease was also planned. In this regards, it is necessary to use a positive control to confirm the integrity of cDNA derived from total RNA. Therefore, a previously reported gene, called *sand*, gene of a nuclear localized protein or a plasma membrane located protein (Cottage *et al.*, 2001) was used as the reference gene.

MATERIALS AND METHODS

Collection of PRSV Infected Materials

A precise PRSV infected *Carica papaya* plant was selected from papaya orchard Horticultural Crop Research and Devolopment Institute (HORDI) in Gannoruwa to use as an inoculum of PRSV.

Preparation of PRSV Inoculums for Mechanical Inoculation Method

Infected young leaves were ground in a sterilized mortar with 2-5 part of phosphate buffer. To prepare phosphate buffer 1.36 g of KH_2PO_4 was dissolved in 1000 mL of H_2O (solution A) and 1.78 g of $Na_2HPO_4.2H_2O$ was dissolved in 1000 mL of H_2O (solution B). Then 51.0 mL of solution B was mixed with 49.0 mL of solution A, to prepare 0.01 M Phosphate buffer solution (pH 7).

Inoculation of Mountain Papaya and Carica papaya by PRSV

PRSV was inoculated by mechanical inoculation method using 0.01 M Phosphate buffer with beta mercaptoethanol as a stabilizing agent (pH 7). As abrasive agent 200 mesh carborundum was dusted over the leaf surface before inoculation to increase the infection by providing wounds for the entry of virus particles. After 5-10 min, inoculated plants were washed with distilled water. Two mountain papaya plants which were four feet in height grown in Shanthipura Nuwara Eliya and *Carica papaya* (var. Red lady) plants that were at 6th leaf stage were inoculated and four plants were placed in the same premises in Shanthipura Nuwara Eliya after inoculation as a positive control. According to our previous experiment mountain papaya dose not survive in Peradeniya and Kurunagala. Therefore, mountain papaya was kept at their native premises at Nuwara Eliya while another inoculated *Carica papaya* plant was kept at HORDI. Symptoms were visually observed seven weeks after inoculation and top part of inoculated parts were harvested for RNA extraction.

Primer Selection for Molecular Detection of PRSV

Three sets of primers were selected from previously published reports to ensure the reliability of molecular detection method. PRSV specific primers designed by Sirinivasulu and Gopal (2012), Poty virus specific primers designed by Zheng *et al.* (2008) and PRSV Sri Lankan strain specific primer designed by Amalka *et al.* (2015) were used in this study. Primers specific for SAND family protein gene designed by Zhu *et al.* (2012) was used in this study as positive control to ensure the integrity of RNA. All primer information are displayed in Table 1.

Molecular Detection of PRSV by RT-PCR

RT-PCR was conducted to detect the infection of PRSV in mountain papaya along with *Carica papaya*. Table 2 indicates the sample types examined by RT-PCR.

Table 2. Sample types examined by RT-PCR

No	Sample
1	Infected *Carica papaya* (original) (Nuwara Eliya)
2	*Carica papaya* inoculated by PRSV
3	*Carica papaya* leaves (un inoculated)
4	Mountain papaya (un inoculated)
5	Mountain papaya 1 inoculated by PRSV
6	Mountain papaya 2 inoculated by PRSV
7	Negative control

PRSV - Papaya ring spot virus

Table 1. Oligonucliotide primers used for molecular detection of papaya ring spot virus (PRSV)

Source of the primers	Primer name	Annealing temperature	Sequence	Reference
PRSV specific	PRSV F1 PRSV R1	45 °C	5'-ATCACAATGTATTACGC-3' 5'-CTCTCATTCTAAGAGGCTC-3'	(Sirinivasulu *et al.*, 2012)
Sand gene specific	SAND F SAND R	55 °C	5'-CGTGGTCTGTCAGTGGGTAG -3' 5'-ATGATGAGAGGCAAGATGG -3'	(Zhu *et al.*, 2012)
Poty virus specific	Nlb2 Nlb3	40 °C	5'-GTITGYGTIGAYGAYTTYAAYAA-3' 5'-TCIACIACIGTIGAIGGYTGNCC-3'	(Zheng *et al.*, 2008)
PRSV Sri Lankan strain specific	PRSVWUF		5'CTCTCATTCTAAGAGGCTC3'	(Amalka *et al.*, 2015)
Poty virus specific	MJ2R	53 °C	5'-TGCTGCKGCYTTCATYGT-3'	(Marie-Jeanne *et al.*, 2000)

