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Research Article

Molecular characterization and infectivity analysis of MYMIV mungbean isolate in various genotypes of mungbean and cowpea

Sanjeev Kumar*, Kiran Vilas Dhobale, Mahesh Das and Lingaraj Sahoo

Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, India

Abstract: Mungbean is an important source of dietary protein and it is mainly cultivated across South-east Asia for food and feed purpose. Worldwide, mungbean production is continuously decreasing due to various biotic factors such as Yellow mosaic diseases (YMD) caused by Begomoviruses. The viral diseases of mungbean remains a major constraint for its production. Despite its major economic importance, much attention is required towards the viral diseases of mungbean in India. In our study we conducted a survey to investigate viral diseases of mungbean in Assam, North-Eastern, India. We report complete sequences of mungbean infecting Begomovirus from Assam, Mungbean yellow mosaic India virus DNA-A and DNA-B genomic components. The nucleotide sequence comparison analysis revealed that mungbean isolate MYMIV DNA-A [OK431079] having highest similarity (99%) with earlier reported of MYMIV segment DNA-A [KU950430] and lowest similarity (95%) with soybean isolate of MYMIV [AJ416349] whereas mungbean isolates MYMIV DNA-B [OK431080] showed highest identity (96%) with MYMIV GUM14 segment [KY556680] and lowest identity (90%) with MYMIV MI8 segment [FM202444]. Our survey revealed MYMIV mungbean isolate of Assam is a variant of other previously reported isolates characterized in north and central regions of India. We therefore characterized the MYMIV infection in 3 different genotypes of mungbean towards their sensitivity and as well as its other potential host, cowpea through agroinoculation of dimeric infectious clones and confirmed the pathogenicity of MYMIV. This is the first report of mungbean MYMIV isolate also infecting to cowpea with YMD symptoms in North-East India which is experimentally cross validated through Koch's postulate.

Keywords: *YMD, Cowpea, mungbean, MYMIV, Agro-inoculation, Begomovirus*

Introduction

Mungbean, [*Vigna radiata (L.*) Wilczek] is considered as one of the most economically important food legume crop of India for valuable digestive protein. In India, mungbean has been considered the third most popular pulse crop after chickpea and pigeonpea. The summer mungbean accounts for substantial proportion of the total mungbean production in India. The total mungbean production in India mainly comes from Punjab, Madhya Pradesh, Gujarat, Haryana, Bihar, West Bengal, Western Uttar Pradesh, Assam and Tamilnadu. Mungbean cultivation is infested by variety of insect pest, nearly 85 insect pests attack mungbean from field to storage, among which whitefly transmission is the major one (Sekar and Nalini, 2017). The yellow mosaic disease (YMD) of mungbean is majorly caused by whiteflies transmitting Begomovirus (family Geminiviridae). The two major Begomovirus strains which infect to mungbean are Mungbean yellow mosaic virus (MYMV) and Mungbean yellow mosaic India virus

***Corresponding Author:**
Sanjeev Kumar;

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(MYMIV) especially in South and Southeast Asia. The overall yield loss has been estimated from 10 to 100%, depending upon climate conditions, the virus susceptibility among the mungbean genotype and stage of crop infection (Singh and Kaur, 1980; Bashir *et al*., 2006).

The major causative agent of YMD of mungbean is whitefly (Bemisia tabaci Gennadius), an insect vector for YMVs (Selvi *et al*., 2006). The YMD symptoms in diseased plants appear in the form of scattered yellow-color patches on all over the leaf surfaces on young leaves which turns into a yellow mosaic pattern at the late stage of infection and finally it turns into complete yellowing, drying and withering of leaves. Affected plants produces less number of flowers, pods and seeds with hampered photosynthetic rate which ultimately leads to the severe yield loss (Malathi and John, 2009).

Geminiviruses are comprised of small circular, single-stranded DNA plant virus containing the monopartite or bipartite genome ranging from 2.6 - 2.8 kb size, encapsidated in twinned icosahedral particles (Thomas *et al*., 1986). The Geminiviridae family has been comprised into nine genera, viz., Capulovirus, Eragrovirus, Begomovirus, Becurtovirus, Grablovirus, Curtovirus, Topucovirus, Mastrevirus, and Turncurtovirus on the basis of their genomic organization of viral DNA components, insect vector, host range, and the phylogeny reconstruction (Varsani *et al*., 2017; Zerbini *et al*., 2017). Among nine genera, the genus Begomovirus is the largest one, transmitted by whitefly Bemisia tabaci, predominant in the tropical and subtropical regions of the world which mainly infects to the dicot plants including beans, tomato, cucurbits, pepper, cassava and cotton (Brown, 2000; Czosnek and Laterrot 1997; Moffat, 1999). The majority of Begomovirus, are either composed of monopartite (single genomic DNA) or bipartite (two genomic DNA components, DNA-A and DNA-B). The DNA-A genomic component comprised of 5-6 ORFs (AC1, AC2, AC3, AC4, AV1, AV2) which mainly codes for replication and pathogenicity proteins like replication-associated protein (Rep), a key protein for viral DNA replication; the replication enhancer protein (REn); the transactivator protein (TrAP) for control of delayed gene expression and the coat protein (CP) for encapsidation and insect transmission. Two major proteins, nuclear shuttle protein (NSP) mainly responsible for suppression of transmembrane receptor kinase activity and movement protein (MP) for cell to cell movement are controlled by the other genomic component DNA-B and both of the proteins plays important role in systemic spread and symptom development (Brown *et al*., 2015; Krenz *et al*., 2015). Both the viral components DNA-A and DNA-B share a common region (CR) which contains highly conserved nonanucleotide TAATATT↓AC that play essential role in viral replication such as nicking of viral strand and control of some important viral genes expression (Hanley-Bowdoin *et al*., 2015; Pant, 2001).

