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MOLECULAR EVALUATION OF POTENTIAL RESISTANCE TO PAPAYA RING SPOT VIRUS IN MOUNTAIN PAPAYA (*VASCONCELLEA CUNDINAMARCENSIS)*

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A B S T R A C T

Papaya Ring Spot virus (PRSV) is a devastating viral disease in papaya cultivation in Sri Lanka. It was reported that mountain papaya (*Vasconcellea cundinamarcensis*) exhibits resistance to PRSV. Therefore, this study was conducted to evaluate the potential resistance to PRSV in mountain papaya to be used in papaya improvement breeding programs. *Carica papaya* and *V. Cundinamarcensis* were inoculated with PRSV by mechanical inoculation technique and symptoms were evaluated visually. In order to detect the latent infection, a molecular method was developed. PRSV RNA genome segments were amplified using three sets of primers by RTPCR and an amplicon was sequenced to confirm the identity of PRSV. Functional ability of the cDNA was examined using SAND family protein gene (*sand*) as the reference gene. According to the results, visual symptoms were not observed in mountain papaya while standard visual symptoms appeared in *Carica papaya*. Three sets of primers were able to amplify the corresponding amplicons in inoculated *Carica papaya* and sequencing data confirmed that the amplicon was from PRSV. Negative amplification was observed in mountain papaya from the three sets of primers although *sand* gene amplification was observed, proving the correct functionality of cDNA. Therefore, it can be concluded that mountain papaya is likely to be resistant to PRSV.

Keywords: *Carica papaya*, Papaya Ring Spot Virus, Mountain papaya, *Sand* gene as reference gene, *Vasconcellea cundinamarcensis*

INTRODUCTION

Papaya Ring spot disease caused by papaya ring spot virus (PRSV) is considered as one of the major diseases of papaya (*Carica papaya*) in the world. PRSV is a member of the Potyviridae family and it has two strains, PRSV type P which infects both papaya and some cucurbits and PRSV type W which infects only cucurbits (Purcifull *et al*., 1984). PRSV-P infection is normally characterized by the production of ringspot symptoms on fruits of infected papaya trees and based on this symptom the name "papaya ringspot" was given by (Jensen, 1949) to this disease. Infected tree and fruit appear ring shape patches. Therefore, it is called as ringspot. Additionally the disease is characterised with, leaf mosaic and chlorosis, water-soaked oily streaks on the petiole and upper part of the trunk, distortion of young leaves that sometimes results in shoestring-like symptoms (Purcifull *et al*., 1984) and finally cause severe yield loss in comparison with healthy papaya.

PRSV can be controlled by different methods such as destroying infected plants, using barrier crops and growing transgenic resistant plants. Resistance against the PRSV is the most effective method of controlling the disease (Gonsalves *et al*., 2006). However, resistance to PRSV-P has not been identified in *C. Papaya* (Gonsalves, 1998). Although genetically engineered *C. papaya* incorporating coat protein gene of PRSV (Tennant *et al.,* 1994) has been commercially released, 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 are no natural

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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 be used to develop resistance in *C. papaya against* PRSV via breeding techniques.

Both *Carica* and *Vasconcellea* are genetically diploids and share the same chromosome number, 2n=18 (Storey, 1976). Hybridization also has been reported between *Carica papaya* and *Vasconcellea* spp (Drew *et al.*, 1998, Manshardt *et al*., 1989, Muthulakshmi *et al*., 2007). Therefore, development of a resistant variety to PRSV via hybridization of *Carica papaya* and *Vasconcellea* might be a useful approach. In Sri Lanka, in hilly areas of Nuwara-Eliya region, there are sparsely distributed, uncultivated papaya plants recognised as mountain papaya. A bar-coding study conducted by Fonseka (2015), on sparsely distributed mountain papaya in Nuwara Eliya, Sri Lanka has proved that the plants belong to the species, *Vasconcellea cundinamarcensis.* Therefore, the present study was planned to detect whether the *Vasconcellea cundinamarcensis* known as mountain papaya is resistant to PRSV infection. Moreover, the mountain papaya has promising organoleptic characters, especially for juices and marmalades. There is a tendency to vanish this species from Sri Lanka, due to the fact that it is not grown as cultivated crop and it has been a neglected crop grown spontaneously in the wild.

