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Characterization of betasatellite associated with the yellow mosaic disease of grain legumes in Southern India

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Summary. – Yellow mosaic disease caused by mungbean yellow mosaic virus (MYMV) belonging to the genus *Begomovirus* (the family *Geminiviridae*) is a major constraint in cultivation of grain legumes in India. The urdbean (*Vigna mungo* (L.) Hepper) and mungbean (*Vigna radiata* (L.) R. Wilczek) samples affected with yellow mosaic disease exhibits yellow mosaic symptoms along with leaf puckering and leaf distortion in Tamil Nadu. Hence the study was performed to find out if there was any association and influence of betasatellite DNA on the symptom expression of MYMV. Full length viral clones of DNA A and DNA B were obtained through rolling circle amplification from YMD infected samples and identified as mungbean yellow mosaic virus. Interestingly, betasatellite was found to associate with MYMV, and its nucleotide sequence analysis showed its 95% identity with papaya leaf curl betasatellite (DQ118862) from cowpea. The present study represents the first report about the association of papaya leaf curl betasatellite with MYMV and represents a new member of the emerging group of bipartite begomovirus associated with betasatellite DNA.

Keywords: yellow mosaic disease; grain legumes; leaf puckering; betasatellite; begomovirus

Introduction

Yellow mosaic disease (YMD) is a major threat to the cultivation of grain legumes in India. The disease affects the four major crops, urdbean, mungbean, cowpea and soybean and several other legumes like field bean, cowpea, horsegram and French bean incurring severe yield loss (Varma *et al*., 1992). YMD in India is caused by four species of begomoviruses, MYMV, mungbean yellow mosaic India virus (MYMIV), horsegram yellow mosaic virus (HgYMV) and dolichos yellow mosaic virus (DoYMV) (Malathi, 2007; Qazi *et al*., 2007b; Ilyas *et al*., 2010). They are typical bipartite begomoviruses belonging to the family *Geminiviridae* and consist of circular single stranded DNA genome encapsidated in twinned or geminate icosahedral particles (18 × 30 nm size) (Stanley, 1985). The bipartite begomoviruses have two DNA components, encapsidated separately, designated as DNA A and DNA B component. The DNA A component encodes for coat protein (ORF AV1/CP) on the viral strand and for a rolling circle replication initiator protein (ORF AC1, Rep), gene expression regulation protein (ORF AC2, TrAP), replication enhancer protein (ORF AC3, REn) and a PTGS suppressor protein (ORF AC4) on the complementary strand (Hanley-Bowdoin *et al*., 1999; Rojas *et al*., 2005; Stanley *et al*., 2005; Fauquet *et al*., 2008). The begomoviruses belonging to Old World (Harrison and Robinson, 1999) have an additional ORF, AV2 on the viral strand upstream of the coat protein gene. The DNA B component encodes for nuclear shuttle protein (NSP,

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Abbreviations: FbLCB = French bean leaf curl betasatellite; MYMV = mungbean yellow mosaic virus; MYMIV = mungbean yellow mosaic India virus; PaLcuB = papaya leaf curl betasatellite; RCA = rolling circle amplification; SCR = satellite conserved region; ToLCB = tomato leaf curl betasatellite; ToLCNDV = tomato leaf curl New Delhi virus; YMD = yellow mosaic disease

ORF BV1) in viral sense strand and the movement protein (MP, ORF BC1) in the complementary sense strand. The DNA A and DNA B components of a virus have a highly conserved non-coding intergenic region referred to as common region (CR), which contains a stem-loop structure with the loop containing the invariant nonanucleotide sequence TAATATTAC that represents the origin of viral strand replication (Hanley-Bowdoin *et al*., 1999).

In a majority of the Old World begomoviruses DNA A components are associated with satellite DNA, referred as betasatellites (earlier as DNAβ). Betasatellites are approximately half the size of the helper begomovirus (~1360 nt long). Betasatellites are dependent on their helper virus (DNA A) for replication, encapsidation and movement within plants and are required in many cases for symptom induction in the primary host from which they have been isolated (Briddon *et al*., 2001, 2003; Jose and Usha, 2003; Mansoor *et al*., 2003; Briddon and Stanley, 2006). Wherever the begomovirus is associated with betasatellites, another satellite is also found, and it is referred to as alphasatellite (Briddon *et al*., 2004; Nawaz-ul-Rehman *et al*., 2009).

The betasatellite DNA components characterized so far show certain typical characteristics (Briddon *et al*., 2003). They contain a highly conserved non-coding region called satellite conserved region (SCR) with ~150 nt encompassing a hairpin structure with the loop containing the nonanucleotide sequence TAATATTAC. Positionally conserved open reading frame is also present on the complementary strand, coding for a ~13 kDa protein called βC1 protein. An adenine rich (A-rich) region is also present upstream of βC1 coding region.