In order to investigate the YMD incidences in mungbean cultivation in North-eastern region such as Assam, a survey was conducted in major mungbean growing areas during 2019–2020 and found the disease incidence around 60–70%. The disease prevails throughout the mungbean cultivation season both spring and summer. A mild to severe yellow mosaic patches, leaf chlorosis, necrosis in the various plant parts with stunted growth were the major symptoms observed in mungbean cultivated in the field. The high incidences of whiteflies around the mungbean cultivated field prompted us to undertake the survey and find the severity of the disease and its

possible association of Begomoviruses with mungbean. Rolling Circle Amplification (RCA) method was implemented to detect the presence of Begomoviruses in mungbean infected plant samples. Our results revealed the presence of MYMIV as the most prevalent viruses infecting mungbean in major cultivated area of Assam, India. We also found that agroinfectious dimeric clones of MYMIV mungbean isolate induced severe infection in three widely cultivated mungbean genotypes and also to the other non-host plants such as cowpea. Here we report that MYMIV mungbean Assam isolate is infecting various widely cultivated mungbean genotypes as a major host and also to the closely related non-host cowpea and causing Yellow Mosaic disease (YMD) in Assam, India and we have artificially simulated the disease under the controlled laboratory conditions..

Material and Methods

Collection of mungbean infected symptomatic plant material and genomic DNA extraction

To investigate the viral disease occurrence in mungbean cultivated field of Assam, North-East India, a field survey was conducted in some of major locations where mungbean were massively grown during 2019–2020 at different time intervals and field locations in the month of March to June. We surveyed at around 5–10 distant fields located approximately 5–10 km apart of mungbean growing fields. The samples were collected from various locations in this study to determine whether any viral strain variations occurred amongst various field locations. The mungbean infected plants exhibiting yellow mosaic, yellowish vein, reduced leaf size, severe leaf curling, and mottling of leaves with stunted growth were considered for sample collection (Fig. 1 &2). The infected plant materials were collected and stored properly at deep freezer -80C to reduce the dryness and any further damage. Total genomic DNA was extracted from both infected and healthy mungbean samples by using Cetyltrimethylammonium bromide (CTAB) method (Srivastava *et al*., 1995). Symptoms of infected mungbean plant materials collected from the field were also reproduced under controlled glasshouse conditions to examine symptom variability.

Viral DNA amplification and cloning of full-length viral genome

To obtain the full length viral DNAs (DNA-A and DNA-B) from mungbean infected plant materials, we employed Rolling Circle Amplification (RCA) method (Haible *et al*., 2006) using TempliPhi™ DNA amplification kit (GE Healthcare) as per the manufacturer's instruction. Further the RCA product were digested with various common restriction enzymes such as EcoRI, BamHI, PstI, HindIII and SalI (Thermo Scientific FastDigest, USA). The RCA digested products were separated on 1% agarose gel and visualized under UV transilluminator. The bands corresponding to ~ 2.7 – 3.0 kb of the viral genomes either of DNA-A/ DNA-B components) were purified using SureTrap Gel extraction kit (GCC Biotech Ltd. India). All the bands corresponding to 2.7-3.0 kb monomers of either of DNA-A/DNA-B (pUC18-DNA-A/DNA-B) were successfully cloned into the cloning vector pUC18. The ligated clones were confirmed by PCR and restriction digestion. The PCR positive clones were purified using SureTrap Plasmid Mini Kit (Genetix, India) and processed for sequencing commercially (Eurofins, Bangalore). The full-length sequences were obtained, either through the primer walking strategy.

Viral DNA Sequence analysis and phylogenetic study

The full length sequences of all the monomeric clones were analyzed initially through NCBI-BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the similarity index with the other reported isolates were determined. The detailed analysis of the viral sequences were performed by using Mega 5.2.2 (5130611), BIOEDIT version 7.0 programs (Hall, 1999) software. CLUSTALW program and Mac Vector software (v11.1.2; MacVector Inc., USA) were used to align the nucleotide (nt) sequence and amino acid (aa) sequence for

each viral DNA components from various other worldwide reported Begomovirus isolates. On the basis of similarity index, phylogenetic trees were constructed using sequence of complete DNA-A and DNA-B of mungbean isolated Begomovirus and other legume infecting Begomoviruses by the neighbour-joining method using MacVector suite program 10.5 (Mac Vector Inc, USA). The other legumes infecting Begomoviruses were also taken into the account for this study.

