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P1 protein (Partial) sequence comparison of South Indian isolates of *Papaya ringspot virus*

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Abstract
Isolates of *Papaya Ring Spot Virus* (PRSV) from South India (Andhra Pradesh, Karnataka, Kerala and Tamil Nadu) were collected and maintained on Papaya *var.* Red Lady. Upon inoculation, all the PRSV isolates developed severe symptoms of mosaic, puckering, shoestring, leaf distortion, stunted growth, leading to the deterioration and death of the plants. Part of P1 region of four PRSV isolates were sequenced, and variability in the P1 region was obvious from the results of BLAST analysis. PRSV Bengaluru (Karnataka) isolate showed highest nucleotide identity of 88.80 % with PRSV isolates Pune (Aundh) PM-I (Accession no. MF405296), while Coimbatore (Tamil Nadu) isolate showed highest nucleotide identity of 94.47% with PRSV Bengaluru isolate (MK604243). Similarly, PRSV Ernakulam (Kerala) isolate shared highest nucleotide identity of 88.97% with Pune isolate (MF405299) whereas PRSV Tiruapti (Andhra Pradesh) isolate showed maximum nucleotide identity of 93.41 with PRSV Bengaluru isolate (MK604243). The nucleotide sequences of the PRSV isolates were compared to each other and with the sequences of seventeen other PRSV isolates from different geographical locations. The close relationship among Indian isolates was noticed from the phylogenetic tree while clustering pattern of PRSV isolates correlated with their geographical origins. Indian PRSV isolates formed a distinct clade and were grouped separately from isolates of other countries.

Keywords: *Papaya ringspot* virus, genetic diversity, P1 protein and phylogenetic tree

Introduction
Papaya (*Carica papaya* L.) is an important plant, common in the home gardens of tropical and sub-tropical areas. It is also a major fruit crop in different parts of the world. Papaya is one of the few crops that bear fruit around the year and the fruits can be harvested within a short time, offering quick and attractive returns when compared to many other fruit crops (Chauhan and Chatterjee, 2005) [6]. Papaya fruit ranks second only to mango as a source of beta carotene. A decent source of natural sugars, vitamin C, Calcium and phosphorus, papaya is a favorite of many people (Saravanan *et al.,* 2004) [23]. *Papaya ringspot virus* (PRSV) causes devastating disease in tropical and subtropical regions including India, resulting in unacceptable yield losses (Gonsalves, 1998) [7]. The disease is a major bottleneck in papaya production throughout India and leaves affected gardens unproductive. *Papaya ringspot virus* belongs to the genus *Potyvirus* of the family *Potyviridae* which is non-persistently transmitted by a number of aphids. Typical of potyviruses, PRSV has a monopartite linear single-stranded positive sense RNA genome, about 10,326 nucleotides long, excluding a poly-A-tract found at its 3' end. PRSV genome encodes a single large protein (3,344 amino acids) which is then cleaved into smaller proteins with various functions. The proteins include P1, HC-Pro, P3, CI, 6K, NIa-Pro, NIb and CP. Among these P1 codes for a 63 KD protein which is a Proteinase and also likely involved in cell-to-cell movement (Gonsalves *et al.,* 2010) [8] Valli *et al.* (2007) [27] suggested that P1 is also related to infectivity, disease resistance, diversification and evolution.
Information on sequence diversity among different isolates of a virus and their distribution is vital in broadening the knowledge on viral origins, evolution, dispersal and disease development. In countries like India, where the geographical and climatic conditions varies to a great extent, additional sequence data need to be generated from isolates from different regions to decipher the complete PRSV population profile, and allow enhanced assessment of the sequence divergence within the PRSV populations. Such knowledge is beneficial in evolving prospective management strategies. The genome sequence comparative studies of isolates will also provide data about the intricacy in PRSV populations in the subcontinent, and assist to draw phylogeny for improved understanding of the evolution and molecular epidemiology. With PRSV, a number of earlier studies have been concentrated on examining sequence variation in the coat protein sequences

(Jain *et al.,* 1998; Hema and Prasad, 2004; Jain *et al.,* 2004 and; Srinivasulu and Sai Gopal, 2011) [11, 10, 12, 26]. Data on the evolution and molecular epidemiology of PRSV based on P1 sequences are limited, as variability analysis has been performed mainly on north Indian isolates and a few south Indian isolates. Hence, it is essential to have the sequence data of P1 region, and to determine the level of variability among PRSV isolates. The present study reports the partial nucleotide sequences of the P1 region of PRSV isolates from four South Indian states (Andhra Pradesh, Karnataka, Kerala and Tamil Nadu) in south India and sequence comparison analysis to determine the genetic diversity among PRSV isolates.

