RESEARCH ARTICLE

Molecular evidence for association of *papaya ringspot virus* with papaya from North East Hill region of India

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ABSTRACT: Papaya ringspot virus (PRSV) is a major bottleneck in papaya production globally. The incidence of PRSV was as high as 51% in West Tripura district of Tripura followed by Imphal West of Manipur (41.6%) exhibiting the typical symptoms of yellowing, vein clearing, mottling, severe blistering with distorted leaves and concentric rings on the fruits. The papaya cultivars RCTP 1, Red Lady and Manipur local exhibited typical symptoms of PRSV under sap inoculation. The typical flexuous particles of size 800 X 12 nm were observed in the electron microscope. The amplicons of size 350 bp were observed in RT-PCR using the primers derived from the conserved NIb region of *Potyvirus* genome. Two PRSV isolates from North East India (PRSV-Mnp1 and PRSV-Trp1) had maximum identity of 83.1 to 84% with PRSV isolates from Thailand, Taiwan and Korea for conserved NIb genomic region. Phylogenetically PRSV isolates from North East India segregated to a distinct cluster compared to other isolates from different papaya growing regions of the world. It is the first molecular evidence for the association of PRSV with papaya from North East Hill region of India.

Keywords: NIb region, North East India, papaya ringspot virus, papaya, RT-PCR

Papaya (*Carica papaya*) is mainly grown in tropical region and fruit has high nutritive and medicinal value. Papaya is cultivated in an area of 1.26 lakh ha and with production of 55.08 lakh tonnes. Globally India ranks first in papaya production with annual production of 5,508 metric tonnes (NHB, 2015). In North East India, area under papaya is 0.1682 lakh ha and annual production is 2.6283 lakh tonnes (NHB, 2015). Several fungal and viral diseases attack papaya and among the viral diseases, papaya ringspot is an important disease causing significant losses to the crop yield and has become a major threat to the papaya cultivation throughout India by rendering papaya orchards unproductive (Varma, 1988). Associated virus i.e. papaya ringspot virus (PRSV) was first reported in the year 1948 (Capoor and Varma, 1948). PRSV leads to crop yield losses upto 85-90% (Hussain and Varma, 1994). PRSV belongs to super kingdom *viridae*, kingdom ss RNA, family *Potyviridae*, genus *Potyvirus* and species *Papaya ringspot virus*. Virus particles are flexuous rod measuring 760-800 nm X 12 nm (Yeh *et al.*, 1998). The virion contains a positive sense, single stranded RNA genome of approximately 10.3 kb length, surrounded by a coat protein (CP) and the molecular weight of single coat protein subunit is 36 kDa (Yen *et al.*, 1992). PRSV is transmitted by several species of aphids in a non-persistent mode and mainly infect cucurbits and papaya. PRSV has two biotypes; Type-P (PRSV-P) infecting cucurbits and papaya, and Type W

(PRSV-W) infecting only cucurbits and not papaya (Purcifull *et al.*, 1984). PRSV infection leads to diverse kind of symptoms on papaya (Brunt, 1996). The most prominent kind of symptoms associated with PRSV infection are leaf mottling and malformation, ring spots on fruits with prominent streaks on stems and petioles of plants. This leads to overall stunting of the plants making it unproductive and eventually plant dies (Tripathi *et al.*, 2008).

Several strategies have been adopted for management of PRSV; however complete control has not been achieved (Tripathi *et al.*, 2008). Selection and use of PRSV strains having attenuated pathogenicity in cross-protection based management of PRSV has been attempted (Fermin *et al.*, 2010). In the last decade, a major focus has been on the use of RNA interference (RNAi) technology in developing resistance to PRSV (Azad *et al.*, 2014; Krubphachaya *et al.*, 2007). For employing these strategies accurate detection of associated virus and its molecular characterization is a prerequisite. Molecular diagnosis of PRSV (P and W) has been initially achieved by targeting coat protein (CP) region amplification by reverse transcription-polymerase chain reaction (RT-PCR) (Jain, 1998; Kumar, 2003). However, mutations in the primer binding sites may lead to false negative results, hence primers targeting more conserved genomic regions like NIb has been employed (Gulsiri *et al.*, 2003; Sharma, 2004). Reports on the detection and molecular characterization of PRSV from

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different geographical regions of India are available, however, no systematic study on the occurrence, incidence and molecular characterization of PRSV from North Eastern Hill (NEH) region of India have been undertaken. With this background, the present study was undertaken to assess the incidence papaya ringspot disease and confirmation of association of PRSV in infected papaya through RT-PCR technique.

