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Partial Sequence Comparison of P1 Proteinase Gene of PRSV Infecting Papaya from Southern Karnataka

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Abstract

GKVK-Bengaluru isolate of Papaya Ring Spot Virus (PRSV) showed highest nucleotide identity of 88 per cent with isolate from Hyderabad (Accession KP743981) and 87 per cent nucleotide identity with PRSV P isolate from Delhi (Accession EF017707) when the partial sequence of P1 proteinase gene was compared. Close relationship among Indian isolates was noticed from the phylogenetic tree constructed based on available sequences and Indian isolates were separated from isolates of other geographical regions forming a different sub-cluster in the phylogenetic tree. The clustering pattern of isolates correlated well with their geographical origins except for one isolate PRSVR3 (Accession KJ755852).

Keywords: Papaya ring spot virus (PRSV), proteinase, phylogeny and genetic diversity

Papaya (*Carica papaya* L.) belongs to family Caricaceae, is an important fruit crop because of its great economic potential. Papaya Ring Spot Virus disease (PRSV) caused by PRSV-P has gained global importance in all the papaya growing countries. The ring spot virus disease is posing a major threat to papaya cultivation throughout India by rendering orchards economically unproductive. Infected Papaya plants shows yellowing, leaf distortion and severe mosaic. Oily or water-soaked spots and streaks appear on the trunk and petioles. The fruits exhibit bumps and the classic ringspot symptoms. Severe isolates are known to cause tissue necrosis (Gonsalves *et al.,* 2010). PRSV infection drastically reduces fruit yield, fruit size and quality. The disease is found to cause losses up to 100 per cent in some parts of the globe (Tennant *et al*., 2007). There is a great scope to increase the papaya productivity if the ring spot virus disease is combated.

PRSV has a monopartite linear single-stranded positive sense RNA genome and is about 10,326 nucleotides long, excluding a poly-A-tract found at its 3' end. PRSV genome encodes a single large protein which is subsequently cleaved into smaller proteins *viz.,* P1, HC-Pro, P3, CI, 6K, NIa-Pro, NIb and CP. P1 codes for a 63 KD protein which is a proteinase and also possibly involved in cell-to-cell movement (Gonsalves *et al.,* 2010).

Knowledge of sequence diversity among isolates of a virus, their distribution has the potential to deepen our understanding of viral origin, disease etiology, development and dispersal. This information would be useful in the development of effective management programmes against viral diseases. Sequence data needs to be generated from isolates of different regions of the country to provide the complete PRSV population profile which could allow critical assessment of sequence divergence within the PRSV population. The comparative studies of isolates in respect to genome sequences would provide data about the complexity in PRSV populations in the country and also help to trace strain phy-logeny for better understanding of the evolution and molecular epidemiology of PRSV. With PRSV, most of the studies have been focused on examining variation in the coat protein gene sequences and data on the evolution and molecular epidemiology of PRSV based on P1 proteinase gene are limited. Hence, the present study reports sequence comparison analysis P1 proteinase gene to ascertain the ge-netic diversity of PRSV isolate in the Southern Karnataka (Bengaluru).

Material and Methods

Virus isolate and maintenance: Papaya seeds (Red lady) were sown in plastic sprouting trays. Twenty days after germination, seedlings were transplanted

to polyethylene covers containing soil + sand + coir pith and FYM (1:1:1:1) and were maintained inside insect proof cages at Department of Plant Pathology glass house. The leaf samples with characteristic PRSV symptoms were collected and sap inoculated to healthy seedlings using standard mechanical transmission procedure. Papaya leaves infected by PRSV were collected, washed under tap water, blot dried and ground with 0.1M phosphate buffer (pH 7.5 at 1gm / ml) using mortar and pestle. The sap was filtered through double layered muslin cloth and filtrate was mixed with a pinch of celite powder (600 mesh). A piece of sterile non-absorbent cotton pad was dipped in filtrate and was rubbed unidirectionally from petiole towards the margin of papaya leaves. The inoculated leaves were washed after 5 minutes with a jet of water to remove the traces of celite. Sap inoculated seedlings were brought to glass house and individually maintained under insect proof cages. Plants were observed daily for symptom development and symptoms expressed were recorded.

