CHARACTERIZATION OF YELLOW MOSAIC VIRUS (YMV) INFECTING GRAIN LEGUMES AND DEVELOPMENT OF DIAGNOSTICS

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COIMBATORE-641 003

2013

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Thesis submitted in part fulfillment of the requirements for the award of Degree of

DOCTOR OF PHILOSOPHY IN PLANT PATHOLOGY

to the Tamil Nadu Agricultural University, Coimbatore - 3

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CERTIFICATE

This is to certify that the thesis entitled **"CHARACTERIZATION OF YELLOW MOSAIC VIRUS (YMV) INFECTING GRAIN LEGUMES AND DEVELOPMENT OF DIAGNOSTICS "** submitted in partial fulfillment of the requirements for the Degree of **DOCTOR OF PHILOSOPHY** in **PLANT PATHOLOGY** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Ms. V.K. SATYA** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles. However, part of the thesis work has been published in peer reviewed scientific journal of national/international repute (copy enclosed).

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ACKNOWLEDGEMENT

I deem it a pleasure and privilege to have worked under the esteemed guidance of my chairman ***Dr. D. Alice****, Professor and Head, Department of Plant Pathology, TNAU, Coimbatore, for her keen interest, patient hearing, able insightful inspiring scholarly, affectionate guidance, constructive criticism and encouragement given which entitled in me the spirit of confidence for successful completion of this study. She has been very kind giving me an opportunity to explore my skills by providing friendly environment.*

I express my deepest sense of gratitude and sincere thanks to ***Dr. V. G. Malathi****, CSIR Emeritus Scientist, Advanced Center for Plant Virology, Indian Agricultural Research Institute for having taught me the basics of research and for having given me a working environment with total freedom in her laboratory at IARI, New Delhi. Her infectious enthusiasm and spirit towards subject had served as a constant source of inspiration and for support throughout the research.*

With immense pleasure I thank the member of advisory committee, ***Dr. P. Jayamani****, Professor (PB&G), and Principle Investigator of the scheme in which I am working for providing the opportunity to explore my skills and for her keen interest, patient hearing and valuable guidance for completion of this study.*

I am bound to thank member of advisory committee, ***Dr. R.Velazhahan,*** *Professor, Department of Plant Pathology, TNAU, Coimbatore for his support and suggestions during research.*

I place my gratitude and thanks to the members of Advisory committee ***Dr. R. Rabindran,*** *Registrar, TNAU, Coimbatore* ***Dr. C. Durairaj****, Professor, Department of Agricultural Entomology, TNAU, Coimbatore for their kind concern, timely help and guidance.*

I extend my heartfelt thanks to the P.G. coordinator ***Dr. T. Raghuchander,*** *Professor (Plant Pathology) for his guidance and care.*

I wish my gratitude and thanks to ***Dr. AR. Muthaiah,*** *Director Incharge, Centre for Plant Breeding and Genetics, TNAU, Coimbatore for his kind concern, timely help and guidance.*

I thank ***Dr. V. Paranidharan, Dr. G. Karthikeyan, Dr. V. Prakasam, Dr. T. Ganapathi, Dr. K, Prabakaran, Dr. Eraivan, Dr. S. Nakkeeran, Dr. Thirubhuvanamala, Dr. E.Rajeshwari,*** *for their help during the course of study.*

The peerless guidance and suggestions given by ***Surya, Jabili*** *and* ***Vijay (IARI)*** *deserves gratitude from the innards of my heart.*

My pen has no power to express pleasure and due regard to my lovely sisters ***Bhuvana, Adhithya, Valar, Shanmugapriya;*** *brothers,* ***Kannan, Madhavan and Deivamani, Mani;*** *my best friend* ***Renuka*** *who always helped me in all possible means by providing valuable moral support time by time in order to take the challenges of life in a best possible way.*

I thank my classmates, labmates, seniors and juniors for their help throughout the study period and for making these two years ever cherishable. I am very much thankful to ***Vinoth, Sowmini, Surya, Indra ka, Jamuna Ka,*** *Plant Breeding for their encouragement, timely help and their support throughout the field work. My special thank to* ***Ramya*** *and her husband* ***Mr. Sathyaraj*** *for their encouragement, timely help and their support throughout my research.*

I am very much thankful to ***Mahes Akka, Vinoth*** *(IARI) and all the labourers from pulses for their help during my research period.*

I wish to thank all teaching staff and non teaching staff members of the Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore for the timely guidance and help given by them throughout the study period.

No words to express my profound thanks to my dear lovable sweet son ***Nithish*** *for his affection, encouragement, prayers, love and care rendered to achieve this goal. And my special thanks to all kids* ***Harsha, Shailesh, Mugunthan, Yeshwant, Govarthanan*** *and* ***Kavitha****.*

On a personal note, words seem to be inadequate to express my affection, appreciation and indebtedness to my mother ***Vijayalakshmi****, my father* ***Kothandaraman,*** *my sweet and lovable Sister* ***Chitra****, Uncle* ***Kathir,*** *Brothers* ***Karthikeyan and Sivaraman,*** *Anni* ***Thulasi and Shanthi*** *for their unstinted encouragement, help and inspiration throughout my study period.*

The financial assistance by the Department of Biotechnology, Government of India is duly acknowledged.

I offer my prayers as thanks to God for having blessed me with the right kind of opportunities in my careers.

(V.K. Satya)

ABSTRACT

CHARACTERIZATION OF YELLOW MOSAIC VIRUS (YMV) INFECTING GRAIN LEGUMES AND DEVELOPMENT OF DIAGNOSTICS

By

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Degree : **Doctor of Philosophy in Plant Pathology**

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Yellow mosaic disease is a major threat to the cultivation of legumes in Tamil Nadu and causes yield loss upto 100 per cent. The prime focus of this study is to characterize the yellow mosaic virus in blackgram, development of diagnostics and screening for resistance source. Leaves of blackgram showing severe yellow mosaic symptoms were collected during summer from three hot spot areas of Tamil Nadu, *viz*., Coimbatore (Coimbatore), Vamban (Pudukottai) and Panpozhi (Tirunelveli) which represents three districts of Tamil Nadu. Five DNA A and eight DNA B clones were cloned and sequenced from all the three locations. The sequence analysis clearly showed that the DNA A clones showed 97-98 per cent identity with *Mungbean yellow mosaic virus*-Vam-Bg (MYMV-Vam;DQ400848) and DNA B clones exhibited 95-98 per cent identity with the DNA B components of *Mungbean yellow mosaic virus* (MYMV-Vam; DQ400849). On the basis of threshold value of 89 per cent set for the begomovirus species demarcation by the geminivirus taxonomy study group, the virus isolates are identified to belong to the species, *Mungbean yellow mosaic virus* (MYMV). The sequences of five DNA A clones and eight DNA B clones have been deposited in the NCBI database under the GenBank accession numbers KC911721, KC911718, KC911722, KC911723, KC911717, KC911724, KC911730, KC911729, KC911728, KC911727, KC911726, KC911725 and KC911731.

The genome organization of MYMV revealed to be typical of Old World bipartite begomoviruses. DNA A components encode two open reading frames (ORFs) on viral sense strand; the ORF AV1 (30 kDa) encodes coat protein (CP) which overlaps with the small ORF AV2 (14 kDa) encodes pre-Coat protein and five ORFs seen on the complementary sense strand are ORF AC1 (Replication initiator protein, Rep ~40 kDa), ORF AC2 (Transcription activation protein, TrAP ~17 kDa), ORF AC3 (Replication enhancer protein, REn ~15 kDa), ORF AC4 (PTGS suppressor, ~11.5 kDa) and ORF AC5 (~11.1 kDa). DNA B components encode ORF BV1 (Nuclear shuttle protein (NSP), ~29 kDa) on the viral sense strand and ORF BC1 (Movement protein (MP), ~32 kDa) on the complementary sense strand. In DNA A component, among various open reading frames, ORF AV1 and AC1 was highly conserved when all MYMV isolates were compared. In DNA B component, both ORFs were conserved amongst MYMV isolates The most interesting feature is that more than 15 per cent divergence was observed between the common region (CR) of DNA A and DNA B compared to other begomoviruses in which CR is highly specific for a virus and it is expected to be near identical between DNA A and DNA B component of the same virus.

The analysis of percent identity of nucleotide sequence of five DNA A components in the present study with other yellow mosaic virus isolates and begomoviruses of Indian origin revealed that they shared the highest identity of 97-98 per cent with MYMV-vam and less identity with MYMIV (81 per cent). MYMV isolates of present study shared 95-99 per cent identity between themselves and less than 56 per cent identity with New World, *Bean golden yellow mosaic virus* (BGYMV).

The comparison of complete nucleotide sequence of eight DNA B components obtained in the present work with other yellow mosaic virus isolates and selected begomoviruses revealed interesting features. All the eight DNA Bs showed 95-98 per cent identity with MYMV-Vam (DQ400849) and they shared maximum identity with MYMIV (87-89 per cent). These DNA B variants shared 93-99 per cent identity between themselves.

Phylogenetic analysis of complete DNA A nucleotide sequence of YMV isolates including the sequence data available in the database revealed that the distinct separation of YMV from other begomovirus of Indian subcontinent and China. The YMVs are well separated suggesting that they do not share any common evolutionary events. As expected, the begomovirus from the New World, BGYMV deviates from branches of both YMVs and begomoviruses from Indian subcontinent. Phylogenetic analysis based on alignment of complete nucleotide sequence of DNA B components clearly revealed that the DNA B component of individual species of yellow mosaic viruses branched into separate clades. From the analysis of recombination breakpoints using recombination detection programme, statistically significant recombination events were detected among MYMV DNA B components in the intergenic region.

Partial tandem dimers of DNA A and DNA B of MYMV isolate of blackgram (Coimbatore) were constructed by directional cloning of a bitmer followed by further cloning of full length of DNA A or DNA B appropriately. Then the dimeric constructs were mobilized into *Agrobacterium tumefaciens* EHA105*via* triparental mating using pRK2013 as a helper. The agroclones were highly infectious on blackgram and infectivity percentage was 63 per cent in blackgram Co5. And the MYMV DNA A and DNA B specific probes have been developed from the Coimbatore isolate, MYMV-CA1 and MYMV-CB1 to detect the MYMV in many crops.

The betasatellites DNA associated with yellow mosaic virus infected blackgram plants were cloned and sequenced from Coimbatore and Vamban. Complete nucleotide sequence analysis of two betasatellite clones (one each from Coimbatore and Vamban) in BLAST search revealed 95 per cent identity with *Papaya leaf curl betasatellite*, PaLcuB - [India:Chinthapalli:2005, DQ118862]. The identity observed is higher than 78 per cent identity kept as threshold value for demarcation of betasatellite species, the name *Papaya leaf curl betasatellite* - [India:CBE:BG] and *Papaya leaf curl betasatellite* - [India:VBN:BG] are proposed for the new betasatellites characterized in this study and the sequences have been deposited in the NCBI database under the GenBank accession numbers KC959933 and KC959935 respectively.

The alphasatellites DNA associated with yellow mosaic virus infected blackgram plants were cloned and sequenced from Coimbatore and Vamban. Complete nucleotide sequence of two alphasatellites each from Coimbatore and Vamban samples were analysed in BLAST search programme in the NCBI. The alphasatellites shared 98-99 per cent identity with *Vernonia yellow vein Fijian alphasatellite*, VYVFA, JF733780. Since the sequence identity is more than 98 per cent, the threshold value for demarcation of alphasatellites species, the alphasatellites of the present study are named as *Vernonia yellow vein Fijian alphasatellite* and the sequences have been deposited in the NCBI database under the accession numbers, KC959932 and KC959931. The satellite DNA, alpha and betasatellites were found associated with MYMV, which constitutes the first report for MYMV.