MATERIALS AND METHODS

Incidence of PRSV in Manipur and Tripura

A roving survey was conducted in four districts of Tripura and four districts of Manipur. In each district, different places were visited where papaya was cultivated and during the survey observations on percent disease incidence, stage of the crop and symptoms were recorded. Percent disease incidence was calculated as:

Percent disease incidence = Number of plants infected/
Total number of plants observed X 100

Mechanical transmission

The mechanical transmission was undertaken using papaya cultivars RCTP-1, Red Lady and some Local varieties of Papaya of Manipur and *Chenopodium amaranticolor*. The PRSV infected leaf samples were finely ground in a snap-chilled mortar and pestle using sodium phosphate buffer pH 7.5 (1:1 w/v). The finely ground sap was filtered through double layered cheesecloth and filtrate was mixed with a pinch of celite Powder (600 mesh). For sap inoculation a piece of sterile cotton pad dipped in filtrate was rubbed unidirectional from petiole towards the margin of the leaves on the test plants (20 days old). The leaves were supported with a piece of cardboard under the lower surface to avoid injury. The inoculated leaves were washed with a jet of water to remove traces of celite. The plants were labeled and kept for observation under insect-proof conditions and were observed at weekly intervals post-inoculation.

Electron microscopy

Sodium Phosphate buffer 1:1 (w/v) was used for macerating 100 mg of infected leaf sample. The macerated solution was centrifuged at 1200 rpm for 5 minutes and the clear supernatant was separated. Few drops of this clear solution was placed on copper grids coated with formavar. After 5 minutes, these grids were washed with double distilled water. The staining was done with 2% uranyl acetate and excess stain was removed. The grids were examined in the transmission electron microscope (JEOL 100 CX-11) at Advanced Centre for Plant Virology, Division of Plant Pathology, ICAR-IARI, New Delhi.

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA preparations of healthy and symptomatic leaf tissue (100 mg) of field collected samples of two

representative isolates (PRSV-Mnp1 from Manipur and PRSV-Trp1 from Tripura) was extracted using RNeasy Plant Mini kit following the manufacturer's protocol (Qiagen Inc.). Isolated RNA was incubated at 70°C for 5 min and snap-cooled before using as template. One step RT-PCR was performed in the same tube following standard method. The PCR reaction mixture (100 µl) contained 2 µl of primers (100 pmol/µl) Nlb 2F and Nlb 3R (Zheng *et al.*, 2008), 5 units RNase inhibitor (Invitrogen), 10 units of reverse transcriptase (Qiagen Inc.), 5 units Taq polymerase (Qiagen Inc.), 1X PCR buffer, 10 mM dithiothreitol, 1XQs solution and 10 µM each of the dNTPs. The amplification conditions were: one cycle of 45 min at 42°C, 40 cycles of 30 s at 94°C, 2 min at 46°C and 1 min at 72°C and one cycle of 5 min at 72°C. Following PCR, 10 µl of reaction products was analysed on 1% agarose gel and visualized using UV transilluminator.

Cloning and sequencing of amplicons

PCR amplified amplicons were gel purified using commercial kit (Qiagen Inc.) and cloned in pGEM-T Easy vector (Promega). These clones were sequenced bidirectionally at commercial facility (Xcelris Genomics, Ahmedabad, India). Sequences were initially analyzed using NCBI-BLAST.

In silico sequence analysis

The sequences of two PRSV isolates were compared with the corresponding partial Nlb nucleotide sequences of diverse PRSV isolates (both P type and W type) reported from different geographical locations of the world (accession number of PRSV isolates are mentioned in Fig. 3, 4). Sequence identity matrix for pairwise combination of aligned sequences was calculated with BioEdit Sequence Alignment Editor 7.1.3 (Hall, 1999). Phylogenetic analysis was performed using MEGA6 (Tamura *et al.*, 2013) and p-distance method was used to compute evolutionary distances. Trees were constructed with phylogeny test by bootstrapping 1000 replicates. The evolutionary history was inferred by neighbor-joining (NJ) methods available in MEGA6.

RESULTS AND DISCUSSION

The PRSV incidence was recorded in all the districts of Tripura and Manipur which were surveyed for the disease. The typical symptoms of yellow mottling, vein-clearing of young leaves, blistering and leaf distortion were observed. Dark-green streaks and rings in the leaf stalks as well as stems of plants were observed in some parts of Tripura. Typical concentric rings along with C-shaped markings and mixture of light and darker green colour on fruits (mosaic-like symptoms) were also observed in some cases (Fig. 1).

The disease incidence varied from 22% to 51%. The incidence was highest in papaya orchards of West Tripura district (51%) followed by Imphal West (41.6%). Two selected PRSV isolates from Tripura and Manipur

Fig. 1. Symptoms of PRSV on papaya at different locations of NEH region

Fig. 2. RT-PCR amplification of symptomatic papaya samples amplifying 350 bp PRSV specific fragment of Nib region; M: 100 bp ladder, lane 1: Healthy control, lane 2: papaya sample PRSV-Mnp1, lane 3: papaya sample PRSV-Trp1

designated as PRSV-Mnp1 and PRSV-Trp1 respectively were mechanically transmitted to *Chenopodium amaranticolor* and papaya varieties (Red Lady, Manipur Local and RCTP-1). Mechanically inoculated papaya plants of all three varieties exhibited symptoms of mottling and shoestring on leaves after 20-24 days post-inoculation, however, symptoms were less prominent on the variety RCTP-1. A successful mechanical transmission of PRSV to *Carica papaya*, *cucurbita pepo*,

Cucumis stivus, and *Citrullus vulgaris* was reported by Zetter *et al.* (1968). Martinez *et al.* (2014) have also reported the mechanical transmission of PRSV on papaya and *Cucurbita pepo* with typical symptoms. The symptomatic young leaves in leaf dip preparation under electron microscopy revealed the presence of filamentous flexuous particles of size 800 x 12 nm (figure not shown). EM assay further confirmed the association of PRSV with infected papaya samples collected from Tripura and Manipur.