Matrial and method

Virus isolates and maintenance: Virus isolates used in this study were collected from commercial papaya orchards (Andhra Pradesh, Karnataka and Tamil Nadu) and kitchen gardens (Kerala) during 2015. Leaves from papaya plants showing typical symptoms were collected from different locations *viz*., Tirupati (Andhra Pradesh), Bengaluru (Karnataka), Ernakulam (Kerala) and Coimbatore (Tamil Nadu). The virus isolates were maintained in the glasshouse inside insect proof cage by repeated mechanical inoculation.

Symptomatology and confirmation of PRSV infection in isolates: The identity of the virus was initially established by pathogenicity tests on papaya based on the typical symptoms developed. The presence of PRSV was confirmed by performing double antibody sandwich (DAC-ELISA) technique with anti-PRSV capture antibody and ALP labelled anti-PRSV detection antibody (Agdia, USA).

Isolation of RNA from PRSV infected papaya plants: PRSV isolates were further characterized by sequencing part of the P1 region to assess the variability among the isolates. Isolation of total RNA from selected isolates of PRSV was done using Trizol method. All the plastic wares and glasswares were washed thoroughly, dried and treated with 0.1 per cent Diethylene pyrocarbonate (DEPC) by dipping for 24 hours and used after sterilization. The PRSV infected papaya leaf samples 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 using mortar and Pestle. Immediately 1ml of trizol was added to the homogenized tissue and the tubes were centrifuged at 9,000 rpm for 10 minutes. Without disturbing the pellet, supernatant was transferred to fresh tube and kept at room temperature. 200 µl of chloroform + phenol (1:1) 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 the top layer was transferred to fresh tubes. 0.5 ml of isopropanol was added to each tube, followed by 10-minute 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 ˚C on a water bath to dissolve the pellet. RNA thus obtained was stored at -20 ˚C, till further use.

Reverse Transcription: Total RNA from healthy and PRSV infected samples were taken for reverse transcription along with positive control and negative control (distilled water). 20 µl RT mixture was prepared by adding 5x RT buffer-4 µl, 25 mM $MgCl_2$-1.0 µl, 10.0 mM dNTP mixture-2.0 µl, reverse primer - 5'CCCCACACATTGTAACGTCCA3' (10 µM)- 2.0 µl, reverse transcriptase 25 units (TaKaRa primescript reverse transcriptase), 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˚C for 60 minutes and then at 94 ˚C for 5 minutes. The c-DNA thus obtained was used for performing PCR.