Usually confirmation of the disease by visual observation of PRSV symptoms is not reliable and molecular detection of the disease is necessary to ensure virus infection. Since, PRSV is a RNA virus, it is necessary to amplify viral cDNA fragments and to perform a positive control to confirm the integrity of cDNA derived from total RNA. As described by Cottage *et al*., (2001), a gene, called *Sand* gene of a nuclear localized protein or a plasma membrane located protein could be employed as the reference gene to confirm the functionality of RNA of papaya. This paper describes the experimental procedure of molecular detection of PRSV in *C. papaya* and *Vasconcellea cundinamarcensis* plants and corresponding results proving the potential resistance to PRSV in *Vasconcellea cundinamarcensis* accessions.

MATERIALS AND METHODS

Preparation of *PRSV* inoculums for mechanical inoculation method: Infected young leaves with PRSV symptoms collected from papaya orchard of Horticultural Crop Research and Development Institute (HORDI), Gannoruwa, Sri Lanka were ground in a sterilized mortar with cold 0.01M potassium phosphate buffer. The 0.01M Phosphate buffer (pH 7) was prepared by dissolving, 1.36g of KH_2PO_4 in 1000 ml of H_2O (solution A) and 1.78 g of $Na_2HPO_4.2H_2O$ in 1000 ml of H_2O (solution B) and then 51.0ml of solution B was mixed with 49.0ml of solution A.

Inoculation of *Vasconcellea cundinamarcensis* and *Carica papaya* by PRSV*:* PRSV was inoculated by mechanical inoculation method using 0.01M potassium phosphate buffer with beta mercapto ethanol 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. Inoculated leaves were finally washed with distilled water.

Three mountain papaya plants which were 4 feet in height naturally grown in Shanthipur, Nuwara-Eliya, Sri Lanka were selected for the inoculation as they were the youngest plants found and three *Carica papaya* (var., Red lady) plants that were at 6th leaf stage were inoculated together as mentioned above. *C. papaya* plants were placed in the same premises in Shanthipura, Nuwera-Eliya, Sri Lanka after inoculation to observe as a positive control. According to our previous experiment mountain papaya does not survive in Peradeniya and Kurunagala. Therefore, mountain papaya plants did not move to HORDI, but they were kept at their native premises at Nuwara Eliya while few inoculated *Carica papaya* plants were kept at HORDI. Symptoms were visually observed 7 weeks after inoculation and top part of inoculated parts were harvested for RNA extraction due to systematic visual observation of PRSV in top part.

Primer selection for molecular detection of PRSV*:* Two sets of primers, PRSV specific primers, PRSV-F1 and PRSV-R1 designed by (Sirinivasulu *et al.*, 2012) and Potyvirus specific primers, Nib2 and NIb3 designed by (Zhu *et al*., 2012) were selected from previously published reports to detect the PRSV infection at molecular level. Also, a forward primer PRSVWUF was designed based on the coat protein gene sequence of PRSV Sri Lankan strain (Accession: U14741) using NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and it was used with the reverse primer of the MJ2-R (Zheng *et al*., 2008) which is a potyvirus specific primer. All these three sets of primers and primers specific for SAND family protein gene as

positive control, were used in this study. All primer information is displayed in Table 1.

Table 1. Oligonucleotide primers used for the PRSV detection and reference gene amplification.

S.No.	Source of the primers	Primer name	A.T.*	Sequence	Ref.
1	PRSV specific	PRSV F1 PRSV R1	45°C	5'-ATCACAATGTATTACGC-3' 5'-CTCTCATTCTAAGAGGCTC-3'	(Sirinivasulu*et al*., 2012)
2	Potyvirus specific	Nlb2 Nlb3	40°C	5'-TITGYGTIGAYGAYTTYAAYAA-3' 5'-TCIACIACIGTIGAIGGYTGNCC-3'	(Zheng *et al*., 2008)
3	Potyvirus specific	MJ2R	53°C	5'-TGCTGCKGCYTTCATYGT-3'	(Marie-Jeanne *et al.,* 2000)
	PRSV Sri Lankan strain specific	PRSVWU F		5'-CTCTCATTCTAAGAGGCTC-3'	
4	*Sand* gene specific	SAND F SAND R	55°C	5'-CGTGGTCTGTCAGTGGGTAG -3' 5'-ATGATGAGAGGCAAGATGG -3'	(Zhu *et al*., 2012)