The role played by betasatellites in the viral pathogenicity is yet to be fully understood. As inoculation with betasatellite results in severe symptom expression (Jose and Usha, 2003; Saunders *et al*., 2004; Li *et al*., 2005), it is considered to be a symptom modulating molecule and a pathogenicity determinant (Saeed *et al*., 2005). It leads to helper viral DNA accumulation (Mansoor *et al*., 2003; Saunders *et al*., 2004) and can replace DNA B of a bipartite begomovirus required for systemic movement (Saeed *et al*., 2007). The βC1 protein has been shown to act as viral suppressor capable of knocking out the host RNAi defense (Cui *et al*., 2005; Gopal *et al*., 2007; Sharma *et al*., 2010).

Till date, betasatellites have been found to be associated with many members of monopartite begomoviruses. In recent years, number of betasatellites have been identified in association also with bipartite begomoviruses (Bull *et al*., 2004; Rouhibakhsh and Malathi, 2005; Qazi *et al*., 2007a; Guo *et al*., 2008; Sivalingam *et al*., 2010; Jyothsna *et al*., 2013b). The association of betasatellite with the bipartite begomovirus, MYMIV was reported in cowpea (Rouhibakhsh and Malathi, 2005) and subsequently with tomato leaf curl New Delhi virus (ToLCNDV). In both cases the presence of betasatellite led to more severe symptoms than inoculation of DNA A and DNA B components. This survey was conducted on yellow mosaic disease of urdbean and mungbean in different districts of Tamil Nadu during the year 2011–2012. The collected plants exhibited yellow mosaic, leaf distortion, enation and puckering symptoms. Research carried out on these plants revealed the association of betasatellite DNA with MYMV for the first time in naturally infected samples from South India.

Materials and Methods

Sample collection. The samples of urdbean and mungbean showing the symptoms of yellow mosaic, puckering and leaf distortion were collected in the districts of Coimbatore, Pudukottai and Tirunelveli, Tamil Nadu in the year 2011–2012.

Genomic DNA isolation and PCR. The total DNA was extracted from 100 mg of infected leaf tissues using the method developed by Rouhibakhsh *et al.* (2008) with 2% β-mercaptoethanol. The PCR was performed to detect the presence of begomovirus and betasatellites using begomovirus-specific degenerate primers, PALIc1960 (5′-TGGACTGCAGACNGGNAARACNATGTGGGC-3′) and PALIr772 (5′-ATATCTGCAGGGNAARATHTGGATGGA-3′) that flank the sequence of 772 to 1960 nt in the DNA A component (Rojas *et al*., 1993) and universal betasatellite-specific primer β01 (5′-GGTACCACTACGCTACGCAGCAGCC-3′) and β02 (5′-GGTACCTACCCTCCCAGGGGTACAC-3′) (Briddon *et al*., 2002). Reactions were performed in 25 µl mixture containing approximately 50 ng of genomic DNA, 5 mmol/l each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India). The reaction was carried out in Eppendorf epgradient S Master cycler (Eppendorf, Germany) programmed with initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final extension step at 72°C for 10 min. Amplicons were analyzed by electrophoresis in 1.5% agarose gel in TAE buffer and visualized by staining with ethidium bromide and recorded with an Alpha Imager 2000 (Alpha Innotech, USA). The sizes of the PCR products were determined by comparison with standard 1 kb DNA ladder (Fermentas, Lithuania).

Rolling circle amplification. Samples which were PCR positive with universal primers for begomoviruses were subjected to rolling circle amplification (RCA). In order to characterize the genomic components of the begomovirus, 70 ng of total nucleic acid was subjected to RCA with 10 units of phi29 DNA polymerase (Fermentas) 500 µmol/l of exo-resistant random hexamer primers (Fermentas)and 0.1 U of pyrophosphatase (Fermentas) (Haible *et al*., 2006).

Cloning of viral genome. RCA product (about 500 ng to 1µg) was subjected to restriction digestion with different endonucleases, *Hind*III, *Bam*HI, *Pst*I, and *Xba*I (Fermentas) to identify the