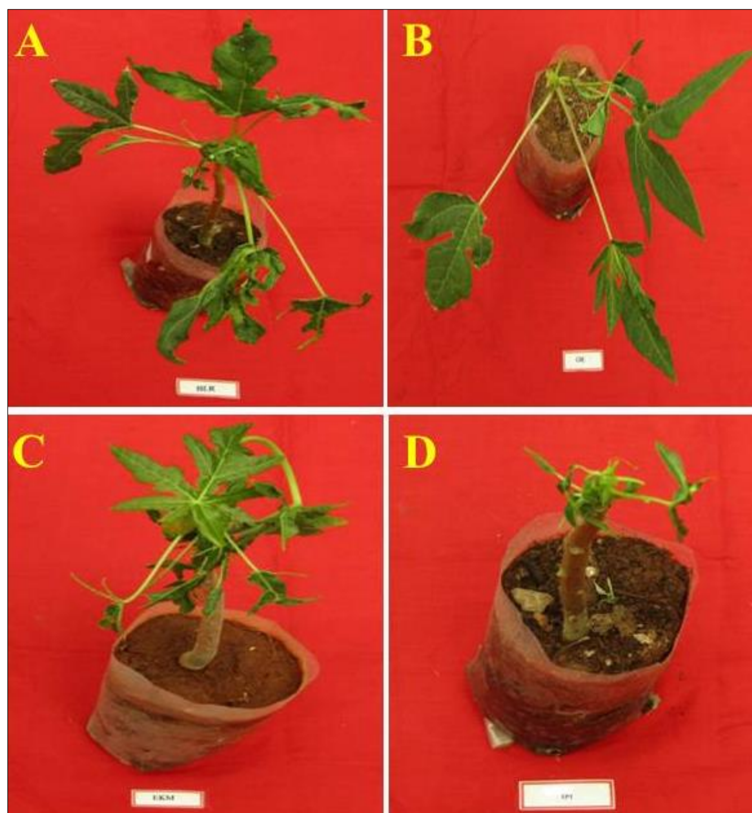
Amplification using Polymerase chain reaction: The c-DNA obtained was subjected to PCR amplification using Eppendrof thermo-cycler in 15.0 µl reaction mixture that contained c-DNA-2.0 µl, Taq DNA polymerase (1 unit)-0.20 µl, 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$)-2.5 µl, of 25 mM $MgCl_2$-0.5 µl, forward primer 5'TTGGAGTGCTAGCCTTGAGTT3' (10 µM)- 2.0 µl, dNTPs mix (2.5 mM each)-2.0 µl and DEPC treated distilled water to make up the volume. The mixture was subjected to one cycle of initial denaturation at 94˚C for 4 min followed by 35 cycles of denaturation at 94 ˚C for 60 sec, annealing at 54 ˚C for 45 sec, extension at 72 ˚C for 90 sec and a final extension at 72 ˚C for 10 min. After the completion of the reaction, the products were kept at 4 ˚C till agarose gel electrophoresis.

Analysis of PCR products by agarose gel electrophoresis: Amplification by polymerase chain reaction was confirmed by performing agarose gel electrophoresis [2% (w/v) in TAE buffer at 15 V cm^{-1}] of 5 µl of amplicon and 5 µl DNA marker (Fermentas Life Sciences, Germany). The gel was stained in Ethidium Bromide solution (0.5 µg ml^{-1}) and visualized in a gel documentation system (GelDoc EZ imager of Biorad).

Sequencing of amplified PCR product and sequence analysis: After successful confirmation, the amplified PCR product was directly sequenced using ABI 3730Xl DNA analyzer by outsourcing to Scigenome labs Pvt. Ltd., Cochin-Kerala, India. Sequencing was done in both directions using forward and reverse primers. 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

Symptomatology and confirmation of PRSV infection in isolates: All the four isolates induced typical symptoms on Papaya *var.* Red Lady. Upon sap inoculation all the four isolates developed mosaic, severe puckering, shoestring, leaf distortion and stunted growth (Fig. 1). None of the isolates showed mild symptoms and all of them finally lead to the deterioration and death of the inoculated plants. The isolates could not be differentiated based on the symptoms they produced. All isolates strongly reacted in DAC-ELISA with PRSV-P polyclonal antibody, thus confirmed the presence of PRSV in all collected isolates. After confirming their identities, isolates were maintained on their natural host papaya in an insect-proof glasshouse by mechanical inoculation. The isolates were designated with code derived from alphabets of the places from where they were collected.



A. Bengaluru (BLR), B. Coimbatore (CBE), C. Ernakulam (EKM) and D. Tiruapti (TPT)

Fig 1: Development of symptoms upon inoculation of PRSV isolates on papaya variety Red lady

Amplification and sequencing of P1 region of PRSV isolates: Total RNA from PRSV infected papaya leaves of different isolates *i.e.,* Bengaluru (BLR), Coimbatore (CBE), Ernakulam (EKM) and Tirupati (TPT) were extracted using Triazole method and cDNA was synthesized through RT-PCR. Isolates were amplified using specific PRSV forward (5'-TTGGAGTGCTAGCCTTGAGTT-3') and PRSV reverse (5'-CCCCACACATTGTAACGTCCA-3') primers and an expected ~ 900 bp band was confirmed in electrophoresis (Fig.2). The amplified products were sequenced.