Attempts were made to study the seed borne nature of the yellow mosaic virus. The viral genome and virion particles were seen in whole seed extracts, seed coat, cotyledons and embryo axes from ten seeds through PCR, ELISA and ISEM. The PCR amplicons produced from seed DNA are of viral origin was further confirmed through Southern blot analysis using the MYMV DNA A specific probe of the present isolate, MYMV-CA1. The presence of MYMV in seed was further confirmed through sequencing. The sequence analysis showed maximum identity of 99 per cent with the present isolate, MYMV-CA1 and sequence from databank MYMV-Vam, DQ400648 and MYMV-Vig (Mad), AJ132575. However the seedlings failed to show symptoms and also the virion particles and viral DNA were not at all detectable in seedlings, both in ELISA and PCR. Preliminary results revealed that MYMV is seed borne but not seed transmitted.

Phenotyping of Recombinant Inbred Lines (RILs) (195 nos) derived from *Vigna mungo* (VBN (Bg) 4) × *Vigna mungo* var *silvestris* 22/2 were screened against MYMV under natural hot spot areas (Coimbatore, Vamban and Panpozhi). VBN(Bg) 4 was completely resistant, whereas *Vigna mungo* var. *silvestris* 22/2 was highly susceptible in all the three hot spot locations. A total of 101 RILS were resistant and 94 RILs were susceptible under the field condition at Coimbatore whereas 95 RILS were resistant and 100 RILs were susceptible at Vamban. In case of Panpozhi, 95 RILS were resistant and 100 RILs were susceptible under field condition. For further confirmation, phenotyping of RILs were done under artificial condition using agroinculation technique. The results revealed that a total of 93 RILs were found to be resistant and 102 RILs were susceptible.

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LIST OF PUBLICATIONS

S.No.	Journal ID	Name of the Journal	NASS Rating	Topics	Authors
1	A031	Acta Virologica	7.1	Characterization of betasatellite associated with the yellow mosaic disease of grain legumes in Southern India	V.K. Satya V.G. Malathi R. Velazhahan R. Rabindran P.Jayamani D. Alice
2	T038	The Madras Agricultural Journal	4.0	Molecular detection of *Mungbean yellow mosaic virus* and their associated satellite DNA from field samples of Tamil Nadu	V.K.Satya D.Alice

LIST OF SYMBOLS AND ABBREVIATIONS

ºC	Degree celcius
bp	Base pair
cm	Centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribo Nucleic Acid
dNTPs	Dinucleotide tri-phosphate
Fig.	Figure
g	Gram
h	Hour
kDa	Kilo Dalton
M	Molar
µl	Microlitre
µM	Micromolar
µg	Microgram
mg	Milligram
mL	Millilitre
min	Minutes
ng	Nanogram
rpm	Rotations per minute
sec	Seconds
U	Enzyme units
viz.,	Namely

CHAPTER I

INTRODUCTION

Pulse crops also known as grain legumes are the second most important group of crops grown essentially in South Asia. In India, they form the important constituent in the vegetarian diet and are cultivated under different agroclimatic conditions. They are cultivated round the year in all the three crop seasons as a short duration crop either as sole crop or intercrop. India holds the first rank in pulses production and consumption in the world. India grows the largest varieties of pulses in the world accounting for about 32 per cent of the area and 23 per cent of the world production. It is grown in an area of 10.84 million hectares in India with a production of 17.09 million tonnes (productivity – 699 kg/ha). Among pulses, blackgram (*Vigna mungo* L. Hepper) occupies a prominent place in India, covering an area about 3.29 million hectares with the production of 1.83 million tonnes and productivity is 555 kg/ha (AICRP report, 2012-13).

Viruses belonging to *Como-*, *Gemini-*, *Ilar-*, *Poty-* and *Tospovirus* groups are known to infect pulses under natural conditions in various parts of the world. The amount of damage they cause varies, depending on the particular virus or combination of viruses present, the virulence of the virus strains, the susceptibility of the variety, the timing of infection, the abundance of insect vectors and environmental conditions. Major damage is due to the yellow mosaic disease (YMD), caused by whitefly transmitted geminiviruses belonging to the genus *Begomovirus* of the family *Geminiviridae.* This disease is a serious malady of the pulses and is the major threat to cultivation of pulses in India (Malathi, 2007).

The family *Geminiviridae* comprises virus members having characteristic twinned icosahedral particles (18 × 30 nm) that encapsidate circular single stranded DNA genome. The members of the family are further classified into four genera (*Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*) based on their host range, insect vector and genome organization (van Regenmortel *et al*., 2000; Fauquet and Stanley, 2003; Fauquet *et al*., 2003). The genus *Begomovirus* comprises viruses that infect dicotyledonous plant species and are exclusively transmitted by the vector whitefly *Bemisia tabaci* Genn. The members of the genus *Begomovirus* are either monopartite or

bipartite genome. In the bipartite begomoviruses, DNA A encodes proteins required for encapsidation, replication and transcription regulation and DNA B encodes for intra and intercellular movement proteins and nuclear shuttle protein.

The YMD was first reported by Nariani (1960) in greengram (*Vigna radiata*) in Northern India and subsequently was observed throughout India in almost all the legume crops. The typical symptoms are small specks or yellow spots in the leaf lamina initially, which later enlarge to yellow and green patches which coalesce and result in complete yellowing of leaves. The affected plants produce less number of flowers and pods and size of the seeds are reduced considerably. The disease causes 85-100 per cent yield loss if the plants are infected at early stage (Nene, 1973). The disease was recognized as a major threat in Tamil Nadu as early as 1970 (Murugesan and Chelliah, 1977a). A lot of information on the varietal resistance, host range, vector transmission, epidemiological aspects and management strategies on YMD are available (Murugesan and Chelliah, 1977b; Sethuraman *et al*., 2001; Venkatesan *et al*., 2010). However, molecular characterization of the YMV received very less attention.

Association of begomovirus with the YMD in Tamil Nadu was shown by Vanitharani *et al*. (1996); subsequently Balaji *et al*. (2004) and Karthikeyan *et al*. (2004) cloned the virus. On the basis of its identity with the species *Mungbean yellow mosaic virus* (MYMV) from Thailand (Honda and Ikegrami, 1986) the virus isolate from Tamil Nadu was identified as MYMV. Interestingly this virus isolate had one DNA A (MYMV - KA30) associated with five different DNA B clones (KA21, KA22, KA27, KA28 and KA34). The DNA B components differed in sequence between each other over 5 per cent and differed in the severity of symptoms (Karthikeyan *et al*., 2004).

The YMV infecting legumes from the Old World are very distinct from legume infecting begomovirus of America and are considered to represent genetically isolated viruses well separated from other begomoviruses and even to be recognized as a separate group as *Legumovirus* distinguishing from other begomoviruses (Qazi *et al*., 2007a; Fauquet *et al*., 2008; Ilyas *et al*., 2010).

Recently two additional type of DNA satellites associated with begomovirus have been described; they are betasatellites and alphasatellites, approximately half the size of

the helper begomovirus (~1360 nt in length). 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 hosts from which they have been isolated (Briddon *et al.,* 2001; Briddon and Stanley, 2006; Kumari *et al.*, 2011). Betasatellites encode a ~13.5 kDa protein known as βC1, an 'A' rich region and a 150 nt length sequence, known as a satellite conserved region (SCR), which is conserved between all betasatellites (Briddon *et al.,* 2002). The positionally conserved βC1 is a pathogenicity determinant (Saunders *et al.,* 2004; Saeed *et al.,* 2005; Guo *et al.,* 2008; Yang *et al.,* 2008) that suppresses RNA silencing (Cui *et al.,* 2005; Gopal *et al.,* 2007; Sharma *et al.,* 2010), enhances viral DNA levels in plants (Mansoor *et al.,* 2003; Saunders *et al.,* 2004) and possibly involved in virus movement (Saeed *et al.,* 2008) but not required for its own replication (Li *et al.,* 2007).

In addition to the *Begomovirus* and DNA-β components, some whitefly transmitted diseases like Ageratum yellow vein disease, Cotton leaf curl disease and Okra leaf curl disease are associated with an additional ss DNA components called DNA 1, now referred to as alphasatellites (Briddon *et al*., 2004). They are actually not true satellite molecules, because they are capable of self replication and are thus designated as satellite-like molecules. These molecules are somewhat larger in size than betasatellites. They have a potential coding region that encodes Rep gene in the virion-sense orientation, an A-rich region and a predicted hairpin structure with a nonanucleotide sequence, TAGTATTAC forming part of the loop (Mansoor *et al*., 1999; Briddon *et al*., 2004). The Rep gene encoded by them is similar to Rep gene of ssDNA molecules of *Nanoviridae* and shares 78% identity in the gene with them (Saunders and Stanley, 1999). Alphasatellites are independently replicating molecules, dependent helper virus only for encapsidation and vector transmission.

The betasatellites are known to contribute to symptom expression but the alphasatellites is dispensible for infectivity and symptom expression. The occurrence of association of satellite DNA with begomoviruses is increasing everyday giving an indication of the widespread nature and importance (Briddon *et al.,* 2008). Thus, an attempt was made to study and characterize the beta and alphasatellites associated with yellow mosaic disease.

Successful plant breeding programs for disease resistance depends on the correct identification of sources of resistance and accuracy in resistance assessment technique (Pico *et al*., 1998; Akhtar *et al*., 2010a). Sources of resistance to MYMV in mungbean and blackgram have been identified mostly on the basis of the field screening and occasionally under glasshouse conditions using varying number of viruliferous whiteflies for inoculation (Kausal and Singh, 1988; Chhabra and Kooner, 1993; Chhabra *et al*., 1993). Vector population declines when the climatic conditions are unfavourable, to overcome these problem development of agroclones and artificial screening through agroinoculation are becoming recognizable in recent years (Biswas, 1996; Biswas and Varma, 2001; Karthikeyan *et al*., 2011). Knowing the distribution patterns of legume infecting viruses is crucial for the management of the diseases for developing resistant cultivars. Therefore the present study was undertaken to understand the identity and diversity of legume infecting virus in the major legume cultivating areas of Tamil Nadu, South India and develop the agroclones for artificial screening. Keeping these lacunae in view the below mentioned objectives are envisaged to study the characterization of legume infecting yellow mosaic virus, association of satellites DNA and identification of resistance source.

Objectives

- To establish the yellow mosaic virus isolates and study the biological characterization of yellow mosaic virus and seed transmission.
- To clone DNA-A and DNA-B components of YMV and prove the infectivity of the clones through *Agrobacterium* mediated delivery using resistant and susceptible variety.
- To elucidate the genome organization of YMV and to study the phylogenetic relationship between YMV isolates and other YMV isolates infecting legumes.
- To develop the specific DNA probe for diagnosis.
- Field / artificial (phenotyping) screening of Recombinant Inbred Lines derived from *Vigna mungo* × *Vigna mungo* var *silvestris* 22/2.