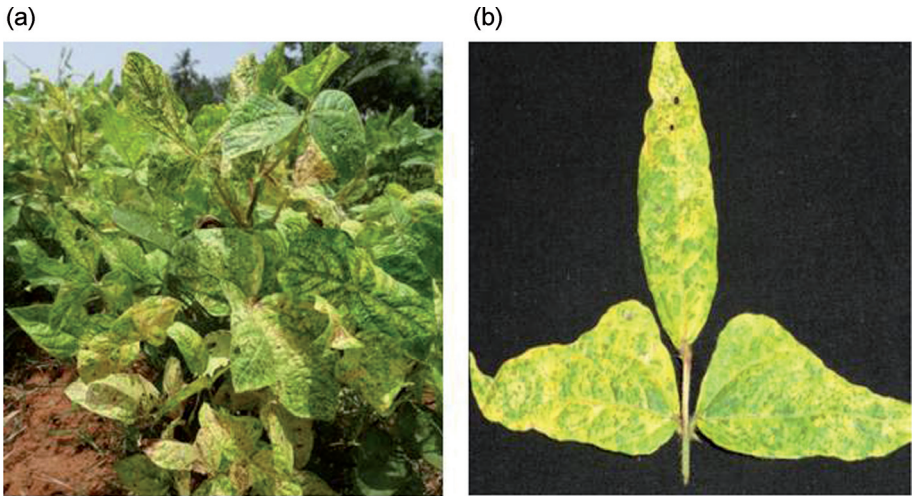

Fig. 1

Yellow mosaic disease affected plants showing yellow mosaic and leaf puckering symptoms

unique sites for cloning of viral genome. Restriction products of ~2.7 kb from *Hind*III and *Bam*HI were purified and cloned into pUC18 vector (Fermentas) and sequenced. For cloning of betasatellites, the RCA product was subjected to PCR using universal betasatellite-specific primers β01 and β02. PCR amplicons of ~ 1.3 kb were separated on 1% agarose gel and purified from the gel using QIAquick Gel extraction kit (QIAGEN, USA) in accordance with the manufacturer´s protocol. The purified PCR fragments were cloned into the pGEM-T easy vector (Promega, USA) according to the manufacturer´s protocol and the recombinant plasmids were used to transform *Escherichia coli* strain DH5α. The insertion of betasatellite was analyzed through colony PCR. The selected clones were sequenced.

Sequence analysis. Sequencing of the selected full length clones was done at Scigenom labs, India using primer walking method. Nucleotide similarity searches were performed by BLAST at NCBI. Complete nucleotide sequences of the full length genomes were aligned and percentage pair-wise identity matrix was generated in BioEdit program. Multiple sequence alignment was done using Clustal W (www.ebi.ac.uk) followed by phylogenetic analysis using MEGA 5.05 (www.megasoftware.net) and phylogenetic tree was constructed with the neighbor-joining algorithm, bootstrapped with 1000 replicates. The nomenclature for DNA A, DNA B and the betasatellites used here is according to the recommendation in the ninth report of ICTV (Kings *et al.*, 2011; Brown *et al.*, 2012).

Results

DNA was extracted from 74 infected plant samples showing severe yellow mosaic along with leaf puckering symptoms (Fig. 1). Twenty two samples out of 74 samples tested gave expected amplicon of ~1.1 kb with PALIc1960 and PALIr772 primers indicating the presence of begomovirus. When genomic DNA was subjected to PCR using betasatellite – specific primers, the amplification (~1.3kb) was seen in a single sample. Failure of amplification with DNA A-specific and betasatellite-specific primers was attributed to extremely low concentrations of the virus. Therefore the viral DNA components in the samples were enriched by RCA. The expected amplicons of ~1.3 kb were obtained in all the samples when RCA product was subjected to PCR (Fig. 2).

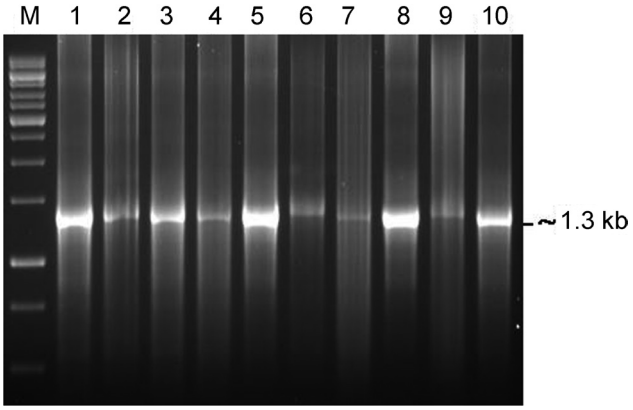

Fig. 2

Agarose gel electrophoresis showing detection of betasatellites from yellow mosaic disease affected plants

PCR amplicons of ~1.3 kb represent the presence of betasatellites. Lane M: 1 kb ladder; lane 1–10: YMD infected samples.

Cloning of viral genome

The RCA products obtained from urdbean samples were digested with five different restriction enzymes. Digestion of RCA product with *Hind*III and *Bam*HI yielded 2.7 kb fragments which were cloned and sequenced. Clones of betasatellite molecules were obtained by PCR mediated amplification from urdbean and mungbean samples and the selected clones of Coimbatore samples (CBE-BG; CBE-GG) and Vamban samples (VBN-BG) were sequenced.