Construction of MYMIV agroinfectious dimers and virus infectivity analysis

To check the molecular infectivity of MYMIV mungbean isolate, Agroinfectious dimers of both DNA-A and DNA-B components were constructed using a high fidelity PCR-based strategy as described in our recent report (Kumar *et al*., 2017a). Two different sets of abutting primers targeting to the sequence of DNA-A and DNA-B to get full amplification were designed. To amplify the DNA-A component of MYMIV, Fw primer 5′- AAATTT GAGCTC ACCTGAGTGCCCCGCGAC- 3′ and the Rv primer 5′- GGGCCC CTGCAG AATATTATAGCTGAGTGC-3′ were commercially synthesized (Eurofins Bangalore India). The underlined nucleotides depicts respective restriction sites of SacI in the Fw and PstI in the Rv primer respectively as per the cloning requirements. PCR reactions was performed as described in (Kumar *et al*., 2017a) and PCR products were gel purified and subsequently cloned into TA cloning vector pTZ57R/T (Thermo Scientific, USA) vector. The pTZ57R/T recombinant clones were confirmed through PCR and sequencing. Further, the 2.7 kb SacI-PstI fragment was subsequently cloned into a plant binary vector pCAMBIA3300 as SacI-PstI insert, and it was named as pC-A′. In the next step, the 2.7 kb PstI digested viral DNA from pUC18-DNA-A plasmid DNA was recloned in pC-A′ to generate a complete dimer of DNA-A in pCAMBIA3300 in head to tail fashion (names as pC-2.0 A). The orientation of the DNA-A dimeric clones was confirmed by restriction digestion using the unique cutter, with DraI present inside the viral DNA sequence.

In the similar way, DNA-B dimeric clone were constructed as described above of DNA-A except use of different primers and restriction sites. We used Fw primer 5′- AAATTT GAGCTC ACC TGA GAG CCC CGC GAC -3′ and the Rv primer 5′- GGGCCC GGATCC AATATTATAGCTGAGAGC -3′. The underlined nucleotides in the primer sequences represent SacI and BamHI restriction site in the forward reverse primer respectively. The 2.6 kb of DNA-B SacI-BamHI fragment were finally cloned into pCAMBIA3300 and named as pC-B′. In the next step, the 2.6 kb BamHI viral DNA fragment was re-cloned at the BamHI site of pC3300-A′ to generate DNA-B dimeric clone (names as pC3300-2.0B). Orientation of the gene were confirmed by restriction digestion usingunique cutter DraI (present inside the DNA-B genome). Further, both the dimeric clones pC3300- 2.0A and pC3300-2.0B were mobilized into Agrobacterium tumefaciens strain EHA105 through Freeze thaw method. The Agrobacterium transconjugants were confirmed through colony PCR using the DNA-A and DNA-B sequence specific internal primers.

Agroinoculation of dimeric constructs in mungbean and cowpea

The Agromoblized dimeric constructs of MYMIV DNA-2A and DNA-2B were inoculated in YEP medium (pH-7.0±0.2) supplemented with kanamycin (50 µg/ml), rifampicin (20 µg/ml) and incubated at 28 °C with shaking at 180 rpm (OD600 = 0.8). The rapidly grown overnight cultures were harvested at 5000 rpm for 15 min at 4 °C and further resuspended in MES buffer as described in (Kumar *et al*., 2017a) and used for agroinfiltration assays in mungbean and cowpea. The agroinoculation was performed in the combination of both pCDNA-2A and pCDNA-2B for the viral infection and their systemic movement inside the host cells. Three genotypes of mungbean (*Vigna radiata*) cv. *K-851*, cv. *PUSA VISHAL*, cv. *PUSA-105* and one genotype of Cowpea (*Vigna unguiculata*) cv. *PUSA KOMAL* plants were grown in soil: compost (1:1) inside the greenhouse and maintained as described in

(Kumar *et al*., 2017a). The agroinoculation of dimeric constructs were performed at the stage of 6–8 trifoliate leaves (Kumar *et al*., 2017a). A total of five plants each from three genotypes of mungbean and one genotypes of cowpea was used for each agroinfiltration experiments. After agroinoculation, plants were maintained under greenhouse conditions and the viral disease symptoms were recorded periodically.

Viral DNA accumulation analysis in mungbean and cowpea

The agroinoculated symptomatic plants of both mungbean and cowpea were molecular analyzed. To detect the presence of MYMIV-DNA-2A and DNA-2B in the inoculated plants, total genomic DNA was extracted (inoculated with DNA-2A+ DNA2B, mock and non-inoculated) and RCA reaction was performed. The RCA amplified products were further digested with unique cutter restriction enzymes DraI of DNA-A and DNA-B. The digested product were resolved on 1% agarose gel and visualized the viral DNA fragments under UV transilluminator. To check the accumulation of MYMIV viral DNA transcripts in the test plants mungbean and cowpea, semi-quantitative RT-PCR was performed. Total RNA was extracted from the RCA positive agroinoculated plants using RNeasy Plant Mini Kit (Qiagen, Venlo, Limburg, Netherlands) as per the manufacturer's instructions. The cDNA was prepared by taking 500ng of total RNA from each tested plants and followed all the cDNA synthesis protocol (Kumar *et al*., 2017b; Kumar *et al*., 2017c). The semi-quantitative RT-PCR was performed using an internal primers of pre coat protein (AV2) of MYMIV DNA-A (forward primer 5′-TAT ACA GTC GGT AAA ACC GAG GTT-3′) and reverse primer (5′-CTAATT CTC GTG GTT TTATGT ACC-3′). We used the PCR condition as follows: 95 °C for 3 min; 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min for 34 cycles and a final extension of 72 °C for 5 min to amplify 270 bp internal fragment of AV2. To normalize the transcripts two sets of housekeeping Vu-ubiquitin primers (forward primer 5′-GTCTCGAAGCACGCTGAGGT-3′ and reverse primer 5′-AGAGAGTGGGAAGCCGATCC-3′) and Vr-tubulin 5′-ACTGCATCTGCTATGTTCAG−3′and reverse primer 5′GAATATCACACACACTCGAC-3′ were used and the PCR condition was kept same as described above except the annealing temperature of 58 °C for 1 min.