RNA Extraction

About 75 mg of fresh leaves were ground in 1 mL of TRIzol® reagent (Invitrogen, life Technologies). Then sample was centrifuged at 12,000 rpm for 10 min. After centrifugation, supernatant was separated and incubated for 5 min at room temperature. Then 200 µL of chloroform was added to TRIzol mixture. Sample was shaken vigorously and was incubated for 3 min at room temperature. Then sample was centrifuged at 12 000 rpm for 15 sec. The uppermost aqueous layer was separated from lower two layers and was transferred to new eppendorf tube. Absolute iso-propanol (0.5 mL) was added to each sample and was incubated for 10 min at room temperature for precipitating RNA. Pelleted RNA was washed with 75% ethanol and was centrifuged at 7500 rpm for 5 min. The pellet was air dried and dissolved in 30 µL of nuclease free water while incubating at 55-60 °C in water bath. Resulted RNA was immediately used for cDNA synthesis.

cDNA Synthesis

Two micro liter of total RNA was incubated with 2 µL (10 mM) of reverse primer and 4 µL of water at 65 °C for 5 min and was snap-chilled on ice for 2 min. The cDNA was synthesized using 100 units of Maxima reverse transcriptase (Thermo scientific, life technologies, USA), 4 µL of 5X RT-buffer, 2.5 M of dNTPs, 10 units of RNase inhibitor in 20 µL of total volume in thermal cycler at 42 °C for 1 h followed by heating at 90 °C for 5 min. Finally cDNA was diluted by adding 30 µL of water.

PCR Assay

Polymerase chain reaction amplification was done in 10 µL of PCR mixture. Each reaction contained 5 µL of PCR master mix (2X PCR master mix, Promega), 0.8 µL (10 mM) of forward primer and reverse primer, 1.4 µL water, 2 µL of cDNA. The PCR program was used with one cycle of initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at optimized temperature for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. The amplified PCR products were visualized by 1% agarose gel, containing 0.5 µL/mL Ethidium bromide.

RESULTS AND DISCUSSION

Visual Observation of Inoculated Carica papaya and Mountain Papaya

C. papaya plants, which was the positive controls placed in Nuwara Eliya did not show prominent PRSV symptoms instead leaf tip twisted probably due to the cold weather. The second leaf from the top was in distorted condition. There were no any visual symptoms appeared in mountain papaya (Table 3). The inoculation of mountain papaya was done by mechanical inoculation method assuming that *C.papaya* spp. are similar to *Vasconcellea* spp. as reported by Amalka *et al.* (2015).

Table 3. Symptom appeared in PRSV inoculation

Sample	Symptoms
Carica papaya (HORDI)	Distortion of young leaves. Oily patches in petioles
Inoculated *Carica papaya* (Nuwara Eliya)	Leaf tip twisted and about to dry
Inoculated *Carica papaya* (Nuwara Eliya)	Leaf tip twisted
Inoculated mountain papaya 1 (Nuwara Eliya)	No any visual symptom
Inoculated mountain papaya 2 (Nuwara Eliya)	No any visual symptom

PRSV-Papaya ring spot virus

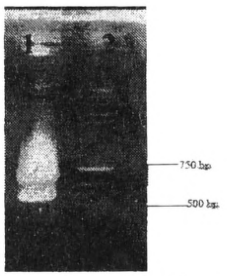

Figure 1. Agarose gel electrophoresis of RNA obtained from mountain papaya plant. *Lane 1-mountain papaya, Lane 2-1 kb ladder*

RT-PCR Amplification Obtained for the Detection of PRSV

Amount of RNA extracted from the tested plants was satisfactory and figure 1 shows the RNA band obtained from one mountain papaya sample. As PRSV was single stranded RNA virus RT-PCR was conducted to detect the viral gene using previously reported primers designed based on cDNA sequences (Table 1). A 350 bp band was amplified from infected *Carica papaya* by poty virus specific primers while there was no amplification in mountain papaya. Also, according to the results of RT-PCR, band size of 1024 bp was observed with PRSV specific primer pair (PRSV WU F1/MJ2 R primers) which was designed for Sri Lankan strain of PRSV (Amalka *et al.*, 2015), in infected *C. papaya* while no any amplification was observed in inoculated mountain papaya. Moreover, exact band size of 1.7 kb was observed with PRSV specific primer pair in infected samples of *C. papaya* while no any amplification was observed in mountain papaya (Figure 2).