Identification of genome components

Sequences of *Hind*III clone, *Bam*HI clones, and three betasatellite clones were analyzed in BLAST search program. The analysis clearly showed that the *Hind*III clone represented DNA A component and *Bam*HI clone belonged to DNA B component. The DNA A component showed 98% identity with MYMV (MYMV-Vam DQ400848) whereas the DNA B component exhibited 98% identity with DNA B component of MYMV-Vam, DQ400849 and MYMV-[KA34], AJ439057. Hence the virus in the present study was identified as MYMV.

Complete nucleotide sequence analysis of three betasatellite clones (one each from urdbean from Coimbatore, mungbean from Coimbatore and urdbean from Vamban) in BLAST search revealed 95% identity with papaya leaf curl betasatellite, PaLcuB – [India:Chinthapalli:2005, DQ118862]. The identity observed is higher than 78% identity kept as threshold value for demarcation of betasatellite species. The name papaya leaf curl betasatellite – [India:CBE:BG], papaya leaf curl betasatellite – [India:CBE:GG], and papaya leaf curl betasatellite –[India:VBN:BG] are proposed for the new betasatellites characterized in this study. The complete sequences of DNA A and DNA B and three clones of betasatellite have been deposited in the NCBI database under the

Table 1. Percentage nucleotide identity of papaya leaf curl betasatellites from legumes with other selected betasatellites

Betasatellites	PaLCuB-[IN:CBE:BG]	PaLCuB-[IN:CBE:GG]	PaLCuB-[IN:VBN:BG]
PaLCuB-[IN:CBE:BG]	100	99	99
PaLCuB-[IN:CBE:GG]	99	100	100
PaLCuB-[IN:VBN:BG]	99	100	100
PaLCuB-[IN:Cp:Chi:05] DQ118862	95	94	94
PaLCuB-[IN:MRT:05] EF043234	93	92	92
PaLCuB-[IN:ND:Ipo:09]JX050199	92	92	92
PaLCuB-[IN:ND:Pumpkin:10] JX040472	92	92	91
PaLCuB-[IN:ND:Papaya:03] AY244706	91	91	91
PaLCuB [IN:Jab:03] AY230138	88	88	88
FbLCB-[IN:Kan:11] JQ866298	53	53	53
MYMIB-[IN:Fai:Cp:12] JX443646	52	52	52
ToLCMaB-[IN:Pun:04] AY838894	75	75	75
ToLCB-[IN:Cp:04] AY728263	58	58	58
ToLCB-[IN:ND:Papaya:09] HM143911	59	59	59
ToLCB-[IN:Bhu:13] JN663851	57	57	57
ToLCB-[IN:Bih3:10] GU732205	53	53	53
CLCuB [IN:Luc:10] HM143916	59	59	59
CLCuB-[IN:Luc:10] GU440581	59	59	59
CLCuB-[IN:Sri:08] GQ370388	44	44	44
ChLCB-[Pk:Fai62:04] AM279672	52	52	52
ChLCB-[PK:Si:04] AM279662	58	58	58
TbLCB-[PK:Bah:99] AJ316034	57	57	57
AYVB-[IN:Mad:03] AJ557441	55	55	55
AYLCB-[IN:Luk:11] JQ408218	49	49	49
BYVMB-[IN:WB:07] EF417919	40	40	40
BYVMB-[IN:Tha:OY158:06] GU111971	40	40	40
BYVMB-[IN:Tri:OY118:06] GU111970	40	40	40
BYVMB-[IN:Mad:03] AJ308425	40	40	40
BYVMB-[IN:Coi:OYCO1:05] GU111975	39	39	39
VYVB-[IN:Mad:10] FN435836	36	36	36

Betasatellites in the present study are bold.

GenBank Acc. Nos. KC911718 and KC911724, KC959933, KC959934 and KC959935, respectively.

Sequence comparison

The complete nucleotide sequence of these three betasatellite isolates was determined to be 1351 to 1359 nt long. The complete nucleotide sequence of betasatellites in present study was compared with other PaLCuB sequences and other betasatellites available in the GenBank database. They exhibited 91 to 93% identity with PaLCuB from papaya, pumpkin and ipomoea and 88% identity with PaLCuB from tomato. The comparison with betasatellites reported from cowpea and French bean showed only 52 to 58% identity. The lowest percentage of identity was seen with Vernonia yellow vein betasatellite and Bhendi yellow vein mosaic betasatellite (36% and 39%, respectively) (Table 1).