Results

Cloning of mungbean infecting Begomoviruses and allied components

The field cultivated mungbean plants showing viral disease symptoms were collected from various places of Assam, India (Fig. 1). The virus infected mungbean plants exhibited symptoms of stunted growth, with sever yellow patches on upper leaf surface, yellowish veins on leaves, reduced leaf size, chlorosis, necrosis and leaf curling symptoms (Fig. 1). The disease incidences were observed maximum 60-70% during the period of April to June 2019-2020. We successfully cloned 12 viral DNA fragments through RCA method from 15 number of samples collected. The RCA results depicts the presence of 2.6–3.0 kb band either of DNA-A or DNA-B, which is the typical characteristic of bipartite genomes of Begomoviruses and samples collected from asymptomatic healthy mungbean plants were uniformly negative with absence of RCA products. (Fig. 2).

Fig. 1 Virus infected mungbean plant materials collected from diverse locations of Assam, North-East, India

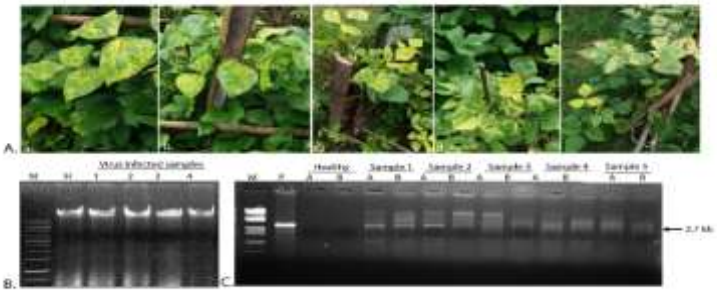

Fig. 2 A. Virus infected mungbean plants in field of Assam, India (a-e); B. Genomic DNA isolated from the healthy (lane H) and infected mungbean leaf samples (lanes 1-4; C. Rolling circle amplification followed by restriction digestion of healthy and infected mungbean samples (1-5). Lanes A, B represent digestion with BamHI and PstI respectively. The appearance of 2.7 kb bands on digestion with above enzymes indicates the presence of Begomovirus in the infected samples. Lane marked M represents the molecular mass marker. Lane marked P represents the positive control (pUC19 RCA kit supplied).

Molecular characterization of mungbean infecting Begomoviruses and allied components

The detailed sequence analysis for the determination of potential open reading frames (ORFs) using ORF Finder (NCBI; http://www.ncbi.nlm.nih.gov/gorf/gorf.html) of the viral components showed 07 clones to have an typical arrangement of genes of bipartite (DNA-A components of bipartite) Begomoviruses, whereas 05 clones had an typical arrangements of the DNA-B components of bipartite Begomoviruses. The pairwise sequence comparison of the viral DNA-A and DNA-B genomic components suggested that, the majority of viral DNA components had 96–99% nt sequence similarity and, when it was compared with the NCBI-GeneBank database for the viral genomic sequences, it showed high similarity (94–99%) to sequence of other isolates of MYMIV. These results indicated that they might be not considered as of novel Begomovirus but they might get considered as the variants of earlier reported Begomoviruses (Table 1 &2). We observed that from all 12 viral DNA sequenced components, MYMIV is most prevalent strain of Begomovirus which is infecting to the mungbean cultivation in the field of Assam India.

We obtained full-length sequences of three clones each of DNA-A [OK431079] and DNA-B [OK431080] from each samples and one of the clone was used for characterization of the full genome of DNA-A and DNA-B (Fig. 2).

We found DraI was the unique cutter of DNA-A and DNA-B in the viral genome. We also compared the sequences of MYMIV-DNA-A and MYMIV-DNA-B Assam isolate with the other NCBI GeneBank reported Begomovirus isolates infecting to legumes which showed the similarity index ranging from 95-98%.

The MYMIV Assam isolate were comprised of 2724–2767 nt of DNA-A and 2639–2682 nt of DNA-B respectively which is much identical to the previously characterized bipartite Begomoviruses (Fig. 3). The genome of DNA-A comprised a total of five to six open reading frame (ORFs) of which four ORFs, i.e., 1088 nt of AC1 (2626–1538), 452 nt of AC2 (1228–1680), 404 nt of AC3 (1086–1490), 296 nt of AC4 (2176–2475) and 224 nt of AC5 (760-984) are in complementary sense strand and two ORFs,773 nt of AV1 (316–1089) and 341 nt of AV2 (156–497) are in antisense strand of the virion. The common/intergenic region (IR) of 282 nt long were found to be located between the ORFs AC1 and AV2. Similarly two ORFs were denoted in the sequences of DNA-B component which is mainly responsible for cell to cell movement of viron particles. Among 2 ORFs one is of 770 nt of BV1 (420–1190) on the sense strand and 896 nt of BC1 (1222–2118) on the complementary strand (Fig. 3). We did not observed any significant similarity between the genome of DNA-A and DNA-B except for some sequences (174 nt) in common regions (CR). A conserved nonanucleotide sequence TAATATTAC were present in the CR region which is known to guide replication initiation of amongst all geminiviruses. On the basis of full length sequences of both viral DNA components a phylogenetic dendogram of our MYMIV mungbean isolate [MYMIV-IN[IN:-ASSAM:MUNG] Begomovirus were compared with other Begomovirus isolates reported worldwide. (Fig. 4 & 5). The MYMIV mungbean isolate identified from Assam India, were found most similar to the other MYMIV isolates reported from various regions of India, Nepal and Pakistan, Bangladesh and Indonesia and the similarity index were between 94–98%, which also indicates that this MYMIV Assam isolate has close relationship with other reported strains.