CHAPTER II

REVIEW OF LITERATURE

Tropical grain legumes (also known as pulses) are of prime importance to developing countries as a major source of dietary protein in areas where protein intake from meat and fish is low. India is one of the major pulse producing countries of the world, grain legumes rank third behind cereals and oilseeds. As the world's population rises, the demand for grain legumes is also rising and agriculture is struggling to meet increasing demands. Both biotic and abiotic constraints limit grain legume production and across Southern Asia, diseases due to viruses are one of the major biotic causes of losses to production (Ilyas *et al*., 2009). Yellow mosaic diseases are one of the major limiting factors for the low productivity of the pulse crops. The affected crops are blackgram, greengram, french bean, pigeonpea and soybean and causes an annual yield loss of about US $ 300 million in a year (Varma *et al*., 1992). The disease was recognized as a major threat in Tamil Nadu as early as 1970 (Murugesan and Chelliah, 1977a; Murugesan *et al*., 1977). A lot of information on the varietal resistance, host range, vector transmission, epidemiological aspects and management strategies to contain the disease are available (Murugesan and Chelliah, 1977b; Sethuraman *et al*., 2001; Venkatesan *et al*., 2010). However, molecular characterization of the YMV received very less attention. Literatures related to yellow mosaic diseases are reviewed below.

2.1. History and distribution of yellow mosaic disease

Yellow mosaic disease (YMD) was first reported from western India in the late 1940s in Lima bean (Capoor and Varma, 1948) and later Nariani (1960) reported in greengram in the experimental fields at Indian Agricultural Research Institute, Pusa, New Delhi. Since then the YMD was observed subsequently throughout India in almost all major legume crops. Similarly, in the regions of the subcontinent now forming part of Pakistan, YMD was first reported in cowpea in Faisalabad (Vasudeva, 1942). Across the subcontinent, including India, Bangladesh, Pakistan and Sri Lanka, YMD is a major constraint to the production of most of the major legume crops (Jalaluddin and Shaikh, 1981; Malik, 1992; Sivanathan, 1977). A severe outbreak of YMD in greengram occurred

in northern Thailand in 1997. This caused major losses to production and initiated a shift in cropping practices (Morinaga *et al*., 1993). Several variants of these viruses causing YMDs in grain legumes in different geographical regions of India have been detected (Varma *et al*., 1998; Biswas and Varma, 2000; Qazi *et al*., 2007a; Ilyas *et al*., 2010).

2.2. Symptoms

Symptoms caused by YMV are largely dependent on host species and susceptibility. In blackgram, greengram and soybean, YMV causes irregular yellow and green patches on older leaves and complete yellowing of young leaves of susceptible varieties. Affected plants produce fewer flowers and pods; pods often develop mottling, remain small and contain fewer and smaller seeds. In blackgram two types of symptoms viz., yellow mottle and necrotic mottle can be distinguished (Nair and Nene, 1974; Nene, 1973). The necrotic mottle is usually associated with resistance. In pigeon pea, initially the symptoms appeared as yellow specks on the newly developed leaves. The specks coalesced and formed yellow patches against the green background of the lamina (Williams *et al*., 1968). In cowpea, affected plants showed a yellow mosaic with downward leaf curling, vein swelling, vein enations and severe leaf distortion (Rouhibakhsh and Malathi, 2005). Infections of french bean usually did not produce a mosaic but instead induced a downward leaf curling (Qazi *et al*., 2007a).

2.3. Host range

Yellow mosaic virus species mostly infect dicotyledonous plants especially leguminous crops and weeds. The crop species affected include; lima bean (Capoor and Varma, 1948), dolichos (Capoor and Verma, 1950), greengram, blackgram and soybean (Nariani, 1960; Nene, 1972), pigeon pea (Williams *et al*., 1968), horsegram and french bean (Muniyappa *et al*., 1976), groundnut (Sudhakar Rao *et al*., 1979), moth bean (Satyavir, 1980), cluster bean (Rao *et al*., 1982) and cowpea (Varma and Reddy, 1984). The host range of YMV in Thailand appears to be restricted to plants in the family *Leguminosae* (Honda *et al*., 1983). Differences in the host range of YMV isolates have been studied by various workers, but the results continued to be ambiguous (Varma and Malathi, 2003). The difficulty was mainly due to vector feeding behaviour, the host genotypes used and the environmental conditions provided. The difficulty in vector

transmission of the virus from cowpea was also experienced by Thottapilly *et al*. (1998) when they attempted to transmit the cowpea golden mosaic virus from cowpea to lima bean. Pigeon pea isolate of MYMV (MVMV-Pp) could be transmitted by whitefly to greengram, blackgram, french bean, soybean, lima bean, horsegram and cowpea (Mandal *et al*., 1998). Soybean isolate of *Mungbean yellow mosaic India virus* (MYMIV-Sb) has the ability to infect cowpea, blackgram and greengram. MYMIV-Sb characteristically differed from MYMIV-Bg and MYMIV-Mg isolates in being infectious on cowpea and produce typical golden mosaic symptoms (Usharani *et al*., 2005). Cowpea isolate of MYMIV was transmissible by whitefly to cowpea, yard long bean and french bean and not to any other legumes (Malathi *et al*., 2005).

2.4. Epidemiology

The maximum incidence of YMV was recorded in greengram sown from March-May while low disease incidence was observed in the crop raised from August-December under South Indian conditions (Murugesan and Chelliah, 1977b). High temperature has been found to be positively correlated with *B. tabaci* population in 20-30 days old crop and with disease incidence in 45 days old crop. Multiple regression technique indicated that partial regression coefficient on maximum temperature alone was important in predicting the whitefly population one week in advance, whereas partial regression coefficient on maximum temperature, rainfall and vector population were important to predict the disease outbreak (Murugesan *et al*., 1977). Singh and Gurha (1994) also observed the highest disease incidence in summer sown greengram (March-April) compared to the crop grown in kharif and spring season at Kanpur, under North Indian conditions. Livinder *et al*. (2009) reported that the highest yellow mosaic disease incidence was observed when the temperature was maximum (34-35ºC), whereas no disease incidence was observed when there was heavy and wide spread rain during August. Khan *et al*. (2012) studied the correlation of environmental factors (maximum and minimum temperature, relative humidity and rainfall) with yellow mosaic disease and found significant correlation of environmental variables with yellow mosaic disease incidence.

2.5. Transmission

The YMV isolates of Indian subcontinent are not sap transmissible (Nariani, 1960), however a *Mungbean yellow mosaic virus* isolate from Thailand is sap transmissible (Honda *et al*., 1983). Yellow mosaic viruses are transmitted by whitefly, *B. tabaci* Genn. in a persistent circulatory manner. Even a single whitefly can transmit the virus and cause 25 per cent infection after Acquisition Access Period (AAP) and Inoculation Access Period (IAP) of 24 h, and 4-10 whiteflies/plant are required for 100 per cent transmission (Nair and Nene, 1973). Female whiteflies are better transmitters and also retain the virus for a longer period (10 days) than the male (3 days) (Rathi and Nene, 1974a, 1974b). Manjunath *et al*. (2012) reported that B Biotype whitefly had less AAP and IAP to acquire and transmit the virus when compared to indigenous *B. tabaci*, and female whitefly had high transmission efficiency (53.3 per cent) as compared to male (33.3 per cent).

2.5.1. Seed transmission

Among the different mode of transmission of viruses, seed transmission of viruses is the most important one as the viruses may persist in the seeds for a long period and may spread the diseases into whichever new areas they are transported. About 20 per cent of plant viruses are reported to be seed transmitted (Mink, 1993; Hull, 2002). It is especially true of legume crops. The lists of legume viruses that are known to be seed transmitted are provided in the Table 1. The major seed borne/transmitted viruses are *soybean mosaic virus* in soybean, *Cowpea severe mosaic virus* in cowpea and *Bean common mosaic virus* in mungbean. The majority of the suspected seed transmitted viruses affecting grain legumes belong to *Clostero*, *Poty* and *Tobamo* group (Hull, 2002). Though there is record of large number of viruses belonging to diverse group, present in seed tissue, actual seed transmission and seedling showing infection and expressing symptoms are known only in very limited cases. The lack of high rate of seed transmission in majority of the cases is mainly attributed to the host genotype (Smith and Hewitt, 1938; Couch, 1955) and the virus propensity to multiply and invade the embryo early in developmental stage.

The seed infection of the viruses can be broadly categorised into two types. In the first type, seed borne viruses where the external seed coat is contaminated with the seed borne virus, which immediately infects the germinating seedling and cause infection. In this type endosperm or embryo are not at all infected by the virus. The typical example of this type of seed infection is *Tobacco mosaic virus* contamination of tomato seed coat (Lartey *et al*., 1997). The second and most common type of seed transmission is in which the virus found in embryo and in the seed tissue. The embryo or the gametes (male or female) get infected by the virus or direct invasion of the embryo after fertilization take place (Mandahar, 1981).

Some of the seeds which are infected may show symptoms or may remain normal. When infected seeds germinate, seedlings exhibit symptoms at the very early stage of the germination and seedling establishment, it is considered to have resulted due to seed transmission (Hull, 2002). The proportion of seed infection and seed transmission are governed by various parameters, some of which are briefly discussed below;

2.5.1.1. Virus

From the discussion of Stace-Smith and Hamilton (1988) it is clear that, a minimum of thirty groups of viruses and one group of viroids are reported to be seed transmitted. The highest seed borne nature is seen in *Cryptovirus*, followed by *Potyvirus*, *Nepovirus*, *Ilarvirus*, *Tobamovirus* and *Cucumovirus*. From the above list it can be seen that majority of the viruses are RNA viruses. There are examples of seed borne DNA viruses too. Among *Geminiviridae*, Bennet (1936) showed the presence of the virus in all seed tissue except embryo. In the genus *Begomovirus*, the *Abutilon mosaic virus* was reported to be seed borne by Keur (1934), however this has been disproved by Costa (1955).

In the case of *Caulimoviridae*, the viruses, *Commelina yellow mottle virus* (Scott *et al*., 1992), *Pineapple bacilliform virus* (Thomson *et al*., 1996), *Piper yellow mottle virus* (de Silva *et al*., 2002), *Tarobacilliform virus* (Macanawai *et al*., 2005), *Dahlia mosaic virus* (Pahalawatta *et al*., 2007) and *Cocoa swollen shoot virus* (Quainoo *et al*., 2008) are shown to be seed transmitted in their respective host. The virus members differ in their transmission properties within each family and genus.

2.5.1.2. Viral gene or determinants of seed transmission

To get transmitted through seeds, viral genome should be able to counter many challenges. The virus must systemically spread, during the vegetative stage of the plant, establish itself in developing embryo and withstand the unfavourable condition of seed desiccation and storage. After the period of storage when seeds germinates, they should get activated thus, the virus encounter host changes, (i) genetically from diploid to haploid (gamete) then again to diploid state of plant, (ii) from active vegetative growth to reproductive stage, dormancy and back to vegetative stage. By doing recombination study between actively transmissible and non transmissible viral isolates, the determinants of the transmission have been identified in some virus. Examples are RNA1 as major determinant in *Pea early brown virus*, RNAr in *Barley stripe mosaic virus* (BSMV), 5'NTR, the Hc-Pro and coat protein gene in the case of *Pea seed-borne mosaic virus* (PSbMV) (Johansen *et al*., 1996).

2.5.1.3. The host genotype

The viruses are transmitted at different rates in different hosts for eg. *Abutilon mosaic virus* (AMV) 10 per cent of the seed in *Melitous indica*, 2 per cent in *Stachys arvenus* and 0.1 per cent in *Ornithopus compressus* (McKirdy and Jones, 1994). The difference in genotype is mainly due to multiplication of the virus in the non vascular region, accumulation of it in enough concentration in seed coat near micropylar region so as to gain entry into the embryo. The host resistance may limit these processes and so within the same species, transmission efficiency differs.