A.T*: Annealing temperature

Molecular detection of PRSV by RT-PCR*:* As the PRSV was single stranded RNA virus, RT-PCR was conducted to detect the viral genes as a method of proving infection of PRSV in mountain papaya along with *Carica papaya*. Two plants from each of inoculated mountain papaya and *Carica papaya* were used for RNA extraction. About 75mg of top fresh leaf parts of inoculated plants were ground in 1ml of TRIzol(R) reagent (Invitrogen, life Technologies) and RNA was extracted following the manufactures protocol. Resulted RNA was immediately used for c-DNA synthesis. 2 µl of total RNA was incubated with 2 µl (10mM) of reverse primer and 4µl of water at 65°C for 5min and was snap-chilled on ice for 2 min. The c-DNA was synthesized using100 units of Maxima reverse transcriptase (Thermo scientific, life technologies, USA), 4µl of 5X RT-buffer, 2.5M 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, c-DNA was diluted by adding 30 µl of water. PCR 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 (10mM) of forward primers and reverse primer, 1.4 µl of water, 2 µl of c-DNA. 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 2min and final extension at 72 °C for 10 min. The amplified PCR products were visualized by 1% agarose gel, containing 0.5µl/ml ethidium bromide.

Sequencing of the amplified PCR product and sequence alignment: The PCR product amplified from PRSV- F1 and PRSV-R1 was run in a 0.8% gel and the DNA band was excised under the ultra violet light. The gel band was inserted into a eppendorf tube and the tube was filled with 300µl of binding buffer (Fermentas, GeneJET™ PCR Purification Kit) and kept at 65°C for 10 min in a dry bath. Solution was transferred into a separate DNA binding column. The Column was centrifuged at 13,000 rpm for 1 min. The column was then placed into a fresh tube and 30 µl of distilled water was added. Tube was heated at 65°C for 5 min and finally the solution was centrifuged at 13,000 rpm for 1 min to collect the purified DNA. The purified PCR product was sent to Macrogen Inc. Korea for sequencing.

To confirm whether the amplified DNA obtained from inoculated samples of *C. papaya* was from PRSV, the sequence was aligned with the known sequence of PRSV available at National Centre for Biotechnology Information (NCBI) using MEGA 4 software.

RESULTS AND DISCUSSION

Visual observation of inoculated Carica papaya and mountain papaya: *C. papaya* plants, the positive controls placed in Nuwara-Eliya did not show standard prominent PRSV symptoms instead in all samples leaf tip twisted probably due to the cold weather. However, in all samples, the second leaf from the top was in a distorted condition and therefore, considered as the PRSV infection. However, all inoculated *C. papaya* plants that were kept at HORDI

showed clear PRSV symptoms such as distorted upper leaves and oily patches in petioles generating 100% infection rate. There were no any visual symptoms related to PRSV appeared in any of mountain papaya (Table 2), thereby infection rate was considered as 0%. The inoculated leaves had only few wounds created by carborandom treatment.

The inoculation of mountain papaya was done by mechanical inoculation method assuming that *C.papaya* spp was similar to *Vasconcellea* spp with regards to the mechanical transmission of virus by sap inoculation. In this method, the inoculum was manually applied after conducting an abrasive treatment for all the plants. However, there were no any visual symptoms detected in mountain papaya while 100% infection rate was observed in *C. papaya* thereby statistically proving the success of inoculation method for *C. papaya*. However, there can be latent infection of the PRSV in mountain papaya although visual symptoms were not appeared. Therefore, molecular detection method was employed to confirm the results observed visually.

Table 2. Symptoms appeared after PRSV inoculation.

No.	Sample Type	Symptoms	
1	*Carica papaya leaves* inoculated at original place (HORD	*Distortion of young leaves. Oily patches in petioles*	
2	*Carica papaya* leaves inoculated at Nuwara Eliya	Leaf tip twisted and about to dry. Second leaf showed some distorted nature	
3	Mountain papaya leavesinoculated at NuwaraEliya	*No any visual symptoms of PRSV.*	

RT PCR amplification obtained for the detection of PRSV*:* Amount of RNA extracted from the tested plants was satisfactorily obtained (Figure 1). A fragment of about 246 bp was amplified from the RTPCR conducted with *Sand* gene primers in all *Carica papaya* and mountain papaya samples and size of the amplified band was exactly same as the previous finding reported by Zhu *et al.*, (2012). As PRSV is a single stranded RNA virus, integrity of extracted total RNA was required to be proven. In view of this, *Sand* gene as the reference gene was amplified from the inoculated samples proving the functionality of RNA. Also, amplification of *Sand* gene revealed that the *Sand* gene could be an adoptive positive control for experiments in RNA expression studies of papaya.