Table 1: Percent identities (nucleotide#) of MYMIV-India [India: Assam: Mungbean] with DNA-A of selected begomoviruses reported worldwide.

Name of Begomoviruses	DNA-A	% Identity
MYMIV-IND[IND:JHARKHAND:COWPEA]	KY556679	96.1
MYMIV-IND[IND:ASSAM:MUNGBEAN]	OK431079	-
MYMIV- BANGLADESH[BANGLADESH:BANG:MUNGBEAN]	AF314145	98.4
MYMIV-IND[IND:MEGHALAYA:MUNGBEAN]	KU950430	99.3
MYMIV-INDONESIA[INDONESIA:KERSANA:SOYBEAN]	JN368438	96.9
MYMIV-IND[IND:BENGAL:SOYBEAN]	HF922628	98.2
MYMIV-IND[IND:BIHAR:PIGEONPEA]	KX363947	96.3
MYMIV-IND[IND:JABALPUR:BLACKGRAM]	LC271790	96.2

MYMIV-IND[IND:SAGAR:SOYBEAN]	LC271794	95.9
MYMIV-IND[IND:BHOPAL:SOYBEAN]	LC271792	95.8
MYMIV-PAKISTAN[PAKISTAN:NAWABSHAH:SOYBEAN]	AM992618	96.7
MYMIV-NEPAL[NEPAL:LALITPUR:MUNGBEAN]	AY271895	96.6
MYMIV-PAKISTAN[PAKISTAN:MUZAFAR:MUNGBEAN]	FM208839	96.5
MYMIV-IND[IND:VARANASI:FRENCHBEAN]	KC019304	96.3
MYMIV-IND[IND:VARANASI:COWPEA]	DQ389154	96.3

Table 2-Percent identities (nucleotide#) of MYMIV-India [India: Assam: Mungbean] with DNA-B of selected begomoviruses reported worldwide.

Name of Begomoviruses	DNA-B	% Identity
MYMIV-IND[IND:JHARKHAND:COWPEA]	KY556680	94.6
MYMIV-INDONESIA[INDONESIA:BOGOR:YARDLONGBEAN]	JN368442	94.0
MYMIV-INDONESIA[INDONESIA:BREBES:YARDLONGBEAN]	JN368443	93.9
MYMIV-INDONESIA[INDONESIA:BREBES:YARDLONGBEAN]	JN368444	94.0
MYMIV-INDONESIA[INDONESIA:REMBANG:YARDLONGBEAN]	JN368441	94.1
MYMIV-IND[IND:SAGAR:SOYBEAN]	LC271795	92.8
MYMV-IND[IND:KARNATAKA:BLACKGRAM]	KC911727	94.8
MYMV-IND[IND:TAMILNADU:MOTHBEAN]	DQ865202	94.8
MYMV-IND[IND:TIRUNELVELI:BLACKGRAM]	KC911731	94.8
MYMV-IND[IND:TIRUPATI:BLACKGRAM]	KF947526	96.3
MYMIV-IND[IND:ASSAM:MUNGBEAN]	OK431080	-
MYMIV-IND[IND:MEGHALAYA:MUNG]	KU950431	97.8
MYMV-IND[IND:GUJRAT:MUNGBEAN]	AY937196	93.7
MYMV-IND[IND:BELGAUM:MOTHBEAN]	MN698291	96.5
MYMIV-IND[IND:RAICHUR:URDBEAN]	MT027037	96.5

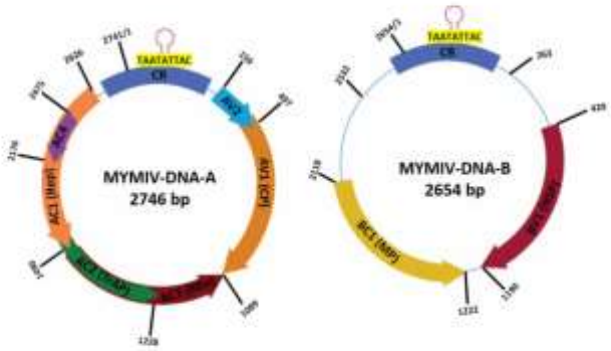

Fig. 3. Schematic viral DNA maps of the MYMIV genomic DNA-A and DNA-B components. The viral components open reading frames are demarcated by bar arrows with the head showing the 3′- terminus. The number denotes the nucleotide position.

Fig. 4. Phylogenetic tree of complete DNA-A of MYMIV-INDIA [INDIA: ASSAM: MUNGBEAN]. The phylogenetic tree was constructed with distance/neighbour-joining method with 1000 bootstrap replications and viewed with the help of MacVector suite program 10.5 (Mac Vector Inc, USA).

Fig. 5. Phylogenetic tree of complete DNA-B of MY MIV-INDIA [INDIA: ASSAM: MUNGBEAN]. The phylogenetic tree was constructed with distance/neighbour-joining method with 1000 bootstrap replications and viewed with the help of MacVector suite program 10.5 (Mac Vector Inc, USA).