There are other parameters like, the time at which the plant is infected, location of the seed on the plant, age of the seed and temperature. Ultimately, the environmental factors play a major role, in deciding how much per cent of seeds harbouring the virus result in seedlings showing symptoms.

2.5.1.4. Distribution of the virus within the seed

Except for one case of TMV, in which only seed coat has viruses and other seed tissue are free from viruses, in most of the cases viruses are distributed in seed coat, endosperm and embryo (Ali and Kobayashi, 2010).

2.5.1.5. Mechanism of seed transmission

The embryo can be infected by two ways, either from the mother plant virus directly invading embryo or entering through infection of gamete. The mechanism by which the virus gains entry into embryo has been studied in various host/virus systems. Of all the studies, the model proposed by Wang and Maule (1994) to describe seed transmission in Pea/PSbMV is more appealing. According to the model, the virus moves through the seed coat and slowly reach micropylar region in enough concentration. The micropyle is in close contact with a special combination of cells called suspensor cells. The suspensor cells function as conduit for the flow of nutrient, through which the viruses can also move and reach the immature developing embryo. The suspensor cells will degrade once the embryo develops and the connection with the maternal tissue is snapped. A very narrow duration of time, the micropyle connects to suspensor cells exist which can allow the virus to enter. The host genotype may differ in the duration of time available. Whether this model proposed will be applicable in all the seed transmission cases will have to be looked into.

2.6. Electron microscopy

The geminate particles were observed in *Mungbean yellow mosaic virus* (MYMV) affected greengram plants under electron microscopy for the first time by Thongmeearkom *et al*. (1981) and aggregates of virus particles and fibrillar bodies appeared in nuclei of phloem cells. Honda *et al*. (1983) purified the virus from yellow mosaic disease affected greengram plants and observed the occurrence of quasi-isometric particles of 18×24 nm size in pairs under electron microscope. Association of geminate particles in YMD infected plants of greengram, soybean, lima bean, french bean, groundnut and bambarra groundnut was shown by Muniyappa *et al*. (1987). Similar geminate particles were also observed in plants inoculated with YMV isolates from blackgram, cowpea and pigeonpea by immunosorbent electron microscopy using polyclonal antibody to *Squash leaf curl virus* (Srivastava, 1989; Varma *et al*., 1992).

2.7. *Geminiviridae*

In tropical and sub-tropical regions of the world, the typical symptoms of geminivirus infections have been observed in plants since the nineteenth century

(Wege *et al.*, 2000). It was in the 1970s when a distinct group of single-stranded DNA viruses was found associated with these symptoms (Harrison *et al.*, 1977). This new group was later named Geminivirus (Goodman, 1977) and later in 1980s classified into a new plant virus family *Geminiviridae* (Rybicki, 1994). The name was derived from *Gemini*, the Zodiac sign symbolized by twins (Harrison *et al.*, 1977). Geminiviruses are a group of viruses which infect economically important agricultural crops and characterized by the circular single stranded DNA, of length ranging from 2.5 to 3.0 kb that is encapsidated in twinned icosahedral particles (18 × 30 mm) (Stanley, 1985). Geminiviruses replicate through an intermediate dsDNA molecule in the nuclei of infected host plant cells and depend upon the host DNA replication machinery (Jeske, 2007). Geminiviruses are independently replicating plant viruses, with the smallest known genome of plant-infecting viruses, utilize bidirectional mode of transcription and overlapping genes for efficient coding of proteins (Rojas *et al.*, 2005). The members of the *Geminiviridae* are grouped into four genera (*Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*) based on their host range, insect vector and genome organization (van Regenmortel *et al*., 2000; Fauquet *et al*., 2003; Fauquet and Stanley, 2003).

2.7.1. The genus *Begomovirus*

More than 80 per cent of the known geminiviruses belong to the genus *Begomovirus*, economically most important and geographically wide spread virus infect only dicotyledonous plant species and are transmitted exclusively by the whitefly *B. tabaci*. A total of 196 member species are there in the genus *Begomovirus*, representing the largest genus of family *Geminiviridae* (Brown *et al.*, 2012). Based on phylogenetic studies and genome arrangement, begomoviruses have been divided broadly into two groups: the Old World (OW) viruses (Eastern hemisphere, Europe, Africa, Asia) and the New World (NW) viruses (Western hemisphere, the Americas) (Rybicki, 1994; Padidam *et al*., 1999; Paximadis *et al*., 1999). Begomovirus genomes have a number of characteristics that distinguish OW and NW viruses. All NW begomoviruses are bipartite, whereas both bipartite and monopartite begomoviruses are present in the OW. In addition, DNA-A of bipartite begomoviruses from the NW lacks an AV2 ORF (Rybicki, 1994; Stanley *et al*., 2005). NW begomoviruses also have an N-terminal PWRsMaGT motif in the CP that is absent from OW viruses (Harrison *et al*., 2002).

Two groups of viruses, those infecting legumes in Africa, India, and South East Asia are called Legumoviruses (Ilyas *et al*., 2010) and viruses that infect sweet potato are distinct from all other OW begomoviruses.

Complete nucleotide sequence information of genomic components of about twenty eight yellow mosaic virus (YMV) isolates from different regions of India and from different hosts is currently available (Malathi and John, 2008). Phylogenetic analysis of YMV characterized, led to recognition of totally seven different YMV species, together referred to yellow mosaic viruses (YMVs). The species affecting cultivated legumes are *Mungbean yellow mosaic virus* (MYMV; Moringa *et al*., 1993), *Mungbean yellow mosaic India virus* (MYMIV; Usharani *et al*., 2004), *Dolichos yellow mosaic virus* (DoYMV; Maruthi *et al*., 2006), *Horsegram yellow mosaic virus* (HgYMV; Barnabas *et al*., 2010), *Velvet bean severe mosaic virus* (VBSMV; FN543425) and *Kudzu mosaic virus* (KuMV; Ha *et al*., 2008) from Vietnam. The two viruses *Rhynchosia yellow mosaic virus* (RhYMV; Ilyas *et al*., 2009) and *Rhynchosia yellow mosaic India Virus* (Jyothsna *et al*., 2011) have been reported from Pakistan and India respectively. Of these MYMIV and MYMV are most important, MYMIV is more predominant in northern, central and eastern regions of India (Usharani *et al*., 2004) and MYMV in southern (Karthikeyan *et al*., 2004; Girish and Usha, 2004) and western regions.

2.8. Genome organization

The begomoviruses associated with YMDs have bipartite genomes (DNA A and DNA B) (Honda and Ikegami, 1986; Mandal *et al*., 1997). The DNA A component contains six open reading frames (ORFs), two in the viral sense strand and four in the complementary sense strand and encodes for coat protein (CP, V1/AV1) and Pre-coat protein (AV2/V2) in viral strand and replication initiation protein (Rep, C1/AC1), transcription activator protein (TrAP, C2/AC2), replication enhancer protein (REn, C3/ AC3) and PTGS suppressor (C4/AC4) on complementary strand (Rojas *et al*., 2005; Seal *et al*., 2006). The product of an additional complementary-sense gene (AC5), which is only conserved amongst legume YMVs, has been shown to have a possible role in viral genome replication (Raghavan *et al*., 2004). DNA-B has two ORFs encoding movement proteins: BV1 (nuclear shuttle protein, NSP) on the virus-sense strand and BC1

(movement protein, MP) on the complementary-sense strand (Rojas *et al*., 2005; Seal *et al*., 2006). 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 nanonucleotide sequence TAATATTAC that represent the origin of viral strand replication (Hanley-Bowdoin *et al*., 1999).

2.8.1. ORF AV1- Coat protein (CP)

Coat protein (CP) is responsible for forming the typical twinned particle structure (Townsend *et al*., 1985; Mullineaux *et al*., 1988). Besides the encapsidation function, CP is also required for transmission of the virus both within and between the plants. The CP of the monopartite geminivirus facilitates the transmission and systemic movement (Boulton *et al*., 1989; Lazarowitz *et al*., 1989; Woolston *et al*., 1989; Liu *et al*., 1999). In contrast, bipartite begomoviruses may not require CP for systemic spread (Stanley and Townsend, 1986; Gardiner *et al*., 1988; Jeffrey *et al*., 1996; Pooma *et al*., 1996), although the disease symptoms are often attenuated and the onset of disease is delayed when the plants are systemically infected with CP mutants (Hayes and Buck, 1989; Sanderfoot and Lazarowitz, 1996; Unseld *et al*., 2004). The CP also protects the viral ssDNA from degradation during transmission (Azzam *et al*., 1994; Frischmuth and Stanley, 1998) and determines the vector specificity (Briddon *et al*., 1990; Hofer *et al*., 1997; Hohnle *et al*., 2001). At some point during rolling circle replication (RCR), CP interferes with nicking of DNA thus limiting the viral DNA copy number (Rojas *et al*., 2005; Yadava *et al*., 2010).

2.8.2. ORF AV2- Pre-coat protein (Pre-CP)

The ORF AV2, overlapping the ORF AV1 in the viral sense strand of DNA A occurs only in the OW bipartite and monopartite begomoviruses. Begomoviruses native to the NW characteristically lack the AV2 gene. A pathogenicity determinant is believed to be involved in virus movement in plants (Rojas *et al*., 2005) and/or act as a suppressor of RNA silencing (Zrachya *et al*., 2007; Yadava *et al*., 2010). It takes part in perinuclear distribution of begomoviruses by association with endoplasmic reticulum (ER) and cytoplasmic strands (Sharma *et al*., 2011).

2.8.3. ORF AC1 - Replication-initiation protein (Rep)

Rep is a multifunctional, oligomerric protein which possesses site-specific DNA-binding, nicking and ligation properties, and executes ATP-dependent topoisomerase I, ATPase and helicase activities (Pant *et al*., 2001; Choudhury *et al*., 2006). Rep is an RCR-initiator protein that recognises the reiterated motifs (*iterons*) and nicks within the nonanucleotide sequence to initiate replication (Hanley-Bowdoin *et al.*, 2004; Nash *et al.*, 2011).

2.8.4. ORF AC2 - Transcriptional activator protein (TrAP)

It transactivates the expression of virion-sense genes from both DNA A and DNA B (for bipartite begomoviruses), functions as a suppressor of RNA silencing in bipartite (Trinks *et al.*, 2005; Yang *et al.*, 2007) and monopartite begomoviruses (Gopal *et al.*, 2007). It also overcomes virus induced hypersensitive cell death (Hussain *et al.*, 2007; Mubin *et al.*, 2010).

2.8.5. ORF AC3 - Replication enhancer protein (REn)

REn is involved in establishing an environment conducive for virus replication (Pedersen and Hanley-Bowdoin, 1994). REn is required for efficient viral DNA replication (Pasumarthy *et al.*, 2011).

2.8.6. ORF AC4 - C4 protein- PTGS suppressor

The function of the C4 protein still remains unclear but for some viruses it is a pathogenicity determinant in cell-cycle control, may counter a host response to Rep expression and a suppressor of PTGS by binding of siRNAs (Vanitharani *et al.*, 2004; Pandey *et al.*, 2009).

2.8.7. ORF AC5

AC5 protein, encoded by a gene not well conserved between begomoviruses, has been shown to have a possible function in viral DNA replication (Raghavan *et al*., 2004).