Symptomatology and confirmation of PRSV infec-tion in isolate: The identity of the virus was initially confirmed by symptoms developed on papaya. The presence of PRSV in the different isolates was further verified by double antibody sandwich ELISA with anti-PRSV capture antibody and ALP labelled anti-PRSV detection antibody.

Isolation of RNA: Isolation of total RNA from infected plants was done by using Trizol reagent. All the plastic wares and glasswares were washed thoroughly, dried and treated with 0.1 per cent DEPC (Diethylene pyrocarbonate) water by dipping for 24 hours and used after sterilization. The PRSV infected papaya leaf samples maintained under glasshouse were brought under ice-cold condition and ground to a fine powder in sterilized and dried pre chilled mortar and pestle using liquid nitrogen. About 100 mg of powdered leaf material was taken into a 1.5 ml micro centrifuge tube and homogenated partially using a homogenizer. Immediately 1ml of Trizol was added to the homogenized tissue. The tubes were centrifuged at 9,000 rpm for 10 minutes to remove extracellular material. Without disturbing the pellet, supernatant was transferred to fresh tube and kept at room temperature. 200 µl of chloroform + phenol (1 ml chloroform : 1 ml phenol) was added to the supernatant. After 15 minutes of shaking, tubes were centrifuged at 12,000 rpm for 8 minutes. Three distinct layers were formed, from which only the top layer was transferred to fresh tubes. 0.5 ml of Isopropanol was added to each tube, followed by 10 minutes incubation at room temperature. The tubes were then centrifuged at 13,000 rpm for 5 minutes and the pellet was collected discarding supernatant. 75 per cent ethanol (750 µl+250 µl H_2O) was added to the pellet and tubes were centrifuged at 12,000 rpm for 2 minutes. After centrifugation, ethanol was discarded, and pellet was vacuum dried for 10 minutes. 20 µl of DEPC treated water was added to each tube and they were incubated at 55-60 ^{0}C on a water bath to dissolve the pellet. RNA thus obtained was stored at -20 ^{0}C.

Reverse Transcription: Total RNA from healthy and PRSV infected samples were taken for reverse transcription along with negative control (distilled water). Primers for amplification of P1 proteinase gene were designed using NCBI primer blast tool with sequences from Hyderabad isolate (Accession No: KP743981). 20 µl RT mixture was prepared by adding the following ingredients into the PCR tube. 5X RT buffer 4 µl, 25 mM $MgCl_2$ 1.0 µl, 10.0 mM dNTP mixture 2.0 µl, reverse primer 5'TCTTTCCGAACTTGAGTTGCT3' (10 µM) 2.0 µl, Reverse Transcriptase 0.5 µl (50 units), Viral RNA 5.0 µl (1:10 diluted with water) and finally volume was made with 5.5 µl DEPC treated distilled water. The RT-PCR mixture was reverse transcribed at 39 ^{o}C for 60 min. and then at 94 ^{o}C for 5 min. The c-DNA thus obtained was used for performing PCR.

Polymerase chain reaction: The c-DNA obtained was subjected to PCR amplification using forward primer designed to amplify PRSV nucleotides from total RNA extracted from infected papaya plants. PCR amplifications were conducted using Eppendrof thermo-cycler in 15 µl reaction mixture that contained 2.0 µl c-DNA, 0.2 µl Taq DNA polymerase (5 U/µl), 2.5 µl of 10X PCR buffer, 0.5 µl of 25 mM $MgCl_2$, 2.0 µl forward primer 5'CAATTCGAAGCAACCAAACAAAT3' (10 µM), 2.0 µl dNTPs mix (2.5 mM each) and DEPC treated distilled water to make up the volume. The mixture was subjected to one cycle of initial Denaturation at

94 ^{0}C for 4 minutes followed by 35 cycles of denaturation at 94 ^{0}C for 60 seconds, annealing at 46.00 ^{o}C for 45 seconds, extension at 72 ^{0}C for 90 seconds and a final extension at 72 ^{0}C for 10 min. After the completion of the reaction, the products were kept at 4 ^{0}C prior to gel analysis.