Virus infectivity analysis using MYMIV agroinfectious dimers in mungbean and cowpea

For the virus infectivity analysis, the agroinfectious dimeric constructs of both MYMIV DNA-A and DNA-B genomic components (Fig. 6) were introduced into the leaves of three genotypes of mungbean and one genotype of cowpea by using the artificial agro-infiltration technique. The agroinfiltration was performed in the combinations of MYMIV DNA-2A and DNA-2B and the consequent disease progression were observed periodically. We observed severe viral disease symptoms in 2 genotypes of mungbean cv. *K-851* (5/5) and cv. *PUSA VISHAL* (5/5) at 28 days post infiltration (dpi) (Fig. 7, Table 3) while no symptoms were observed in cv. *PUSA-105* (0/5) till 56 dpi (Fig. 7C, Table 3). We also observed leaf curling and golden mosaic symptoms at 28 dpi (typical symptoms of Begomovirus infection) in cowpea (5/5) when the MYMIV agroinfectious dimeric constructs were co-infiltrated (DNA-A + DNA-B) in cv. PUSA-KOMAL (Fig. 8, Table 3). However, the severity of symptom was little less in cowpea at 28 dpi. in compare to mungbean. All the infected leaves got drooped in mungbean cv. *PUSA VISHAL* and cv. *K-851* at 64 dpi, while cv. *PUSA-105* plant leaves were looked healthy. The results indicated that the mungbean genotype cv. *PUSA VISHAL* and cv. *K-851* is highly sensitive against MYMIV strain while *PUSA-105* is tolerant against MYMIV (Fig. 7). Similarly the cowpea genotypes cv. PUSA-KOMAL were also found sensitive against MYMIV mungbean isolate which induced severe yellow mosaic symptoms which were progressively increased at 64 dpi (Fig. 8). As expected, the empty vector pCAMBIA3300 (negative control) could not induced any viral disease symptoms in the test plants even after 80 dpi in mungbean and 180 dpi in cowpea. In contrast, both mungbean cv. *PUSA-VISHAL* and cv. *K-851* and cowpea cv. PUSA-KOMAL were observed with stunted growth when the plants were coinfiltrated with the agroinfectious dimeric constructs.

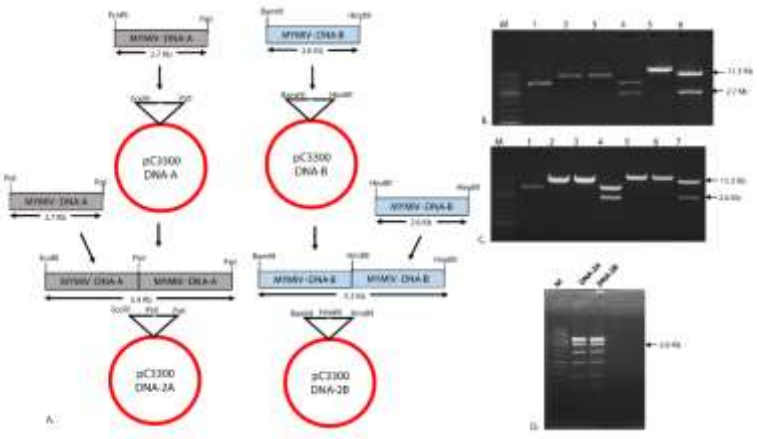

Fig. 6 A. Schematic representation of cloning strategy followed for Agroinfectious dimeric construct preparation of both MYMIV DNA-A and DNA-B components in pCAMBIA3300 vector. B. The dimeric agroinfectious clones of DNA-A and DNA-B, were obtained by

ligating two monomeric viral DNA unit with pCAMBIA3300 in head to tail fashion for each component in sequential step. Lane M: Middle range DNA marker Lane 1: 8.6 kb of linearized pCAMBIA3300, Lane 2-3: 11.3 kb of linearized monomeric DNA-A1 with EcoRI and PstI respectively, Lane 4: 2.7 Kb insert released from monomeric clone of DNA-A1 with EcoRI and PstI, Lane 5: 14 kb of linearized DNA-A2 with EcoRI, Lane 6 monomeric DNA-A1 2.7 kb released from DNA-A2 with PstI; C. Lane M: Middle range DNA marker, Lane 1: linearized 8.6 kb of pCAMBIA3300, Lane 2-3: 11.2 kb of linearized monomeric DNA-B1 with HindIII and BamHI respectively, Lane 4: 2.6 Kb insert released from monomeric clone of DNA-B1 with HindIII and BamHI, Lane 5-6: 13.9 kb of linearized DNA-B2 with HindIII and BamHI respectively, Lane 7: monomeric DNA-B1 2.6 kb released from DNA-B2 with BamHI; D. Orientation confirmation of dimeric clones of DNA-A and DNA-B using DraI uniques restriction enzyme site if both |DNA-A and DNA-B.