To further confirm the PRSV infection, RT-PCR using primer pair targeting conserved partial Nib region of *Potyvirus* genome was employed (Zheng *et al.*, 2008). RT-PCR successfully amplified conserved Nib genomic region of PRSV from naturally infected papaya samples collected from Manipur and Tripura. Amplicons of the expected size (350 bp) were obtained from both the infected samples whereas no amplification was observed in healthy plants samples, indicating the viral origin of amplified products (Fig. 2). The PCR products were cloned and sequenced. Analysis of obtained nucleotide sequences of PRSV-Mnp1 and PRSV-Trp1 revealed that both isolates were identical for conserved Nib region (Fig. 3). PRSV-Mnp1 and PRSV-Trp1 shared an identity of 80.2 to 84% with other PRSV isolates reported from different parts of the world. These isolates had a maximum sequence identity of 84% with Thai PRSV isolate (AY010722), 83.4% with other Thai PRSV isolate (AF405531) and 83.1% with PRSV isolates from Taiwan

Fig. 3. Two-dimensional colour-coded graphical representation of nucleotide sequence identities of PRSV isolates from NEH region and other parts of the world. The origin of PRSV isolates (numbers mentioned in figure); 1: PRSV-Mnp1 (North East India), 2: PRSV-Trp1 (North East India), 3: India, 4: India, 5: India, 6: Mexico, 7: Brazil, 8: Brazil, 9: Colombia, 10: Colombia, 11: Venezuela, 12: Hawaii, 13: Taiwan, 14: Taiwan, 15: Hawaii, 16: South Korea, 17: Thailand, 18: Thailand, 19: China, 20: China, 21: China, 22: China, 23: China, 24: Taiwan, 25: Thailand, 26: Taiwan, 27: Taiwan, 28: Taiwan, 29: South Korea, 30-37: taiwan. A scale with the percent colour code is presented

and Korea (Fig. 3). Interestingly not many variations for nucleotide sequences of partial Nlb genomic region were observed among PRSV isolates originating from different geographical regions of the world. This conserved Nlb region could not be used to differentiate among PRSV-P and W isolates, as the earlier reported PRSV isolates from Delhi (EF017707 and EU475877) which were biologically P and W type respectively were identical for this genomic region (Fig. 3). Earlier primers derived from conserved Nlb region of viral genome were utilized for detection of PRSV from naturally infected papaya samples originating from different locations (Sharma *et al.*, 2004). They also reported the occurrence of PRSV infection from Tamil Nadu based on amplification of CP coding region through RT-PCR. Neighbour joining (NJ) phylogenetic analysis using PRSV isolates (both P and W biotype) from different parts of the world indicated distinct segregation of Indian PRSV isolates (Fig. 4). Interestingly PRSV isolates from North East India (PRSV-Mnp1 and PRSV-Trp1) were in separate cluster distinct from group A (Asian PRSV isolates) and group B (American PRSV isolates) as reported by Ortiz-Rojas and Chaves-Bedoya (2017). Indian PRSV population was demonstrated to be genetically distinct as compared to

other isolates from different parts of the world (Jain *et al.*, 2004; Srinivasulu and Sai Gopal, 2011). Phylogenetic analysis clearly indicated that PRSV isolates from North East India characterized in this study were genetically distinct from those reported from the North and South India, thus adding another dimension to the genetic profile of Indian PRSV population.

Although widespread occurrence of PRSV from North, Central and South part of India have been reported, there was no conclusive evidence for the association of PRSV with papaya in North East India. The present study conclusively provided the molecular evidence for association of PRSV with ringspot disease of papaya in North East India. A high incidence of PRSV in Manipur and Tripura was recorded in the present study. Characterization of conserved Nlb genomic region of PRSV isolates from North East India in present study indicated the existence of genetically distinct isolates in this region. Further, full genome characterization of PRSV from North East India will shed more light on population genetics of PRSV in India. Although symptoms of PRSV disease on papaya were recorded since long back; to the best of our knowledge, this is the first conclusive

Fig. 4. Neighbor-joining (NJ) phylogenetic tree illustrating partial NlB region based relationship of PRSV isolates from NEH region with the PRSV isolates from different geographical regions of world. The evolutionary history was inferred by NJ method based on the Tamura-Nei model. Bootstrap support values from a NJ search with 1000 replicates are shown at the nodes of the branches. Accession number of PRSV isolates is mentioned

molecular evidence for the association of PRSV from NEH region.

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