Analysis of PCR products by agarose gel electrophoresis: Amplification was confirmed by agarose gel electrophoresis.

Sequencing of amplified PCR product and sequence analysis: After successful confirmation, the amplified PCR product was directly sequenced using ABI 3730Xl DNA Analyzer available at Scigenome Labs Pvt. Ltd., Cochin-Kerala, India. Sequencing was done in both directions using forward and PRSV reverse primers.

Construction of Phylogenetic tree: The sequence homology obtained in BLAST (www.ncbi.nih.gov / BLAST) and Neighbor joining phylogenetic tree was generated using MEGA 6.06 software tool. In order to calculate the confidence limits placed in construction of phylogenetic tree, bootstrapping analysis was carried out using 1000 replicates, resulting in a boot strapped Neighbor joining tree.

Results and Discussion

Symptomatology and confirmation of PRSV infection: Virus induced symptoms typical of PRSV infection on papaya with severe mosaic and leaf distortion on papaya cv. Red lady. The isolate strongly reacted in DAS-ELISA with PRSV-P polyclonal antibody confirming the identity of PRSV in the inoculated plants. After confirming the identity, isolate was maintained on their natural host pa-paya in an insect-proof glasshouse by mechanical inoculation.

Amplification and sequencing of P1 proteinase gene: Total RNA from PRSV infected papaya leaves was extracted using Triazole method and cDNA was synthesized through reverse transcription. Part of P1 proteinase gene was amplified and an expected ~1000 bp band was confirmed through electrophoresis (Plate 1). After confirmation further PCR amplifications were carried out with 50 µl reaction mixture and products were send for sequencing.

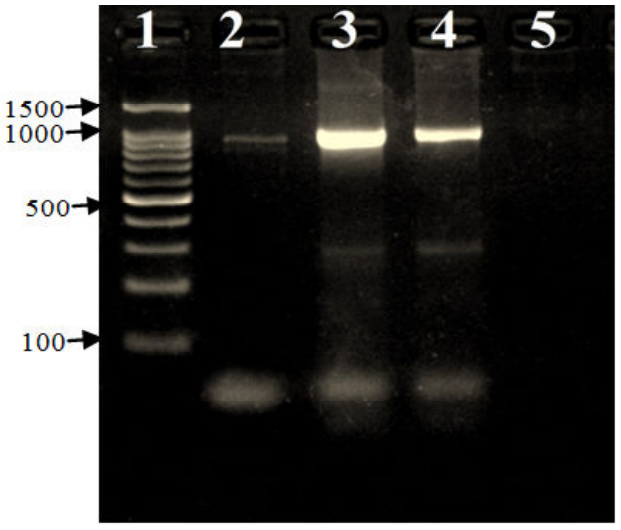

Plate 1: Amplified PCR product of P1 proteinase gene of GKVK-Bengaluru isolate

Lane 1: 100bp DNA ladder, 2: Healthy Papaya sample, 3&4: GKVK Bengaluru isolate and 5: Water Control

The sequence homology obtained in BLAST revealed the query matching with reported PRSV P1 proteinase gene sequences from different geographical locations. GKVK-Bengaluru isolate showed highest identity of 88 per cent PRSV isolate from Hyderabad (Accession KP743981) and 87 per cent identity with PRSV P isolate from Delhi (Accession EF017707). It showed 84 per cent identity with PRSV W isolate from India (Accession EU475877) followed by 79 per cent identity with PRSV strain Leaf deformation (Accession DQ340769) and PRSV isolate pFT3NP (Accession JX448373). BLAST revealed that it was distinct from the isolates of New Delhi and Hyderabad.