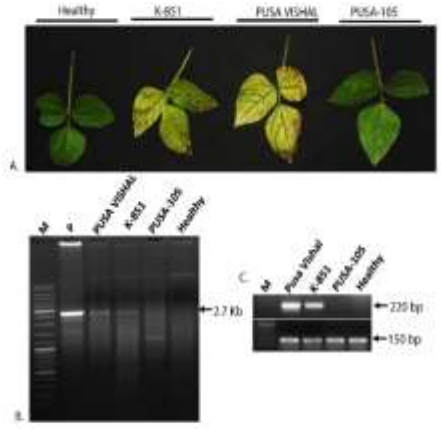

Fig. 7. A. The disease was induced by MYMIV-INDIA [INDIA: ASSAM: MUNGBEAN] in three widely cultivated genotypes of mungbean cv. *K-851*, cv. *PUSA VISHAL*, cv. *PUSA-105* respectively that was inoculated with agronfectius dimeric constructs of MYMIV in the combination of DNA-2A +DNA-2B, the agro-inoculated plants are shown in the top panel to reveal the patterns of disease symptoms at around 42 dpi; B. The level of viral DNA accumulation was checked by semi-qPCR at 56 dpi using DNA-A specific abutting primers MYMIV-INDIA [INDIA: ASSAM: MUNGBEAN]. Appearance of (~ 2.7/2.6 kb) after digestion of RCA product with PstI/BamHI of DNA-A/DNA-B indicates the presence of MYMIV infection in plant. Lane marked M represents the molecular mass marker. Lane marked P represents the positive control (pUC19 RCA kit supplied); C. Expression analysis by semi-quantitative RTPCR in RCA positive plants using pre-coat protein (AV2) specific primer. The appearance of 220 bp amplification in all RCA positive plants with AV2 specific primer confirmed the presence and expression of MYMIV dimer constructs. Vr- tubulin primers were used as internal control. Lane marked M represents 100 bp ladder.

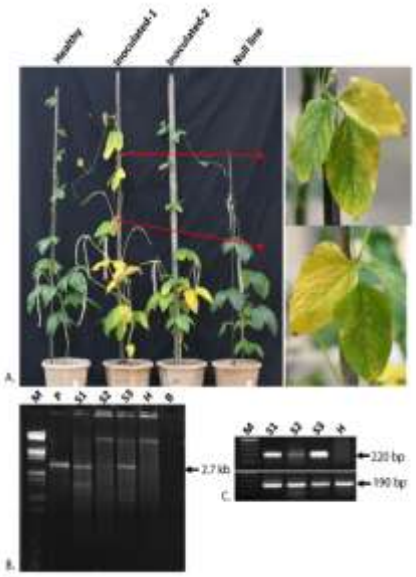

Fig. 8. A. The disease was induced by MYMIV INDIA[INDIA:ASSAM:MUNGBEAN] in widely cultivated genotypes of cowpea cv. PUSA-KOMAL that was inoculated with agronfectius dimeric constructs of MYMIV in the combination of DNA-2A +DNA-2B, the agro-inoculated plants are shown in the top panel to reveal the patterns of disease symptoms at around 48 dpi; B. The level of viral DNA accumulation was checked by semi-qPCR at 56 dpi using DNA-A specific abutting primers MYMIV-INDIA [INDIA: ASSAM: MUNGBEAN]. Appearance of (~ 2.7/2.6 kb) after digestion of RCA product with PstI/BamHI of DNA-A/DNA-B indicates the presence of MYMIV infection in plant. Lane marked M represents the molecular mass marker. Lane marked P represents the positive control (pUC19 RCA kit supplied); C. Expression analysis by semi-quantitative RTPCR in RCA positive plants using pre-coat protein (AV2) specific primer. The appearance of 220 bp amplification in all RCA positive plants with AV2 specific primer confirmed the presence and expression of MYMIV dimer constructs. Vu- ubiquitin primers were used as internal control. Lane marked M represents 100 bp ladder.

Table3- The infectivity and symptom induced by MYMIV-IN[IN:ASSAM:MUNG] and the number of symptomatic plants as confirmed by RCA

Host/Inoculated dimeric agro-constructs	**Symptomatic plants/ Inoculated plants**	**Types of symptoms (60 dpi)**
Mungbean		
PUSA VISHAL	5/5	Yellow mosaic and yellowish vein
K-851	5/5	Yellow mosaic and yellowish vein
PUSA-105	0/5	No symptoms
Cowpea		
PUSA KOMAL	5/5	Yellow mosaic, yellowish vein and severe downward leaf curling

Viral DNA accumulation analysis in the symptomatic mungbean and cowpea

To determine the viral DNA components proliferation in the agroinoculated test plants of mungbean and cowpea, total DNA from the symptomatic plant leaves were extracted and subjected to RCA and semi-quantitative qPCR analyses. RCA reaction was performed with the genomic DNA of both symptomatic

and healthy plants of mungbean and cowpea. The RCA amplified products of MYMIV were subsequently digested with unique cutter DraI of DNA-A and DNA-B. The respective 2.7 kb fragment of MYMIV DNA-A and 2.6 kb fragment of DNA-B were observed in the symptomatic plants of mungbean cv. *PUSA-VISHAL* and cv. *K-851*, while no RCA fragments were observed in cv. *PUSA-105*. Similarly RCA fragment of 2.6 to 2.7 Kb of MYMIV DNA-B and DNA-A respectively were observed in cowpea cv. PUSA-KOMAL, while no such band was observed, when plants were infiltrated with null vector pCAMBIA3300, and non-infiltrated control plant (Fig. 8). The semi-quantitative qPCR was performed also on the agroinfiltrated test plants of mungbean and cowpea to know the level of viral DNA accumulation by using an internal primer specific to MYMIV-DNA-A specific to pre-coat protein (AV2). The semi-quantitative results revealed higher accumulation of viral DNA at 28 dpi in mungbean cv. *PUSA-VISHAL* and cv. *K-851* and at 42 dpi in cowpea cv. *PUSA-KOMAL* (Fig. 7C and 8C). There was no any viral DNA accumulation was observed in the plants infiltrated with empty vector pCAMBIA3300 (negative control) in test plants (Fig. 8). For the normalization of transcripts an internal control Vu-ubiquitin in cowpea and Vr-tubulin in mungbean were used.