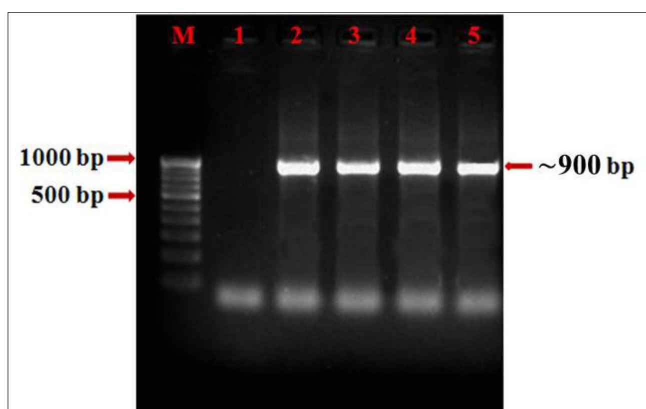


Fig 2: Amplified PCR product of partial P1 proteinase gene of South Indian PRSV isolates (M: 100 bp DNA ladder, 1: Healthy papaya sample, 2: PRSV-BLR isolate, 3: PRSV-CBE isolate, 4: PRSV-EKM isolate, 5. PRSV-TPT isolate)

Blast analysis: The sequence homology obtained in BLAST (www.ncbi.nih.gov /BLAST) revealed the query matching with reported PRSV coat protein sequences from different geographical locations (Table 1) and the sequence was deposited in NCBI GenBank. PRSV-BLR isolate (MK604243) showed highest nucleotide identity of 88.80 %with PRSV isolates Pune (Aundh) PM-I (Accession no. MF405296), followed by 88.69 per cent identity with PRSV isolate Pune (Aundh) VC (MF405299). It also showed 87.48% and 88.42 % nucleotide identity with PRSV isolates PRSV-HYD (KP743981) and Kaladgi K-12 (MF356491). PRSV-CBE (MK604245) showed highest nucleotide identity of 94.47% with PRSV-BLR isolate (MK604243) followed by 88.68 % and 88.42 % identity with PRSV isolate Pune (Aundh) VC (MF405299) and PRSV isolate Pune (Aundh) PM-I (MF405296) respectively. Similarly, it also showed 88.15 % and 87.87 % nucleotide identity with PRSV isolate Santhekellur S-13 (MF356482) and Kaladgi K-12 (MF356491) respectively.

BLAST search revealed that PRSV-EKM (MK604244) shared highest nucleotide identity of 88.97% with Pune (Aundh) VC (MF405299) followed by 88.07% identity with PRSV isolate Hulihyder S-23 (MF356487) while showing 87.13% nucleotide identity with PRSV-BLR (MK604243). PRSV-TPT isolate showed maximum nucleotide identity of 93.41with PRSV-BLR (MK604243) followed by 88.20% and 88.19 % nucleotide identity with PRSV isolates Pune (Aundh) VC (MF405299) and PM-I (MF405296) respectively. Likewise it also showed 87.52 % identity with PRSV isolate Hulihyder S-23 (MF356487) and 87.36 % identity with Kaladgi K-12 (MF356491). Overall nucleotide sequence similarities of isolates under present study varied from 71.77 to 94.47%.

Table 1: Per cent nucleotide identity among PRSV isolates from India

PRSV sequences deposited in NCBI GENBANK	Location	PRSV isolate(s)			
		BLR MK604243	CBE MK604245	EKM MK604244	TPT MK604246
PRSV BLR (MK604243)	Bengaluru, Karnataka	-	94.47	87.13	93.41
Pune (Aundh) PM-I (MF405296)	Pune, Maharashtra	88.80	86.45	87.48	86.54
Pune (Aundh) VC (MF405299)	Pune Maharashtra	88.69	88.69	88.97	88.20
Kaladgi K-12 (MF356491)	Bagalkot, Karnataka	88.42	87.87	86.93	87.36
PRSV-HYD (KP743981)	Hyderabad, Telangana	87.48	87.01	87.29	86.68
Santhekellur S-13 (MF356482)	Raichur, Karnataka	88.15	88.15	87.11	87.36
Bijapur K-20 (MF356495)	Vijayapura, Karnataka	88.01	87.73	86.34	86.94
Hulihyder S-23 (MF356487)	Koppal, Karnataka	87.87	87.75	88.07	87.52
PRSV- DEL (EF017707)	Delhi	87.00	86.31	83.21	85.59
Bheemarayanagudi K-30 (MF356496)	Yadagiri, Karnataka	86.33	86.45	86.21	86.54

Phylogenetic analysis: The nucleotide sequences of four isolates from South India (PRSV-BLR, PRSV-CBE, PRSV-EKM and PRSV-TPT) and sequences of seventeen other PRSV isolates available in the NCBI GenBank were used construct phylogenetic relationship. The close relationship among Indian isolates was noticed from the phylogenetic tree constructed based on available sequences and clustering pattern of isolates correlated with their geographical origins. Indian PRSV isolates formed a distinct clade and were grouped separately from isolates of other countries.