DNA B component contains two ORFs in opposite orientations encoding a nuclear shuttle protein (NSP, ORF BV1) on the virion sense strand and a movement protein (MP, ORF BC1) on the complementary-sense strand

2.8.8. ORF BV1 - Nuclear shuttle protein (NSP)

NSP is responsible for transport of viral DNA from the nucleus into the cytoplasm (Sanderfoot *et al.*, 1996; Malik *et al.*, 2005). NSP is the major symptom determinant (Mahajan *et al*., 2011).

2.8.9. ORF BC1 - Movement protein (MP)

It is also responsible for viral pathogenic properties (Jeffrey *et al.*, 1996). MP increases the plasmodesmal size exclusion limit and promotes cell to cell movement of the viral DNA (Sanderfoot and Lazarowitz, 1996; Hanley-Bowdoin *et al*., 1999).

2.9. Infectivity of cloned components

Agroinoculation has been mostly utilized to study infectivity, replication and movement of cloned viral genomes (Kheyr-Pour *et al*., 1991; Czosnek *et al*., 1993; Buraguhain *et al*., 1994; Mandal *et al*., 1997). Infectivity of cloned DNAs of blackgram, cowpea, greengram, pigeon pea, soybean isolates of MYMIV, MYMIV-Mb (Biswas, 1996 and Chakraborty, 1996), MYMIV-Pp (Chakraborthy, 1996), MYMIV-Bg (Mandal *et al*., 1997), blackgram isolates of MYMV, MYMV-Vig (Karthikeyan *et al*., 2004; Balaji *et al*., 2004), cowpea isolates of MYMIV-Cp (Malathi *et al*., 2005), soybean isolates of MYMIV-Sb (Usharani *et al*., 2005), soybean isolate of MYMV-Sb (Mad) (Girish and Usha, 2005) on leguminous hosts have been established by agroinoculation.

In this strategy more than one copy of DNA A and DNA B components are cloned in a binary vector, which is mobilized to *Agrobacterium tumefaciens* which is used to deliver the viral inoculum. The infectivity of the cloned DNAs of MYMV – Bg was demonstrated by Mandal *et al*. (1997). Agroinoculation of the dimeric constructs of the viral DNA was done in both sprouted seeds and blackgram seedlings; agroinoculation of sprouted seeds resulted in upto 50 per cent infection as compared to 13-20 per cent by agroinoculation of seedlings. Agroinoculation was performed using partial dimers of KA27 and KA22 DNA Bs along with DNA A by Balaji *et al*. (2004). KA22 DNA B and KA27 DNA B caused more intense yellow mosaic symptoms in blackgram and greengram respectively. The results revealed that DNA B of MYMV-Vig plays an important role in determinant of host range between *V. mungo* and *V. radiata*.

Partial dimeric clones of DNA A (KA30) and each five DNA Bs (KA21, KA22, KA27, KA28 and KA34) of MYMV-Vig, each containing two origins of replication as direct repeats, were inoculated into *V. mungo* plants. Typical yellow mosaic symptoms were observed in the trifoliate leaves from DNA Bs (KA21, KA22, KA28 and KA34) inoculated plants, where as KA27 DNA B induced mild yellow mosaic symptoms, in addition to that the plants were stunted, the trifoliate leaves showed downward curling and the leaflets were smaller with shortened petioles (Karthikeyan *et al*., 2004).

Agroinfection of soybean with the partial dimers of DNA A and DNA B of MYMIV-Sb (MP) could produce the typical symptoms of YMD on soybean in spite of the divergence in the *ori* origin (Girish and Usha, 2005). Usharani *et al*. (2005) demonstrated the infectivity analysis of a soybean isolate of MYMIV on blackgram, cowpea, greengram, french bean and soybean through agroinoculation and it produced symptoms on all the hosts. Agroinoculation of cowpea isolate MYMV –Cp systemically infected and produced yellow mosaic symptoms in blackgram and greengram (Malathi *et al*., 2005). Infectious clones of cowpea isolate of MYMIV-(IN::Ana:CpMBKA25:04) was tested on french bean, cowpea, greengram and blackgram. Typical yellow mosaic symptoms were seen in the first trifoliate leaves of cowpea, greengram and blackgram 11– 16 days post agroinoculation. For french bean, downward leaf curl and puckering symptoms appeared within 6– 9 days. The infectivity to blackgram was significantly lower than to the other three plant species (John *et al*., 2008).

Haq *et al*. (2011) studied the genetic reassortment of DNA A and DNA B components of MYMV-(IN:Vam:05) and MYMIV-(II:ND:Bg 3:91). When combinations DNA A of MYMV and DNA B of MYMIV and DNA A of MYMIV and DNA B of MYMV were agroinoculated on blackgram, french bean, cowpea, greengram; the symptoms appeared only in the case of french bean which was similar to the symptoms caused by MYMIV and MYMV in wild type, whereas in cowpea, blackgram and greengram, symptoms were not seen, they appeared similar to mock inoculated control plants (Haq *et al*., 2011).

An improved agroinfection method was developed for bipartite geminiviruses by Jacob *et al*. (2003), A simple strategy involving a single *Agrobacterium* strain harboring

DNA A and DNA B partial tandem repeats in T-DNAs of two compatible binary vectors yielded a high frequency (100 per cent) of agroinfection of the bipartite *Begomovirus* MYMV-Vig. Co-agroinoculation of MYMV-Vig DNA A with both KA27 and KA22 DNA B components ameliorated severe stunting caused by KA27 DNA B and also alleviated the delay in flowering (Mahajan *et al*., 2011). Barnabas *et al*. (2010) demonstrated that the agroinfection of horsegram with the DNA A and DNA B infectious clones produced the typical yellow mosaic disease symptoms, indicating that both these components are essential for disease induction.

2.10. Detection and diagnosis

Detection and diagnosis of begomoviruses and their associated DNA components are essential for managing the devastating diseases caused by them and in studying the epidemiology of the viruses. Detection in the vector and in weeds which serve as alternative host assumes importance when we wish to identify the inoculum source of the viruses. In India, there has been shift from identification of the virus based on biological characteristics like symptoms, host range and transmission characters to serological and nucleic acid based techniques. Most of the yellow mosaic viruses infecting grain legumes in India are not sap – transmissible, share a very narrow host range within legumes and cause biologically indistinguishable symptoms, making specific identification of the viruses difficult.

Based on epitope-profile studies using panels of monoclonal antibodies to *Indian cassava mosaic virus* and *African cassava mosaic virus* (ICMV and ACMV), Swanson *et al*. (1992) classified legume geminiviruses in India broadly into two groups. One group comprises *Dolichous yellow mosaic virus* and the other group includes the yellow mosaic viruses that infect blackgram, cowpea, french bean, horsegram, pigeon pea, soybean and greengram. Thailand MYMV found to be different from other WTGs derived from India in its epitope profile (Harrison *et al*., 1991). Because of the difficulty in purifying begomovirus virion particles, nucleic acid based approaches like PCR and hybridization with radiolabelled probes are being widely preferred for their diagnosis in the place of serological techniques. There is nearly 60 per cent identity in the nucleotide sequence in DNA A of begomoviruses. As a result, radiolabelled probe prepared either for coat

protein or Rep region of any begomovirus can detect other begomoviruses. Contrastingly, the nucleotide sequence identity between begomoviruses in DNA B is only around 40 per cent, so any probe made using DNA B is highly virus specific. This feature of begomovirus was used to detect the presence of begomovirus as a group using DNA A probe and DNA B probe for specific virus detection as in the case of yellow mosaic viruses of legumes (Varma *et al*., 1992; Biswas and Varma, 2000; Roy and Malathi, 2004).

Roy and Malathi (2004) developed nucleic acid hybridization technique to detect the *Cowpea Golden Mosaic Virus* (CPGMV) in many crops and weed species. This study showed that CPGMV DNA A probe detected all the begomoviruses where as CPGMV DNA B probe is specific to CPGMV. Deng *et al*. (1994) designed the degenerate primers to amplify the virus belonging to the family *Geminiviridae*. Rojas *et al*. (1993) designed the degenerate primers from the highly conserved region of DNA A component to amplify the virus belonging the genus *Begomovirus*. Naimuddin *et al*. (2011) designed the specific primers to amplify both the DNA components of MYMV and MYMIV. For DNA A component, the primers were designed from the coat protein region, whereas for DNA B, the primers were designed from the movement protein region.

2.11. Phylogenetic relationship

Based on phylogenetic studies and genome arrangement, begomoviruses have been divided broadly into two groups: the OW viruses (Eastern hemisphere, Europe, Africa, Asia) and the NW viruses (Western hemisphere, the Americas) (Rybicki, 1994; Padidam *et al*., 1999; Paximadis *et al*., 1999).Yellow mosaic viruses are distinct from the numerous legume infecting begomoviruses that occur in the Americas. In phylogenetic analyses, the legume YMVs are always basal to the OW begomoviruses (Qazi *et al*., 2007a; Ilyas *et al*., 2010). Members of the genus begomovirus are known to form clusters according to their geographical origin with distinct branches for viruses from Americas, Africa and Asia (Fauquet and Stanley, 2003). However in the case of legume viruses of South Asia, they do not align with viruses of their geographical or host origin, but form a genetically distant cluster basal to other Old World begomoviruses. This prompted Ilyas *et al*. (2010) to consider these viruses as a separate group designated as *Legumovirus*.

Girish and Usha (2005) determined the distribution and phylogenetic relationship of legume infecting begomoviruses from South East Asia. A phylogenetic tree based on the DNA A of YMVs from South East Asia which formed two distinct branches. The isolates of MYMV from India, Thailand and Pakistan cluster together whereas isolates of MYMIV from India, Bangladesh, Nepal and Pakistan form a separate branch. The topology of the phylogenetic tree of DNA B is different from that of DNA A with three major branches. DNA B components associated with soybean and horsegram isolates of MYMV constitute one cluster, which shows the most divergence. DNA B components associated with MYMV-Sb (Mad) and MYMV-Vig (Mad) and the MYMV-Mg (TH) form the second cluster. The third cluster is formed by the four DNA B components associated with MYMV-Vig (Mad). All the DNA B components associated with MYMIV species cluster together indicating the heterogeneous nature of the DNA B population (Girish and Usha, 2005).

The Common Region (CR) sequence identity between MYMV-Vig DNA A and DNA Bs (KA21, KA22, KA27, KA28 and KA34) was determined by Balaji *et al*. (2004). KA22 DNA B showed the highest CR sequence identity of 95 per cent to that of DNA A, while KA27 DNA B shared the lowest CR sequence identity of 85.6 per cent. In ORF BC1 (movement protein), MYMV-Vig KA27 DNA B differs from other four DNA Bs in 29 aminoacid position whereas in ORF BV1 (Nuclear shuttle protein), it differs by 53 aminoacids (Balaji *et al*., 2004). The KA27 DNA B of MYMV-Vig has 95 per cent identity to DNA B MYMV (TH), while DNA Bs (KA21, KA22, KA28 and KA34) exhibit 71-72 per cent identity. Interestingly, MYMV-Vig DNA Bs (KA21, KA22, KA28 and KA34) exhibit 89 to 90 per cent sequence identity to MYMIV (Karthikeyan *et al*., 2004).