Analysis of satellite conserved region

One of the main universal features of betasatellites is the SCR. By aligning the sequences of non coding region of the PaLCuB, the SCR was computed. The SCR was determined to be 191 nt long in PaLCuB-CBE-BG, 193 nt in CBE-GG and 192 nt in VBN-BG. From the multiple alignment shown in Fig. 3, it is clear that within the SCR, there are blocks of highly conserved regions with variable regions adjoining it. All the three betasatellites described in the study showed deletion compared to PaLCuB from New Delhi and Jabalpur. The SCR contains the loop structure with nonanucleotide sequence that represents origin of replication. The betasatellites from legumes showed 93% identity in this region with PaLCuB from cowpea, ipomoea and pumpkin. Repeat doublets GCTACGC were found to be present in the SCR of all the three betasatellite sequences.

Analysis of A-rich region

A-rich region is typically between 706 to 979 nt with approximately 51 to 53% of betasatellite DNA sequence. The A-rich region is present in all the three betasatellites.

Analysis of potential coding region

The betasatellites encode a single gene named as βC1 in the complementary strand. It is 369 bp long, encodes for a protein of ~14 kDa. Comparison of amino acid sequence of βC1 protein of PaLCuB (Fig. 4) clearly shows that, the βC1 protein of legume betasatellites in the current work are nearly identical in their amino acid composition with other PaLCuBs. They shared 93 to 98% identity with cowpea isolate from Gujarat, pumpkin and papaya isolate from New Delhi and tomato isolate from Jabalpur. The identity is less than 65% with betasatellites from other legumes (Table 2). From the Fig. 4, it is evident that PaLCuBs have conserved motifs, well different from the other three betasatellites, mungbean yellow mosaic India betasatellite (MYMIB), tomato leaf curl betasatellite (ToLCB), French bean leaf curl betasatellite (FbLCB) isolated from grain legumes. βC1 protein of all the PaLCuBs showed N-terminal truncation as compared to other betasatellites.

Phylogenetic relationship

Complete nucleotide sequences of the betasatellites in present study were compared with other betasatellites deposited in GenBank database and the phylogenetic tree was constructed (Fig. 5). All the eight PaLCuB betasatellites (three from this study and five from DataBank) branch out separately from all other solanaceous and malvaceous betasatellites. The betasatellites, MYMIB from cowpea, FbLCB from French bean formed separate cluster. The third major

Table 2. Percentage amino acid identity of βC1 protein of papaya leaf curl betasatellites from legumes with βC1 protein of selected betasatellites

Betasatellites	PaLCuB-[IN:CBE:BG]	PaLCuB-[IN:CBE:GG]	PaLCuB-[IN:VBN:BG]
PaLCuB-[IN:CBE:BG]	100	100	100
PaLCuB-[IN:CBE:GG]	100	100	100
PaLCuB-[IN:VBN:BG]	100	100	100
PaLCuB-[IN:Cp:Chi:05] DQ118862	98	98	98
PaLCuB [IN:Jab:03] AY230138	93	93	93
PaLCuB-[IN:MRT:05] EF043234	93	93	93
PaLCuB-[IN:ND:Ipo:09]JX050199	92	92	92
PaLCuB-[IN:ND:Pumpkin:10] JX040472	90	90	90
PaLCuB-[IN:ND:Papaya:03] AY244706	93	93	93
MYMIVB-[IN:Fai:Cp:12] JX443646	45	45	45
ToLCB-[IN:Cp:04] AY728263	61	61	61
FbLCB-[IN:Kan:11] JQ866298	35	35	35