The nucleotide sequences of the GKVK-Bengaluru isolate (PRSV BLR) was compared to sequences of nineteen other PRSV isolates available in the gene bank (Table I). Indian isolates were separated from isolates of other geographical regions forming a separate sub-cluster in phylogenetic tree (Fig. 1) and clustering pattern of isolates correlated well with their geographical origins. The close relationship among Indian isolates was noticed from the phylogenetic tree constructed based on available sequences and generally clustering pattern of isolates correlated well with their geographical origins except for one isolate PRSVR3 (Accession KJ755852).

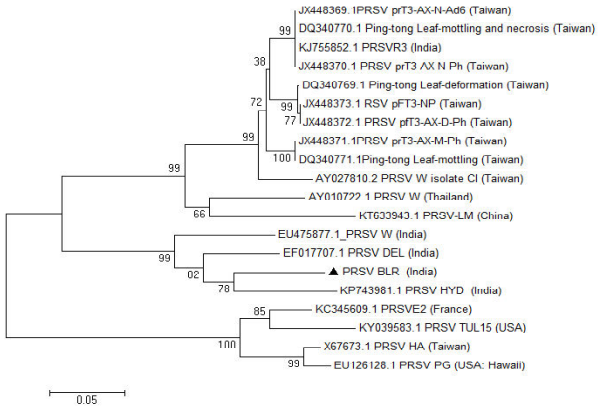

Fig.1: Bootstrapped neighbor joining phylogenetic tree showing the relationship of PRSV isolates

A nucleotide BLAST search using the complete genome of PRSV-W-TUL15 showed 83 to 92 per cent nucleotide sequence similarities with the published PRSV isolates (Ali, 2017). Between the P1 proteins of Taiwanese and Hawaiian PRSV isolates there was only 70.9 per cent nucleotide identity (Wang and Yeh, 1997). The comparative analysis of a severe isolate of papaya ringspot virus (Mex-VrPO) of Mexico and five others reported before showed that P1 was most variable with 13-33 per cent divergence while coat protein showed only 5-9 per cent divergence (Noa-Carrazana *et al*., 2007). High variability in the P1 proteinase gene has also been reported for other

TABLE I

Details of the Papaya ring spot virus isolates obtained from NCBI database

Isolate	Pathotype	Location	Genbank accession no.	Host	References
PRSV-BLR	P	GKVK-Bangalore	-	Papaya	This study
HYD	Not Specified	Hyderabad: India	KP743981	Papaya	Unpublished
DEL	P	India	EF017707	Not Specified	Parameswari *et al.*,2007
PRSV W (India)	W	India	EU475877	Not Specified	Mangrauthia *et al.*, 2008
Ping-tong Leaf-deformation	Not Specified	Taiwan	DQ340769	Papaya	Unpublished
pFT3-NP	P	Taiwan	JX448373	Papaya	Unpublished
pfT3-AX-D-Ph	P	Taiwan	JX448372	Papaya	Unpublished
prT3-AX-N-Ad6	P	Taiwan	JX448369	Papaya	Unpublished
prT3-AX-M-Ph	P	Taiwan	JX448371	Papaya	Unpublished
Ping-tong Leaf-mottling	Not Specified	Taiwan	DQ340771	Papaya	Unpublished
Ping-tong Leaf-mottling and necrosis	Not Specified	Taiwan	DQ340770	Papaya	Unpublished
PRSVR3	Not Specified	India	KJ755852	Papaya	Unpublished
PRSV W (Thailand)	W	Thailand	AY010722	Not Specified	Attasart *et al.*, 2002
PRSV W isolate CI	W	Chiayi: Taiwan	AY027810	*Luffa cylindrica* (Linn.) Roem.	Unpublished
prT3-AX-N-Ph	P	Taiwan	JX448370	Papaya	Unpublished
PRSV-LM	P	Hainan, Lingshui, China	KT633943	Papaya	Unpublished
PRSV E2	Not Specified	France	KC345609	*Cucurbita pepo*	Romay *et al.*, 2014
PRSV HA	Not Specified	Taiwan	X67673	Not Specified	Wang *et al.*, 1994
PRSV PG	Not Specified	USA: Hawaii	EU126128	Not Specified	Unpublished
TUL15	WUSA	KY039583	Gourd	Ali, 2017	