The DNA A and DNA B sequence of HgYMV (IN:Coi) compared with other bipartite begomoviruses revealed that DNA A has 84-85 per cent identity with isolates of MYMV and 98-99 per cent identity with horsegram, lima bean and french bean isolates of HgYMV from Banglore, whereas DNA B has 97 per cent identity with recent accessions of HgYMV isolate and also with one DNA B component of MYMV (IN:Mad:Sb) (Barnabas *et al*., 2010). Recombination continues to be the major driving force in geminivirus evolution and is particularly well documented for the begomoviruses (Padidam *et al*., 1999; Pita *et al*., 2001, Seal *et al*., 2006). For the legume YMVs there is

little evidence for recombination. Girish and Usha (2005) have shown some evidence for limited recombination between isolates of MYMIV in intergenic region and Rep gene. There was no significant recombination among MYMV isolates.

2.12. Betasatellite DNA

Betasatellites (DNA-β) are satellite molecules, approximately half the size of the helper begomovirus (~1360 nt in length). DNA-β satellites affect the replication of their helper virus and alter the symptoms induced in some host plants (Saunders *et al*., 2000; Briddon *et al*., 2001). Betasatellites have a highly conserved region called as Satellite Conserved Region (SCR), an adenosine rich region (A-rich) and a single open reading frame (ORF) in the complementary strand that codes for the βC1 protein (Briddon *et al*., 2003, 2008). The SCR (~200 nt in length) contains a potential hairpin structure with the loop sequence TAA/GTATTAC, similar to the origin of virion strand DNA replication for the geminivirus and nanoviruses (Briddon *et al*., 2003). Pair-wise nucleotide sequence identity of about 78 per cent is an appropriate demarcation threshold for distinguishing between betasatellites (Briddon *et al.,* 2008; Kings *et al.,* 2011).

Betasatellites are thought to be promiscuous that they may associate and function with more than a single helper begomovirus. Previously a single begomovirus, *Tomato leaf curl virus* (ToLCV), originating from Australia was shown to be associated with a ss DNA satellite molecule. This satellite had no discernable effects on either viral replication or on the symptoms caused by ToCLV (Dry *et al*., 1997). Saunders *et al*. (2000) found another satellite-like molecule associated with *Ageratum yellow vein disease* (AYVD), which was essential for infectivity. Similar role of satellite was later demonstrated for Cotton leaf curl viruses (Briddon *et al.,* 2003; Radhakrishnan *et al*., 2004) and *Bhendi yellow vein mosaic virus* (BYVMV) (Jose and Usha, 2003) and *Tomato yellow leaf curl china virus* (TYLCCNV) (Zhou *et al.,* 2003). Acquisition of betasatellites by some of the OW bipartite begomoviruses (Rouhibakhsh and Malathi, 2005; Sivalingam *et al*., 2010) in the north-western region of the Indian subcontinent appears to have occurred recently, resulting in severe and unusual disease symptoms.

Though majority of betasatellites are associated with OW monopartite begomoviruses, there are rare instances where betasatellites have been detected along

with bipartite begomoviruses like *Tomato leaf curl New Delhi virus* (ToLCNDV), *Mungbean yellow mosaic India virus* (MYMIV) and *Tomato yellow leaf curl Thailand virus* (TYLCTHV) (Bull *et al.*, 2004; Rouhibakhsh and Malathi, 2005; Qazi *et al.*, 2007b; Guo *et al.*, 2008; Sivalingam *et al.*, 2010). Experimentally Saeed *et al.* (2007) demonstrated that when ToLCNDV and *Cotton leaf curl multan betasatellite* (CLCuMuB) are co-inoculated, betasatellite encoded protein can mobilize ToLCNDV DNA A from site of inoculation into distal tissue, indicating the possibility of interaction between the bipartite begomovirus and betasatellite. The betasatellites have been shown to be nonspecific and adapt to interaction with new begomoviruses (Nawaz-ul-Rehman *et al*., 2009). Recently a monopartite begomovirus with a betasatellite has been reported to cause a severe leaf curl disease in French bean (Kaamal *et al*., 2012).

2.12.1. βC1

The βC1 protein is a pathogenicity (symptom) determinant (Cui *et al*., 2004; Saunders *et al*., 2004), may mediate virus movement in plants (Saeed *et al*., 2007), binds DNA in a sequence non-specific manner (Cui *et al*., 2005), is a suppressor of RNA silencing (a host defense mechanism targeted against foreign nucleic acids and triggered by double stranded RNA) (Cui *et al*., 2005; Kon *et al*., 2007), forms homo-multimeric complexes in planta (Cheng *et al*., 2011), interferes with host gene expression (Andleeb *et al*., 2010) and has been shown to interact with a variety of host factors (Yang *et al*., 2008; Eini *et al*., 2009).

2.13. Alphasatellites

In addition to the begomovirus and DNA-β components, some begomovirus diseases (Ageratum yellow vein disease, Cotton leaf curl disease and Okra leaf curl disease) are associated with an additional ss DNA components called DNA 1 (now called as alphasatellites (Briddon *et al*., 2004). Alphasatellites are approximately half the size of begomovirus components (~ 1375 nt) and show a common organization consisting of a single ORF coding for a Rep protein with similarity to those of nanoviruses (Mansoor *et al*., 1999; Saunders and Stanley, 1999), an A-rich region and a predicted hairpin structure with the sequence TAGTATTAC (Briddon *et al*., 2004). Consequently, these molecules, unlike typical satellites, are capable of self replication in host plants but

require a helper begomovirus for movement within the plant as well as for insect transmission (Saunders and Stanley, 1999; Saunders *et al*., 2000). At least in one case, an unusual class of alphasatellites has been shown to attenuate begomovirus-betasatellite symptoms by reducing betasatellite DNA accumulation (Idris *et al*., 2011). Recently, two distinct alphasatellites have been found to be associated with two New world bipartite begomoviruses infecting weeds, *Euphorbia mosaic virus* and *Cleome leaf crumple virus* (Paprotka *et al*., 2010). The alphasatellite – like molecule found in Venezuela was associated with the bipartite begomovirus *Melon chlorotic mosaic virus* (Romay *et al*., 2010).

2.14. Taxonomy and Nomenclature

For classification of viruses, certain rules are there and it should be followed to classify viruses into species, strains and variants (van Regenmortel, 2006). *Geminiviridae* is the second largest family among plant viruses and taxonomy and nomenclature of geminivirus is becoming difficult due to the increasing number of viral genomic sequences deposited. The taxonomic list of geminiviruses is regularly updated (Kings *et al*., 2011; Brown *et al.*, 2012) but there should be some criteria to classify and describe the newly identified geminiviruses. Therefore, to make the taxonomic standards more clear and nomenclature guidelines more transparent, ICTV has proposed a recent set of demarcation criteria (Brown *et al.*, 2012) for classification and naming of geminiviruses. It has been proposed that if the pairwise nucleotide sequence identity of a newly isolated geminivirus sequence to the already reported geminiviral sequences, using Clustal W, is <89 per cent, it would be accepted as a new species whereas, a sequence identity >89 per cent would be classified as a member of the same species. By comparing the nucleotide sequence of a geminivirus isolate to all known strains and variants if the pairwise comparison analysis <93 per cent it would be a member of new strain of that species and if it would be >94 per cent it is a variant of that strain of the same species. In case of betasatellites, identity of pairwise comparisons between sequences is about 78 per cent kept as demarcation threshold for distinguishing between betasatellites.

2.15. Screening for resistance source

Successful plant breeding programs for disease resistance depends on the correct identification of sources of resistance and accuracy in resistance assessment technique

(Pico *et al*., 1998; Akhtar *et al*., 2010a, 2010b). Sources of resistance to MYMV in greengram and blackgram have been identified mostly on the basis of the field screening and occasionally under glasshouse conditions using varying number of viruliferous whiteflies for inoculation (Amin *et al*., 1985-90; Kausal and Singh, 1988; Chhabra and Kooner, 1993; Chhabra *et al*., 1993). Biswas and Varma (2001) screened fifteen cultivars of blackgram to YMV variants under field conditions. Of the various cultivars tested, DPU 84-14, DPU 102, UG 218, UG 389, UL 257 and UL 310 were found to be promising as they exhibited resistant to highly resistant reaction to YMV under both field and glasshouse condition. Seventeen varieties of soybean were evaluated for resistance to YMD caused by YMV-Pp1 under glasshouse condition through vector inoculation. Out of these, five varieties of soybean namely, PK 416, PK 1042, PK1046, Pusa 20 and Pusa 40 proved to be resistant (Biswas, 2002). Bashir (2003) screened 276 lines of greengram against MYMV and out of which ten lines showed resistance. Similarly, nine resistant lines were observed in the field conditions from 83 lines against MYMV (Awasthi and Shyam, 2008). Salam *et al*. (2009) screened large number of genotypes including some germplasm, breeding lines in F_1 and F_2 generations and some released varieties against yellow mosaic virus disease of greengram during rabi and summer of 2004 and 2005. The results indicated that two genotypes GG-89 and GG39 appeared to be immune with no disease incidence during 2004. Akhtar *et al*. (2011) screened 162 greengram exotic and indigenous genotypes from eight different geographic regions for resistance to MYMIV and its whitefly vector at Pakistan during the two consecutive years 2008 and 2009. Resistance levels were assessed by visual scoring of symptoms in the field under natural conditions. But none of the genotypes was found to be resistant and also large diversity of response among the genotypes were observed.

Karthikeyan *et al*. (2011) evaluated 203 F_2 progenies obtained from Vamban (Gg) 2 × KMG 189 under field condition through infector row method. The results revealed that 30 F_2 populations were identified as resistant out of 203 against MYMV. Kundu and Pal (2012) evaluated the F_2 population of recombinant inbred lines obtained from the cross T9 × VM1 under field condition and the results showed 35 elite inbred lines resistant against MYMIV.

CHAPTER III

MATERIALS AND METHODS

A detailed account of protocols, techniques and materials utilized in the present study is given below. Information about the reagents, buffers used in molecular analysis is given in Appendix I. The protocols described by Sambrook and Russell (2001) were followed for molecular biology work. Biosafety committee instructions of the institute were followed in handling the plants inoculated with different constructs. The details of begomoviruses, betasatellites and alphasatellites used for sequence analysis and phylogenetic comparison are given in Appendix IV.

3.1. Establishment of virus culture

3.1.1. Source of virus

Blackgram plants affected with severe yellow mosaic disease showing severe yellow mosaic symptoms were collected from widely distributed districts of Tamil Nadu such as Coimbatore (Coimbatore), Vamban (Pudukottai) and Panpozhi (Tirunelveli) (Table 2) where the disease incidence was 90-100 per cent. Young leaf samples showing yellow mosaic symptoms were collected and used for this research work.

3.1.2. Maintenance of whitefly cultures

Adult whiteflies (*Bemisia tabaci*) collected from legume field were maintained on cotton (*Gossipium* sp.) and brinjal (*Solanum* sp.) plants which do not harbor the yellow mosaic virus. Plants were kept in an insect-proof glass house. The culture of whiteflies was maintained on those plants by regular transfer after every six weeks to fresh batch of plants.

3.1.3. Establishment of virus on blackgram plants

Blackgram (Co5) plants were grown in 10 × 25 cm pots in an insect-proof glasshouse. Adult non viruliferous whiteflies were given an Acquisition access period of 24 h on the leaves of naturally infected blackgram plants in a cage and then transferred to healthy blackgram (Co5) plants for an inoculation access period of 24 h. Thereafter the whiteflies were killed by spraying Imidaclorpid @ 0.1 per cent. Healthy plants were

always inoculated at primary leaf stage using 10-15 whiteflies per plant. Plants were maintained in an insect-proof glasshouse.