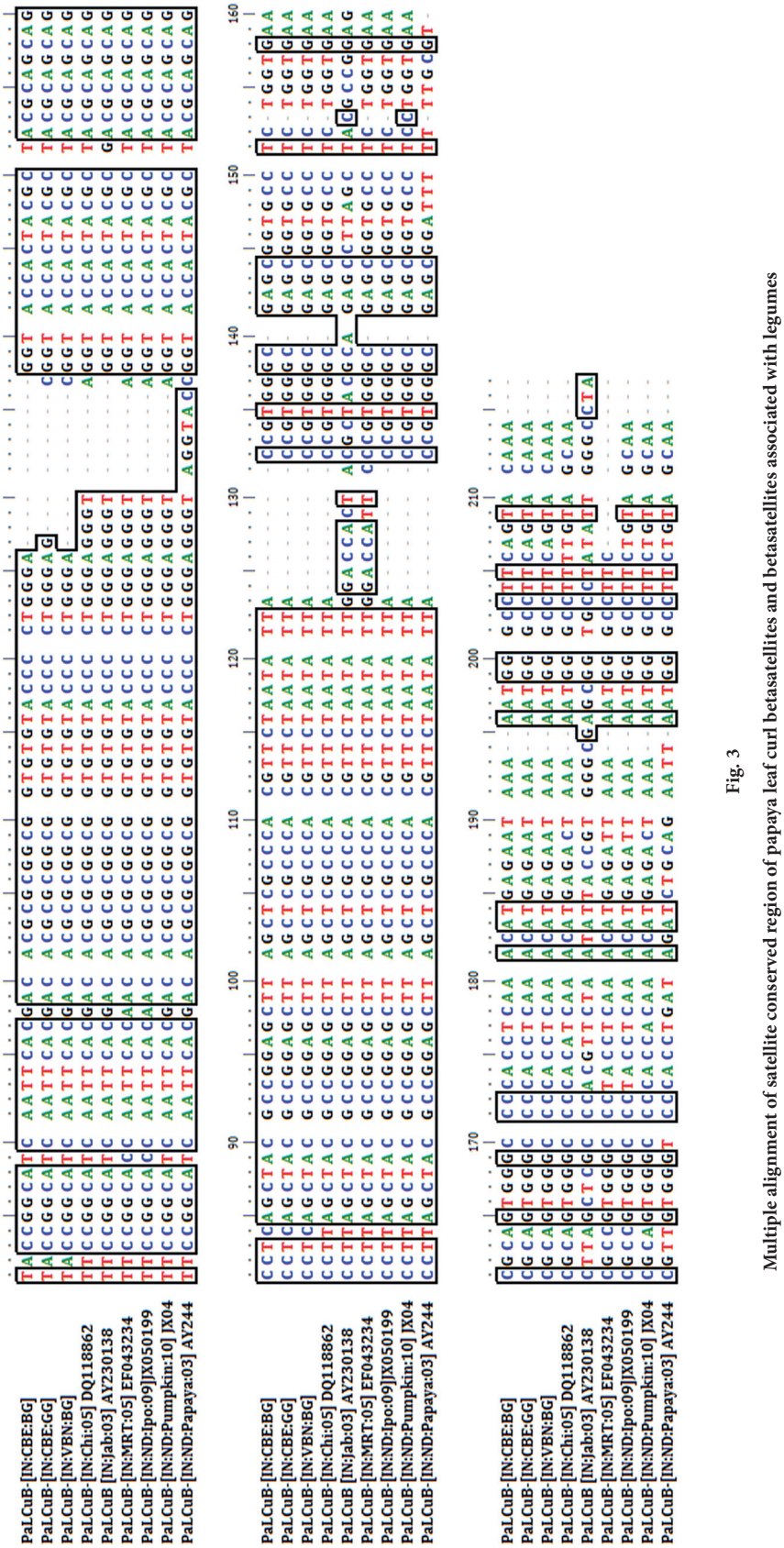

Fig. 3

Multiple alignment of satellite conserved region of papaya leaf curl betasatellites and betasatellites associated with legumes

group of betasatellites consisted of molecules originating from a diverse range of host plants such as chilli, tomato and cotton. The tobacco leaf curl betasatellite (TbLCuB) showed no particular similarity between any other clusters.

Discussion

In recent years, yellow mosaic, leaf curl and leaf distortion symptoms are observed in urdbean and mungbean plants in farmers′ fields of Tamil Nadu. The yellow mosaic symptoms are severe and trifoliate leaves exhibit asymmetry and look distorted. The present investigation was initiated to find out whether any new begomoviruses cause the crinkling symptoms or if any betasatellite components are involved. The results revealed that the PaLCuB was associated with MYMV.

The betasatellites associated with bipartite begomovirus were reported by Rouhibakhsh and Malathi (2005) who investigated the cowpea plants showing severe leaf curl symptoms in northern India and reported the presence of ToLCB species. Subsequently Sivalingam *et al.* (2010) and Jyothsna *et al.* (2013b) demonstrated their association with the bipartite begomovirus, ToLCNDV. Qazi *et al.* (2007a) suggested that severe leaf curl and crumpling symptoms in cowpea plants are caused by MYMIV and betasatellite complex. The tomato yellow leaf curl Thailand virus is another example, where DNA A, DNA B and betasatellite are observed together.

In recent years, the begomovirus-betasatellite complex has been reported in legumes. They are ToLCB associated with MYMIV in cowpea in northern India (Rouhibakhsh and Malathi, 2005), another tobacco leaf curl betasatellite associated with MYMIV in cowpea in Pakistan (Ilyas *et al.*, 2010), MYMIBV associated with MYMIV in cowpea from India (JX443646), and the PaLCuB from cowpea (DQ118862). A new monopartite begomovirus, French bean leaf curl virus (Kamaal *et al.*, 2012) has been identified in French bean and it is associated with FbLCB. In the present study, the bipartite MYMV are associated with PaLCuB. Whether this tripartite association in the yellow mosaic viruses will continue or as the time progresses it will disappear, is an issue which needs to be investigated.