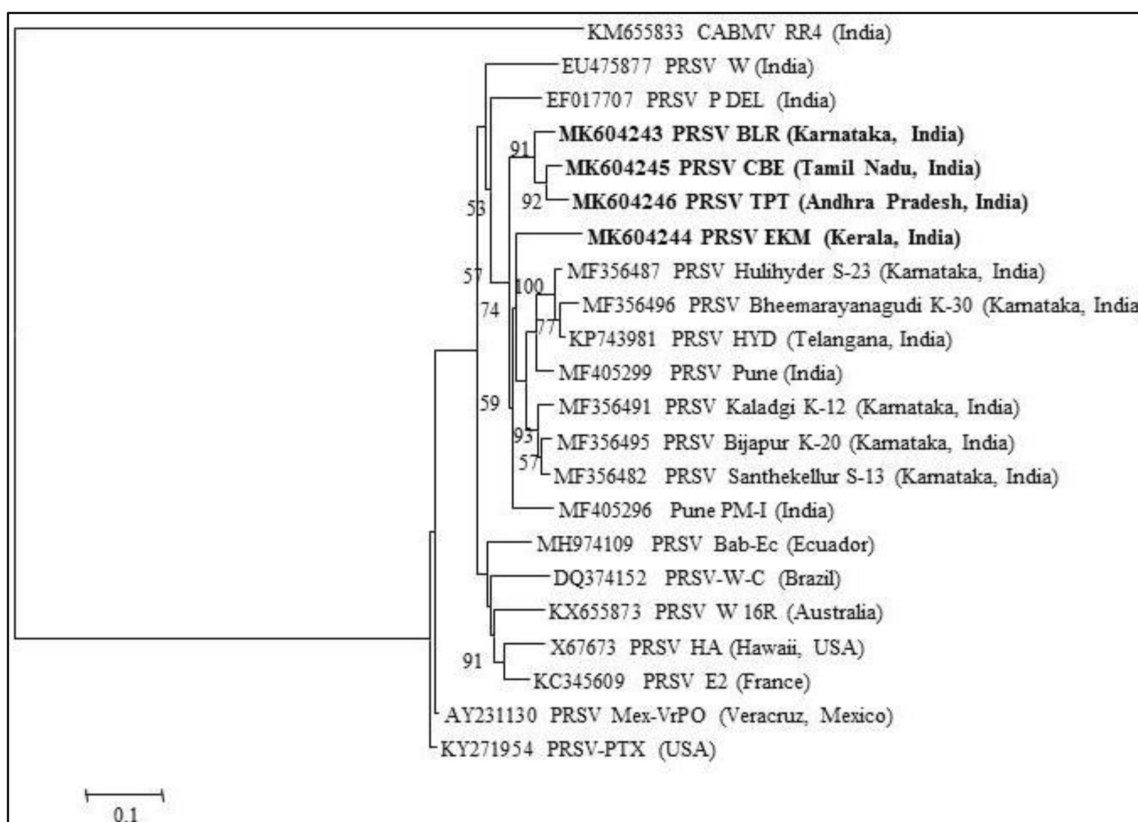


Fig 3: NJ Phylogenetic tree based on P1 sequences of PRSV isolates showing the phylogenetic relationship. Numbers at nodes represent bootstrap percentages. GenBank accession numbers are shown in parentheses. Bar 0.01 substitutions per nucleotide position.