3.2. Electron microscopy

One hundred mg of leaf tissue from artificially inoculated plants was macerated with 0.05 M sodium phosphate buffer in the ratio of 1:1 (w/v). The virus extract was centrifuged at 12,000 rpm for 5 min and the supernatant was collected. One drop of this supernatant was placed on the surface of commercially available formvar coated copper grids. The grids were allowed to dry for 5 min, washed gently with double distilled water and stained with 2 per cent uranyl acetate. Thereafter the excess stain was removed and the mounted grids were examined in a JEO JEM-1011 transmission electron microscope.

3.3. Isolation of genomic DNA

Total DNA was extracted from symptomatic young leaves of yellow mosaic disease affected blackgram from Coimbatore, Vamban and Panpozhi by GEM-CTAB method (Rouhibakhsh *et al.*, 2008) using 2 per cent β - mercapto ethanol. The symptomatic fresh young leaves were collected from field in the early morning. 100mg of leaves were ground to fine powder using liquid nitrogen. Prewarmed DNA extraction buffer (N-cetyl-N,N,N trimethyl ammonium bromide (2 %), 100 mM Tris Hcl (pH: 8.0), 1.4 M NaCl, 20 mM EDTA (pH: 8.0) and β - mercaptoethanol (2%)) was added to the ground leaves and incubated at 65°C for 30 min followed by the addition of 0.7-0.8 vol. of chloroform and isoamylalcohol (24:1 v/v). The contents were gently mixed by inverting the tube for 10 min and then centrifuged at 10,000 rpm for 10 min. The upper aqueous phase containing DNA was transferred to a new 1.5 mL micro centrifuge tube and added with equal volume of isopropanol and mixed well by inverting the tube to precipitate the nucleic acid and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and the DNA pellet was washed with 70% ethanol and air dried. The DNA pellet was resuspended in 50 µl of sterile double distilled water. The genomic DNA was checked by 0.8% agarose gel electrophoresis and stored at -20°C for further use.

3.4. Spectrophotometric quantification of genomic DNA/RNA

The quantity of nucleic acids (DNA) either from plant samples or recombinant plasmids was determined by measuring the absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). DNA concentration was calculated based on the formula, 1 OD_{260} = dsDNA 50 µg/mL, and RNA 40 µg/mL respectively.

µg/µl dsDNA = OD_{260} X 50 X dilution factor / 1000

µg/µl RNA = OD_{260} X 40 X dilution factor / 1000

Quality of the DNA/RNA was judged by taking the A260/A280 ratios. Ratio of 1.8 $\pm$ 0.2 indicates the best quality of the nucleic acids.

3.5. Agarose gel electrophoresis

Total genomic DNA from infected plants or plasmid DNA from recombinant clones were resolved in 1.2% agarose gel. The DNAs were resolved in 0.8 – 1 % agarose gel prepared in 1X TAE (Tris acetate EDTA) buffer, pH 8.8, containing ethidium bromide (0.5 µg/mL). The electrophoresis was performed at ~50V for 2 - 3 h using 1X TAE as electrophoresis buffer. The gel was visualized and results are documented in a UV-Gel Documentation system.

3.6. Preliminary diagnosis for the presence / absence of begomovirus and its associated betasatellites

3.6.1. Detection of YMV through dot blot hybridization

The presence of begomovirus from the infected samples was verified by dot blot hybridization using radio labelled probe to coat protein gene of *Mungbean yellow mosaic virus* (MYMV-IN:vam:Bg) for the detection of DNA A. The total genomic DNA was spotted into nitrocellulose membrane and was fixed onto membrane by baking the membrane at 80ºC for 1-2 h. Then hybridization was done using the protocol as described in following section 3.6.2.

3.6.2. Southern hybridization

DNA fractions resolved in 1.2 % agarose gel were transferred to charged nitrocellulose membrane (NCM, Milipore) or nylon membrane using Southern transfer procedure developed by Southern (Southern, 1975).

3.6.2.1. Southern transfer

- After visualizing agarose gel on the UV transilluminator, the top left hand corner of the gel was cut to fix the current orientation of gel.
- DNA in the gel was alkali-denatured by soaking the gel in denaturation solution for 45min with gentle agitation.
- The gel was then neutralized by soaking in neutralization solution for 30 min with gentle agitation.
- A piece of Whatmann filter paper (3 mm) was soaked in 10X SSC and kept over a glass plate which was in turn placed over a tray containing 10X SSC (transfer buffer).
- The gel was then kept inverted down on the glass plate over the soaked filter paper.
- A piece of nylon membrane cut into the same size as of the gel, soaked in 10X SSC and cut marked the same way as the orientation of gel was placed over the gel, taking care to avoid air bubbles.
- Over the nylon membrane, 3 small pieces (same as the size of gel) of Whatmann filter paper (3mm) were kept and a stack of ordinary filter paper was kept over it, which helps in capillary transfer of DNA from gel to membrane.
- A weight of approximately 750 g was kept on the blotting paper to facilitate efficient capillary action. After 18 h of capillary transfer, the membrane was washed in 10X SSC and air dried.
- The membrane was baked at 80°C for 2 h to fix the DNA on membrane.

3.6.2.2. Pre hybridization

The baked blots were kept in clean hybridization cylinders. Pre hybridization solution was added at the rate of 0.2 mL/sq. cm. The cylinders were then incubated at 65°C for 4h in hybridization oven with gentle rotation.

3.6.2.3. Probe preparation by random primer labelling method (Feinberg and Vogelstain, 1983)

Random primer labelling was done in 50 µl reaction mixture.

Recipe for primer labelling reaction

Template DNA (~100 ng)	1 µl
Random hexanucleotide primer (100 ng/µl)	1 µl
10X labelling buffer	5 µl
dNTPs (excluding dCTP) (2.5 mM each)	2 µl
Dithiothreitol (DTT) 20 mM	2.5 µl
Nuclease free BSA	2.0 µl
(α^{32}P) dCTP (10µCi/µl, 3000 Ci/mmol)	1.0 µl
Klenow (DNA Pol I) (30U/µl)	1.0 µl
Sterile distilled water	34.5 µl
Total volume	50.0 µl

Template DNA and primer was added to SDW and boiled for 1 min followed by immediate plunging in the ice for denaturation. Rest of the components were added into the tube and incubated at 37°C for 2 h to facilitate the synthesis of the new strand. The labelled probe was stored at -20°C until further use.

3.6.2.4. Hybridization

- The double stranded α^{32}P labelled DNA probe was denatured in a boiling water bath for 5min.

- The denatured probe was added to prehybridisation solution at 0.5 x 10^6 cpm/mL concentration.
- It was allowed to hybridize for 18 h at 65°C in hybridization oven with gentle rotation.

3.6.2.5. Autoradiography

- The hybridization solution was discarded and the membrane was washed three times with wash solution (2X SSC, 0.1% SDS) at 65°C, each time for the duration of 15 min.
- The washed membrane was dried on a paper towel, then placed within folds of cling film, placed in the cassette with intensifier and exposed to the phoshphoscreen.
- Hybridization signal detection was carried out using a Storage Phosphor System Cyclone® Plus (Perkin Elmer, Shelton, CT, USA). The hybridization signal intensity was analyzed through densitometric analysis using Opti Quant Version 5.0 (Perkin Elmer, Shelton, CT, USA).

3.6.3. Preliminary detection of *Begomovirus* through PCR

The purified genomic DNA was amplified by polymerase chain reaction (PCR) using universal Begomovirus-specific primers PALIc1960 and PALIr772 (Rojas *et al*., 1993). Reactions were performed in 20 µl mixture containing approximately 50 ng of genomic DNA, 5 mM each dNTPs, 20 pmol of each PALIc1960 forward primer and PALIr772 reverse primer (Table 3) and 0.5 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The reaction was carried out in Eppendorf epgradient S Master cycler (Eppendorf, Hamburg, 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 gels in TAE buffer and visualized by staining with ethidium bromide and recorded with an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). The sizes of the PCR products were determined by comparison with standard 1 kb molecular marker (Fermentas).

3.6.4. Detection of betasatellite through Southern blot analysis

The presence of begomovirus from the infected samples was verified by Southern blot analysis using radio labelled probe to and full-length PaLCuB-[IN:Chinthapalli:08] as probe for the detection of associated DNA betasatellites. The total genomic DNA was spotted into nitrocellulose membrane and was fixed onto membrane by baking the membrane at 80ºC for 1-2 h. Then hybridization was done using the protocol as described in following section 3.6.2.

3.6.5. Detection of associated alpha and betasatellite DNA through PCR

The purified genomic DNA/ RCA product was amplified by polymerase chain reaction (PCR) using universal betasatellites specific primers β01 and β02 (Briddon *et al*., 2002) and alphasatellites were amplified using universal abutting primers UN101/UN102 and DNA101 / DNA102 (Bull *et al*., 2003) (Table 3). Reactions were performed in 20 µl mixture containing approximately 50 ng of genomic DNA, 5 mM each dNTPs, 20 pmol of each forward primer and reverse primer and 0.5 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The reaction was carried out in Eppendorf epgradient S Master cycler (Eppendorf, Hamburg, 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 gels in TAE buffer and visualized by staining with ethidium bromide and recorded with an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). The sizes of the PCR products were determined by comparison with standard 1 kb molecular marker (Fermentas).

3.7. Enrichment of Viral DNA through rolling circle amplification

Samples which were PCR positive with universal primers for begomoviruses were subjected to RCA. RCA utilizes the DNA polymerase of the *Bacillus subtilis* bacteriophage Ø29, which possesses polymerase and strand-displacement activity, allowing circular templates to be amplified preferentially. In order to characterize the genomic components of the begomovirus, 70 ng of total nucleic acid extracted from PCR positive sample was subjected to rolling circle amplification (RCA) using Ø29 DNA polymerase (Haible *et al.,* 2006). The reaction mixture consisted of

Genomic DNA template	0.7 µl (70 ng)
Exo-resistant Random hexamer primer (preferably 3' protected)	2.0 µl (50 µM)
dNTPs	2.0 µl (1 mM)
10X Ø29 DNA polymerase buffer	2.0 µl (1X)
Sterile distilled water (to make up the volume to 20µl)	8.6 µl

- The reaction mixture was incubated at 94^{o}C for 3 min to denature the template DNA.
- Cooled it to room temperature slowly to facilitate the annealing of primers and then added the enzyme.

Ø29 Polymerase (10 units/µl)	0.7 µl
PyroPhosphotase Inorganic (from yeast) (0.1 unit/µl)	4.0 µl

- The whole mixture was incubated at 30^{o}C for 18-20 h in a water bath.
- Reaction was stopped by inactivating at 65^{o}C for 10 min in a dry bath incubator.
- The products were used for construction of multimeric clones as concatamers. Gel electrophoresis of RCA product shows uniform smear of DNA as large amount of replicative concatamers are generated by RCA.

3.8. Identification of viral DNA by restricting the rolling circle amplified product with different endonucleases

500 ng of rolling circle amplified DNA (the replicative concatemers) was subjected to digestion with different endonucleases to identify the unique restriction sites which can be used for cloning. Restriction of RCA product with common endonuclease enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Kpn*I, and *Xba*I) to check for the full-length viral genomic DNA components (~2.7 kb) either DNA A or DNA B and the betasatellite or alphasatellite (~1.3kb).