Betasatellites belonging to PaLCuB showed high degree of conservation in amino acid sequence whereas betasatellites associated with legume viruses showed high divergence. From these results it is clear that MYMV and MYMIV may get associated with diverse betasatellites.

If the betasatellite is present, it is well known that it auguments the viral pathogenicity. It contributes to helper viral

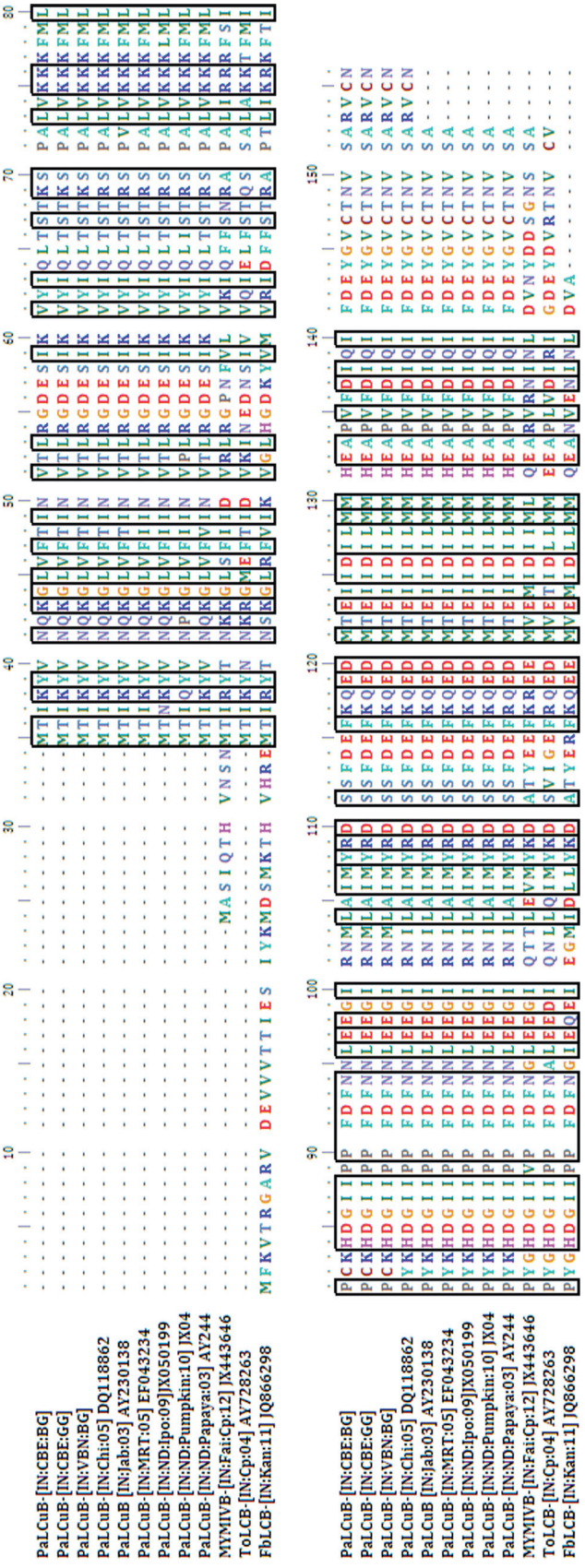

Fig. 4

Multiple alignment of predicted amino acid sequences of βC1 protein of papaya leaf curl betasatellites and betasatellites associated with legumes