RTPCR was conducted to detect the viral genes using previously reported primers designed based on cDNA sequences (Table 1). All three primer sets produced amplification corresponding to the exact size expected. A 350bp band was amplified from infected *Carica papaya* by poty virus specific primers while there was no amplification observed in mountain papaya. Also, according to the results of RT PCR, band size of 1024bp was observed with primer pair, PRSV WU F1 and /MJ2 R, of which forward primer was designed by Sri Lankan strain of PRSV, in infected *C. papaya* while no amplification was observed in inoculated mountain papaya. Moreover, the exact band size of 1.7kb was observed with PRSV specific primer pair in infected samples of *C. papaya* while no amplification was observed in mountain papaya (Figure 2). Amplification of viral DNA fragments were confirmed by using three sets of primers as a validation of molecular detection method.

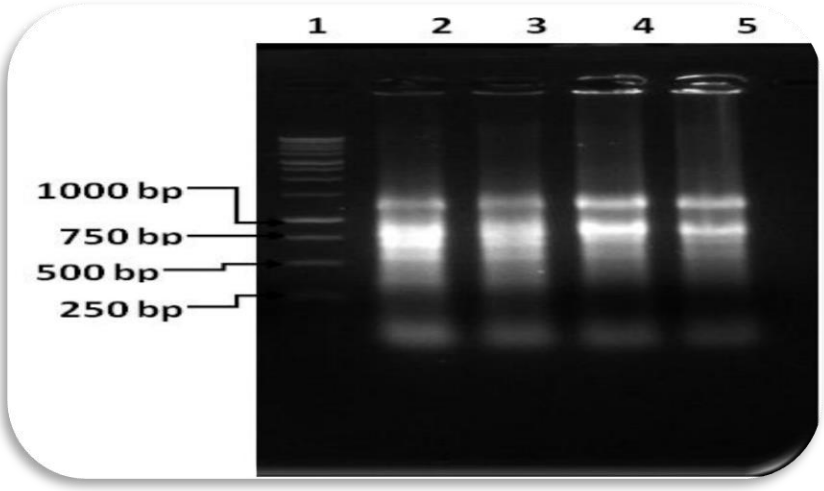

Figure 1. RNA extracted from tested plants
Lane 1: 1 kb ladder (Promega), Lane 2 and 3: inoculated C. papaya, Lane 3 and 4: inoculated mountain papaya