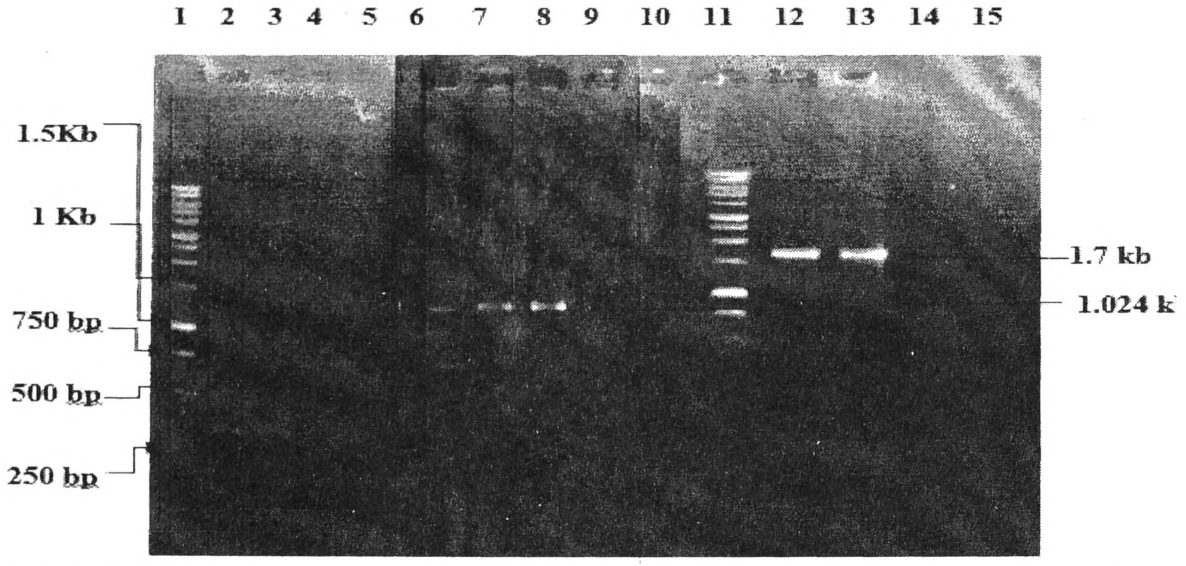

Figure 2. Agarose gel electrophoresis of PCR amplified products obtained from NIBF1/NIBR1 primers, PRSVWUF/MJ2R primers and PRSVF/PRSVR. *Lane 1-6-11-1 kb ladder (Promega), Lane 2- 3 Infected C. papaya with poty virus specific primers, Lane 7-8 Inoculated C. papaya with PRSVWU-F/MJ2R primers, Lane 12-13 Inoculated C. papaya with PRSVF/PRSVR primers, Lane 4-5-9-10-14-15 inoculated mountain papaya*

CONCLUSIONS

Ribose nucleic acid was successfully extracted from the *Carica papaya* and mountain papaya plants and RNA integrity was proved by *sand* gene amplification. Three sets of primers were used to detect the infection of PRSV at molecular level. Reverse transcriptase PCR amplification with papaya ring spot virus specific primers produced 1.7 kb band and Sri Lankan PRSV stain specific primers produced 1.024 kb band in the infected sample of *Carica papaya* while no such amplification was observed in mountain papaya. From these findings it can be speculated that mountain papaya may have the resistant trait to PRSV infection. However, it is necessary to study about the other methods of viral inoculation to mountain papaya to get a strong conclusion about the PRSV resistant trait.

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