The variability in the P1 region of PRSV has been reported earlier by several workers. A nucleotide BLAST search using the genome of PRSV-W-TUL15 exhibited 83 to 92 per cent nucleotide sequence similarities with the published PRSV isolates (Ali, 2017) [1]. Similarly Anil and Nagaraju (2017) [2] reported that PRSV GKVK-Bengaluru isolate showed highest nucleotide identity of 88 per cent with PRSV isolate from Hyderabad and 87 per cent nucleotide identity with PRSV Pisolate from Delhi when the partial sequence of P1 proteinase gene was compared. Between the P1 protein sequences of Taiwanese and Hawaiian PRSV isolates there was only 70.90 per cent nucleotide identity (Wang and Yeh, 1997) [28]. Similarly, Gulsiri *et al.* (2003) [9] reported that among ten putative proteins of PRSV, P1 was most variable (73.90 per cent similarity), whereas cytoplasmic inclusion (CI) protein was the most conserved with 99.10 per cent similarity. The comparative analysis of a severe isolate of PRSV (Mex-VrPO) of Mexico and five others reported before also confirmed that P1 was most variable with 13-33 per cent divergence, while coat protein sequences showed only 5-9 per cent divergence (Carrazana *et al.,* 2007) [5]. Mangrauthia *et al.* (2008) [16] reported that recombination in PRSV mostly take place in the region encoding the P1 protein, P3 protein, cytoplasmic inclusion (CI) and the helper component proteinase. Whereas recombinations in the coat protein of PSRV appear to be less frequent than in other regions (Bateson *et al.,* 2002) [3].

Higher variability in the P1 protein sequence has also been reported for other potyviruses like Zucchini yellow mosaic virus (ZYMV). Lin *et al.* (2001) [15] reported that in ZYMV isolate TW-TN3, P1 protein sequences was most variable with amino acid identities of 59-93.20 per cent. Due to higher

variability, P1 sequences would be a better choice, for assessing the variability among the PRSV isolates when compared to CP sequences. For instance Romay *et al.* (2014) [22] found that, pairwise sequence similarities in the CP coding region failed to unambiguously discriminate Zucchini Tigre Mosaic Virus (ZTMV) isolates from PRSV isolates.

It has been proposed that mutation along with long distance movement could be attributed for the variation observed among different isolates. One more possibility is the recombination events that happened earlier (Hema and Prasad, 2004) [10]. For example, Bedoya and Rojas (2015) [4] noticed recombination in the PRSV isolates from Colombia. Solinska *et al.* (2011) [25] also reported that one of the greatest forces that form the virus genomes are the recombination events that occurred especially in the RNA viruses. Greater sequence divergence within the PRSV population of the Indian subcontinent could also be ascribed to the extensive range of cropping systems and cultivation practices followed in diverse geographical regions. Such vast diversity might have applied different levels of selection pressure on PRSV (Jain *et al.,* 2004) [12]. Introduction of isolates from other geographical areas is another possibility for the natural variation.

Genetic engineering is one of the best choices for the management of viral diseases like PRSV (Kung *et al.,* 2009, Mangrauthia *et al.* 2010 and Yu *et al.,* 2011) [14, 17, 29]. Sequence variability plays crucial role in the exploitation of genes while developing transgenic plants by pathogen derived resistance, since such resistance could be particularly sequence specific (Savenkove and Valkonen, 2001) [24]. It is vital to select regions of at least 90 per cent identity between strains to obtain a durable resistance, while choosing transgenes for developing resistance to potyviruses (Moreno *et al.,* 1998) [19]. Mueller *et al.* (1995) [20] reported RNA mediated resistance against potyviruses with sequence identity of 88 per cent or greater, whereas Jones *et al*. (1998) [13] reported that *Nib* gene required a minimum of 89 per cent sequence identity for the specificity to trigger gene silencing against the pea seed-borne mosaic potyvirus. Rodriguez *et al.* (2008) [21] reported that genetic diversity of PRSV isolates could be large enough to be accounted, while designing management approaches like cross-protection and transgenic resistance. Martinez *et al.* (2014) [18] established that because of the variability among isolates, disease management by cross-protection and transgenic plants need the selection of region specific virus isolates for a particular country. Considering the conserved nature, Srinivasulu and Saigopal (2011) [26] reported that coat protein gene of PRSV TA-Ti isolate to be best choice for developing transgenic resistance to PRSV in south India. These evidences confirm that P1 protein region of PRSV is less conserved than coat protein sequences hence, coat protein would be a better choice to develop transgenic papaya resistant to PRSV using pathogen derived resistance.

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