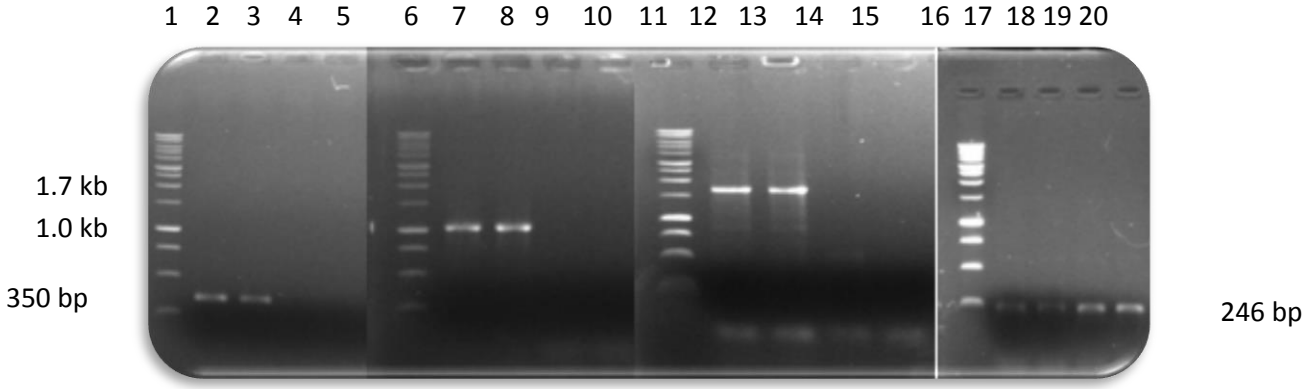

Figure 2. PCR products obtained from different sets of primers for the detection of PRSV
Lane 1, 6, 11and 16 : 1 kb ladder (Promega), Lane 2 and 3 : Inoculated C. papaya with poty virus specific primers, Lane 4 and 5 : inoculated mountain papaya with potyvirus specific primers, Lane 7 and 8: Inoculated C. papaya with PRSVWU-F/MJ2-R primers, Lane 9 and 10: inoculated mountain papaya with PRSVWU-F/MJ2-R primers, Lane 12 and13: Inoculated C. papaya with PRSV-F/PRSV-R primers, Lane 14 and 15 : inoculated mountain papaya with PRSV-F/PRSV-R primers , Lane17 and18:Inoculated C. papaya with SAND-F/SAND-R primers, Lane 19-20 inoculated mountain papaya with SAND-F/SAND-R primers

Confirmation of PRSV by sequence alignment: It was necessary to confirm that that DNA fragments amplified from inoculated *C. papaya* samples were from PRSV genome. Therefore, amplified fragment from PRSV F1 and PRSV R1 primers was sequenced and the results of the sequence alignment revealed that the amplified fragment was from PRSV. Figure 3 a 1. shows a part of sequence obtained from the PRSV Forward primer and Figure 3 b 1. shows a part of the PRSV sequence obtained from the PRSV reverse primer. Both sequences were aligned with two ends of the expected 1.7 bp fragment in complete genomic sequence of a Papaya ringspot virus isolate from Hainan Island, China (Acc. No EF183499.1) with 92 % sequence homology. These evidences confirmed the accuracy of the molecular detection method in *C. papaya*.

Although sand gene was amplified in mountain papaya, PRSV cDNA fragments could not be amplified in any of the mountain papaya samples inoculated by virus. Hence, it can be speculated that negative amplification was due to the absence of PRSV at detectable level in mountain papaya. From these results, it can be hypothesized that there may be potential resistance to PRSV in mountain papaya. However, it is not known whether the 0.01M

phosphate buffer with beta mercaptoethanol composition is suitable to create the pathogenicity in *Vasconsellea* spp or whether PRSV does not cause the disease to *Vasconsellea* spp. The mean annual temperature at the places where the papaya plants were grown in NuwaraEliyais 16^{o}C and it may be the reasons for not detecting prominent visual symptoms of PRSV and having retarded growth in *C. papaya*. However, infection was detected in *C. papaya* by molecular amplification method. Therefore, it can be speculated that mountain papaya could not be a possible source for the survival of PRSV in comparison to *C. papaya*.

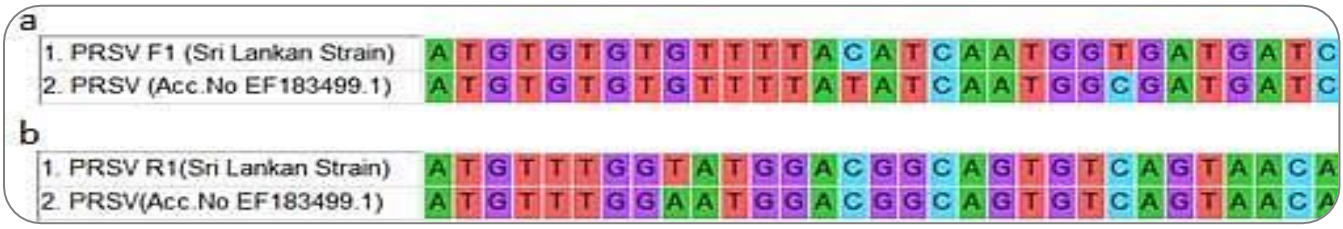

Figure 3. Sequence alignment of amplified DNA fragment

CONCLUSION

RNA was successfully extracted from *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. Potyvirus specific primers produced 350 bp band in infected *Carica papaya* while the band was absent in inoculated mountain papaya. RT-PCR amplification with papaya ring spot virus specific primers produced 1.7kb band and the Sri Lankan PRSV stain specific primers produced 1.024kb band in the infected sample of *Carica papaya* while both amplicons were not observed in inoculated mountain papaya. Results of sequencing of one of the amplified fragment from inoculated *C. papaya* proved that the amplicon obtained was similar to the PRSV cDNA sequence. From the several observations made at morphological and molecular level, it can be concluded that mountain papaya is most likely to be resistant to PRSV infection. However, it is necessary to study other methods of viral inoculation further to examine the possibility of creating the pathogenicity in mountain papaya to confirm the exact resistance to PRSV disease.

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