Potyviruses like Zucchini yellow mosaic virus (ZYMV). Lin *et al*. (2001) reported that in ZYMV isolate TW-TN3 P1 proteinase gene was most variable, with amino acid identities of 59.0-93.2 per cent.

With PRSV most of the earlier works concentrated on the coat protein gene sequences while Romay *et al.,* 2014 observed that pairwise sequence similarities in the coat protein (CP) coding region failed to unambiguously distinguish Zucchini tigre mosaic virus **(**ZTMV) isolates from PRSV isolates. Sequence variability has important implications for the use of genes to develop transgenic plants by pathogen derived resistance and such resistance could be highly sequence specific. The selection of the transgene would be vital step to develop long-lasting virus resistant transgenic papaya. It has been suggested that when designing transgenes for potyvirus resistance, it is essential to select regions of at least 90 per cent identity between strains to obtain a durable resistance (Moreno *et al.,* 1998). RNA mediated resistance to potyviruses has been reported with sequence identity of 88 per cent or greater (Mueller *et al.,* 1995), while Jones *et al*. (1998) reported that 89 per cent identity of the Nib gene was the minimum sequence identity for the specific-ity required to trigger gene silencing in the pea seed-borne mosaic potyvirus.

Genetic engineering is a viable option for managing viral diseases such as PRSV (Kung *et al.,* 2009; Mangrauthia *et al.,* 2010 and Yu *et al.,* 2011). Knowledge of the nucleotide sequence and genetic diversity is necessary to select a virus gene for the development of pathogen derived resistance. Recombinations occur in the majority of RNA viruses, are of great evolutionary importance and constitute one of the greatest forces that shape the virus genomes (Sztuba-Solinska *et al.,* 2011). The hot spots of recombination in PRSV were concentrated in the region encoding the P1 proteinase, P3 protein, cytoplasmic inclusion (CI) and the Helper component proteinase (Mangrauthia *et al.,* 2008). Recombination events in the coat protein of PSRV appeared to be less frequent than in other regions of the genome (Bateson *et al.,* 2002). Zhu *et al*. (2016) reported that nucleotide BLAST analysis of the coat protein sequence of PRSV from China, showed highest identity of 99 per cent with four isolates from Taiwan. According to Srinivasulu and Sai Gopal (2011) coat protein gene of PRSV TA-Ti isolate seemed to be an ideal choice to develop transgenic papaya resistant to PRSV in south India. These evidences confirm that coat protein gene of PRSV is more conserved than P1 proteinase gene hence it is a suitable candidate to develop transgenic papaya resistant to PRSV using Pathogen derived resistance.

Identification of gene sequences in PRSV could provide valuable information on the sequence of events that lead to infection and will lead to a better understanding of the significance of changing hosts during molecular evolution of PRSV, an essential requirement for the development of long-term sustainable management strategies against PRSV. The present study confirms the observation that P1 gene is highly variable, hence it does not seem to be an ideal choice to develop transgenic papaya resistant PRSV using Pathogen derived resistance. However, sequences of more isolates are needed to ascer-tain the complete population profile and to draw strong conclusions on sequence divergence within the PRSV population in the Indian sub-continent.

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