Discussion

Mungbean is an important legume used for high rich protein and mainly cultivated in south East Asian countries (Kumar *et al*., 2017c). Mungbean is growing during early to late summer season in India during which it experiences severe incidence of yellow mosaic disease. Several important Begomovirus strain is known to infect legumes including mungbean. A number of viruses infect mungbean as due to the vector being active and present in appreciable numbers. However, till date, very less studies have been undertaken to identify and characterize viruses infecting mungbean in India (Usharani *et al*., 2004; Usharani *et al*., 2005; Surendranath *et al*., 2005; John *et al*., 2008; Haq *et al*., 2011; Kumar *et al*., 2017a). The yellow mosaic disease is mainly caused by MYMV and MYMIV which are the most prevalent in grain legumes across India (Fauquet *et al*., 2007). In our presented study, in order to identify viral diseases of mungbean in Assam India, a survey was conducted during 2019–2020 in various places of Assam and we observed severe yellow mosaic disease incidences. Our findings confirmed the association of Begomoviruses with yellow mosaic disease in mungbean. The wide incidences of yellow mosaic disease and prevalence of whiteflies in North-Eastern regions prompted us to investigate the disease occurrence and severity index in mungbean cultivation and its association in cowpea. Our finding also clearly revealed appearance of characteristic viral infection symptoms in the various genotypes of mungbean and cowpea plants agro-infiltrated with MYMIV mungbean isolate. The sequence comparison analysis also depicted 95-98% homology of mungbean isolate MYMIV DNA-A and DNA-B with the genomic components of other reported MYMIV strains.

The phylogenetic analysis and the other demarcation criteria revealed that the mungbean isolate MYMIV could be a new variant of the previously reported MYMIV but may not be a novel Begomovirus strain. In recent past, few reports are available on studying the genetic diversity of Begomoviruses in legumes from some specific regions (Ilyas *et al*., 2010) but now and extensive studies is much needed which will provide the main origin, diversity and their host range of the Begomoviruses of agricultural importance.

Any new or variant of Begomovirus isolated from any host plant may or may not cause the viral disease unless it fulfills the criteria of Koch's postulates. Due to a wider host range and their geographical distribution of MYMIV, till date very less number of isolates are known which has been found to be infectious to its natural host or any experimental plants. In our study we found that MYMIV mungbean isolate is able to infect two other genotypes cv. *PUSA-VISHAL*, cv. *K-851* of mungbean and one genotype of cowpea

cv. *PUSA KOMAL* which clearly satisfied Koch's postulates. We also found that the severity of disease symptoms in agroinfiltrated mungbean and cowpea test plants were quiet similar to the natural disease incidence which occurred in the field.

We observed severe yellow mosaic symptoms with stunted growth , necrosis of plants, reduced no of pods, shrunken seeds after agroinfilatration of MYMIV dimeric constructs at 56 dpi which clearly suggested that these two mungbean genotypes are very much sensitive against MYMIV-Assam Begomovirus isolate while absence of viral disease symptoms in mungbean genotype cv. *PUSA-105* agroinfiltrated with MYMIV DNA-2A or DNA-2B, even after 86 days of post infiltration clearly suggested that this genotype is YMV tolerant. In the similar way we observed yellow mosaic and downward leaf curling in cowpea cv. PUSA-KOMAL at 28 dpi which become sever at 56 dpi clearly suggest that this cowpea genotypes is sensitive against MYMIV mungbean Assam isolate. The results also suggested that MYMIV mungbean Assam isolate have wide diversity of virus infectivity in closely related legume species such as cowpea. In a recent report the MYMIV cowpea Jharkhand isolate were also found to infect cowpea with high disease severity at 28 dpi and mild disease severity in mungbean at 56 dpi (Kumar *et al*., 2017a). For the successful virus infection with generation of disease symptoms, it mainly depends upon the productive interactions between various host factors and the viruses at each stage of the infection process and also to the adaptation of various host which differs from each other (Gillette *et al*., 1998; Petty *et al*., 2000).

The appearance of viral disease symptoms in two genotypes of mungbean cv. *PUSA-VISHAL* and cv. *K-851* and one genotype of cowpea cv. PUSA-KOMAL agro-infiltrated with MYMIV mungbean Assam isolate suggested for the first time that MYMIV mungbean isolate could infect host mungbean as well as non-host cowpea and satisfying Koch's postulates. The results also suggests that the MYMIV mungbean Assam isolate has become a potential threat to both mungbean and cowpea production in Assam India. Molecular analysis through RCA and semi-quantitative qPCR supported the extent of virus multiplicity, their systemic movement to the newly generated leaves in the test plants of mungbean and cowpea, co-infiltrated with MYMIV DNA-2A and DNA-2B dimeric constructs. The semi-quantitative qPCR results suggested that the higher relative accumulation of MYMIV DNA components in mungbean in comparison to cowpea at 28 dpi could be mainly due to host specificity for the virus replication and severity of the disease symptoms. The appearance of YMV disease symptoms in non-host cowpea due to artificial inoculation of MYMIV mungbean Assam isolate indicates the possibility of cowpea becoming a new host of this strain which also increases the risk for its cultivation in other parts of India. Therefore, at this scenario some strong practical intervention measures is quiet necessary to combat the disease transmission and also for the development of virus resistant plants.

Author's contributions

SK carried out the survey for the collection of virus infected mungbean plant materials, and execution of all experiments. KVD and MD assisted in cloning of viral DNA fragments. SK and LS conceived and designed the experiments. SK and LS critically analyzed the data. SK prepared the manuscript. All authors read and approved the final version of the manuscript.

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