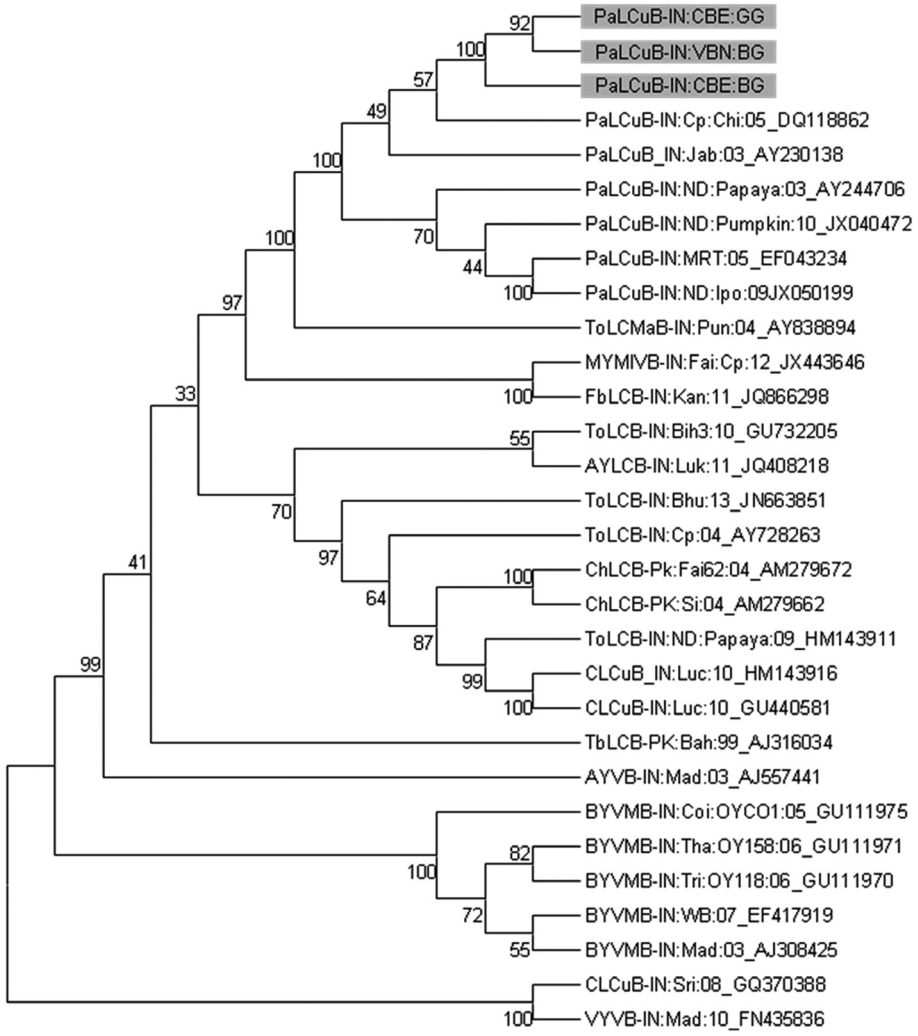

Fig. 5

Phylogenetic dendrogram based upon an alignment of the complete nucleotide sequences of betasatellites with other selected betasatellites associated with begomoviruses

Values at nodes represent the percentage boot-strap scores (1000 replicates). The isolates in the present study are highlighted.

DNA accumulation (Guo *et al*., 2008) and symptom severity in the host plant (Jyothsna *et al*., 2013a). All the betasatellite molecules under study showed several characteristic features in common with other betasatellites reported from other crops. They are, conserved nonanucleotide situated in the stem-loop region, a highly conserved SCR, and conserved βC1 ORF and an A-rich region. In the present study, one conserved repeat sequence was found to be present in the SCR. Previous study with association of betasatllites with yellow mosaic disease of mesta also indicated the presence of such repeat motif in the SCR that could act as cis-acting element needed for binding of Rep-protein (Jose and Usha, 2003; Das *et al.,* 2008). The βC1 protein encoded by the betasatellite has been shown to be a PTGS suppressor, capable of knocking out RNAi defense of plants (Cui *et al*., 2005; Gopal *et al*., 2007; Shukla *et al*., 2013). Interestingly the βC1 was also experimentally proved to facilitate the movement of DNA A of the bipartite begomoviruses ToLCNDV (Saeed *et al*., 2007; Sivalingam and Varma, 2012). It is suggested that βC1 may even alter the environment of the cell creating an advantageous atmosphere for the replication of the virus (Briddon and Stanley, 2006). The position and size of βC1 were found to be conserved in all the betasatellites in the present study.

The potential of MYMIV and MYMV to interact with diverse betasatellite is possible as replication of betasatellites is more relaxed than the DNA B components and it can be facilitated by a set of helper begomoviruses (Briddon *et al*., 2003). It will be necessary to study how frequent is the beta-

satellite/YMV interaction in nature to assess the emerging disease scenario. PaLCuBs are reported from diverse range of hosts such as papaya (Singh-Pant *et al.*, 2012), ipomoea (Swapna Geetanjali *et al.*, 2013) and pumpkin (JX040472). It is very difficult to hypothesize the mechanism by which MYMIV and MYMV could have picked up PaLCuB, as legume infecting begomoviruses do not infect other hosts enlisted above and chances of they occurring in mixed infection with any other monopartite begomovirus are very low. However there was a recent report of tomato leaf curl Karnatka virus in soybean (Raj *et al.*, 2006). It is possible that monopartite begomoviruses of the other host may move to soybean or cowpea aided by the associated betasatellites. Once soybean or cowpea are infected by betasatellites, when YMV infects them, the betasatellites could be replicated and encapsidated by YMV. It is essential to address the problem on how the association of betasatellites with MYMIV and MYMV will affect its host range, virulence and transmission to contain the YMD spread and mitigate the yield loss.

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