3.9. Purification of RCA restricted products

The ~2.7 kb fragments representing full-length DNA A and DNA B components were purified using Phenol:Chloroform:Isoamylaclcohol (PCI) method.

- Equal volume of PCI was added to the RCA restricted products and mixed by gentle inversion for 10 min.
- The mixture was centrifuge at 12000 rpm for 10 min at room temperature.
- The aqueous phase was collected in fresh tube and 2.5 vol. of absolute alcohol and 1/10 vol. of 3M sodium acetate pH 5.2 was added and mixed gently.
- The mixture was incubated at -80ºC for 20 min or 20ºC for 2 h or overnight.
- The mixture was centrifuged at 13,000 rpm for 20 min at 4°C.
- The DNA pellet was washed twice with 70% ethanol to remove the salts.
- The pellet was air dried and dissolved in sterile distilled water and stored at -20°C.

Then the purified full-length fragments were cloned into cloning vector, pUC18 (Appendix III) linearized with the respective enzymes.

3.10. Cloning of the identified begomovirus species

The purified linearized vector and insert were ligated and the ligation mixture was used to transform competent cells of *Escherichia coli* (DH5α). Transformants with the recombinant plasmid were selected on Luria agar (LA) containing nalidixic acid (30 μg/L), ampicillin (50 μg/mL), X-gal (40 μg/mL) and IPTG (0.2 mM). The plates were incubated overnight at 37°C.

3.11. Selection of transformants

3.11.1. Blue/white colony screening

The white colonies were selected and subsequently plated as grid on IXAN (IPTG, X-gal, ampicillin, nalidixic acid) plates. This plate having individual transformants in grid served as master plate. From this master grid, clones were analyzed

and selected. The recombinant transformants were screened by different methods to select the correct recombinant clones. The following methods were used.

3.11.1.1. Colony PCR

The colony PCR was carried out to observe for the presence of the recombinant plasmids in transformed bacterial colonies wherein amplification of the inserted DNA was done using specific primers. Single isolated colony was taken with a sterile toothpick and transferred to a microfuge tube containing 38 µl double distilled H_2O, and following reagents were added into the tube and kept for PCR.

Template DNA	Bacterial colony
Taq DNA polymerase buffer (10X)	5.0 µl (1X)
$MgCl_2$ (25 mM)	4.0 µl (2 mM)
dNTP mix (10 mM each)	1.0 µl (200 nM)
Forward primer (100 ng)	1.0 µl (2 ng)
Reverse primer (100 ng)	1.0 µl (2 ng)
Taq DNA polymerase (5 units/ µl)	0.5 µl (2.5 unit)
double distilled H_2O to make the volume to	50 µl

The conventional PCR was performed in a thermocycler using the conditions of initial denaturation at 94°C for 2 min and 30 cycles of 1 min denaturation at 94°C, 2 min annealing at 55°C and 3 min elongation at 72°C followed by a final extension for 10 min at 72°C.

3.11.1.2. Isolation of recombinant plasmid DNA by miniprep method

Alkali lysis method (Birnboim and Doly, 1979) was followed to isolate plasmid DNA from *E. coli*. Exposure of bacterial suspension to the strong detergent like sodium dodecyl sulphate (SDS), at high pH breaks the cell wall, denature chromosomal DNA and proteins and release plasmid DNA into the supernatant (Sambrook and Russell, 2001).

The isolation of recombinant plasmid DNA was carried out in the following manner:

- Selected colonies, which were presumed to contain viral insert in colony PCR were individually inoculated into 2 mL LB containing 50 μg/mL of appropriate antibiotic.
- The tubes were incubated at 37°C at 200 rpm for 16 h in a shaker incubator.
- Bacterial cells were then transferred to 1.5 mL sterile microcentrifuge tubes and were harvested by centrifugation at 6,000 rpm 7 min.
- Supernatant was removed completely and the pellet was resuspended in 100 μl of Solution I and mixed vigorously by vortexing, for 30 sec.
- 200 μl of freshly prepared Solution II (lysis reagent) was added, mixed by gentle inversion and incubated at room temperature for not more than 5 min for cell lysis.
- 150 μl of ice-cold Solution III was then added and mixed by inversion to precipitate bacterial chromosomal DNA and protein. The mixture was incubated on ice for 15 min.
- The complex of bacterial chromosomal DNA and debris was pelleted by centrifugation at 13,000 rpm for 20 min at 4°C.
- Supernatant was transferred to a fresh tube and again centrifuged at 13,000 rpm for 20 min at 4°C to remove the debris completely.
- Supernatant was collected in fresh tube and 0.8volume of isopropanol was added and mixed by inversion to precipitate plasmid DNA.
- The mixture was centrifuged at 13,000 rpm for 40 min at 4°C.
- The DNA pellet was washed twice with 70 % ethanol to remove the salts.
- The pellet was air dried and dissolved in sterile distilled water and stored at -20°C.

3.11.1.2.1. Restriction analysis of recombinant plasmid DNA

To determine the exact size of the insert, plasmid DNA was restricted with enzyme used for cloning along with *Bgl*I or *Sca*I. All recombinant plasmid DNAs isolated from pGEM-T cloning were subjected to restriction using *Eco*RI endonuclease. The single restriction reaction mixture was as follows:

Plasmid DNA (500-1000ng)	Variable
Restriction enzyme *Eco*RI (5U/µl)	1.0 µl (5units)
10X reaction buffer	2.0 µl (1X)
Double distilled H_2O to make the final volume	20.0 µl

The restriction reaction mixtures were incubated at 37ºC for 3 h. The reaction was stopped by adding 6X loading dye, and electrophoresed on 1% agarose gel. Fragments were separated by agarose electrophoresis to determine the clones having inserts of about 2.7 kb or 1.3 kb which were selected and sequenced.

3.12. Sequencing and sequence analysis

Sequences of the recombinant plasmids were determined at Scigenome Labs, Kerala, India. Nucleotide similarity searches were performed using BLAST at NCBI (www.ncbi.nlm.nih). Nomenclature of identified clones has been done according to Fauquet *et al.* (2008). Nucleotide sequences of distinct and related viral species were obtained from NCBI GenBank database. Nucleotide sequence comparisons were performed aligning with ClustalW, MUSCLE and phylogenetic analyses (neighbor-joining trees with 1,000 bootstrap replicates) using BioEdit 7.0.9.0 with Mega 5.0 and SDTv1.0 and subsequently dendrograms were prepared. RDP3 Beta42 (Martin *et al*., 2010) was used to understand the recombination events on subjecting the aligned sequences to RDP, GENECONV, Maxchi, Bootscan, SiScan and Chimaera methods using selected begomovirus species. Default RDP3 settings with a 0.05 *P*-value cutoff with standard Bonferroni correction for multiple testing were used throughout. Both internal and external references were allowed, with parental cutoff of 70-100 per cent and a window size of 30. Open reading frames (ORFs) within DNA A, DNA B, alphasatellites and betasatellites were confirmed by GENERUNNER. In addition,

complete sequence analysis for each open reading frame (ORF) and intergenic region (IR) was also done for the closest virus species and respective betasatellites and alphasatellites.

3.13. Agroinoculation

3.13.1. Construction of partial tandem dimers in binary vector

Partial tandem dimers of DNA A and DNA B of yellow mosaic virus isolate of black gram were constructed by directional cloning of a bitmer followed by further cloning of full length of DNA A or DNA B appropriately. *Hind*III and *Eco*RI digestion of DNA A gave two fragments of 2 kb and 0.7 kb where as *Bam*HI and *Xba*I digestion of DNA B gave 1.5 kb and 1.2 kb fragments. The 2 kb fragment of DNA A and 1.5 kb fragment of DNA B were cloned in the binary vector pBIN19 (Appendix III) separately after digestion with respective enzymes to give 0.7 mer of DNA A and 0.5 mer of DNA B. Digestion of DNA A with *Hind*III and *Bgl*I released the full length 2.7 kb fragment which was gel purified and ligated in to the 0.7 mer of the DNA A in pBIN19. DNA B was digested with *Bam*HI and *Sca*I to release the 2.7 kb fragment and it was ligated into the 0.6 mer of DNA B in pBIN19. The orientation of construct was determined through restriction analysis.

3.13.2. Triparental Mating

The parital tandem dimeric constructs of the viruses were mobilized from *E.coli* strain DH5α to *A. tumefaciens* strain EHA 105 (Hood *et al.,* 1993) using pRK 2013 as helper plasmid in a triparental mating system (Ditta *et al.,* 1980).

- *A. tumefaciens* strain EHA 105 was grown in LB broth containing rifampicin (30 μg/mL) at 28°C for 48 h at 200 rpm.
- *E. coli* strain DH5α containing dimeric constructs and helper plasmid (pRK2013) were grown in LB broth at 37°C at 200 rpm for 14-15 h.
- 500 μl of *A. tumefaciens* strain EHA 105 and 200 μl each of *E. coli* containing helper plasmid and dimeric constructs were mixed together in an eppendorf tube.

- 50-100 µl of this mixture was spread on sterile Whatmann filter paper pieces placed on a plain LA plate and was incubated at 28°C for 48 h.
- Whatmann filter paper strips with the bacterial growth was suspended in 5 mL LB broth and mixed with gentle shaking to release bacterial culture into LB broth.
- A serial dilutions (1:10) were prepared from 100 µl of bacterial suspension.
- 100 µl of last two dilutions (10^{-3} and 10^{-4}) were spread on LA plates containing rifampicin (30 µg/mL) and kanamycin (50 µg/mL) and incubated at 28ºC for 48 h.
- The distinct colonies that appeared were confirmed to be *trans*-conjugants through colony PCR using universal abutting primers of DNA A and DNA B (Table 3) and the confirmed one was maintained in LA plates containing rifampicin (30 µg/mL) and kanamycin (50 µg/mL).

3.13.3. Agroinoculation on Plants

Agrobacterium mediated delivery of the viral tandem repeats into plant is one of the most routine techniques used by many scientists to prove the infectivity of cloned genomic components. To facilitate the release of unit genome length viral DNA, it is necessary to introduce more than one copy of the viral genome component. Infectivity was checked by stem pricking method using fine entomological needle. The blackgram plants were agroinoculated according to Mandal *et al*. (1997) by seed-sprout method. The plants were maintained at 16/8 h light/dark periods, 18,000 lx, 85% relative humidity for 3-4 weeks at 28-30^{o}C in pathology glass house until they were scored for symptoms and analyzed for viral DNA. The symptoms were recorded every day after the day of inoculation. Following were the parameters considered:

a. Number of infected plants/Number of plants inoculated to calculate percent infectivity.

b. Type of symptoms

Symptoms were scored periodically at regular point of times. For all the experiments empty vector pBin19 without any construct served as negative control (mock inoculated plants).

3.13.3.1.Preparation of *Agrobacterium* slurry

- *Agrobacterium* harbouring viral DNA components were inoculated in LB medium and grown for 48 h at 28°C shaking at 200 rpm.
- 200 µl of this culture was spread on LA plates and incubated at 28°C for 48 h.
- The lawn of bacterial growth in each LB agar plate was collected with a spatula and transferred to an eppendorf tube containing 500 µl of B5 medium and the density of suspension was adjusted to OD_{600} = 0.8.
- This 48 h old *Agrobacterium* cultures containing constructs were mixed in equal proportion according to the combination of viral genomic components to be inoculated and 30 µl of culture was inoculated per seedling.

3.14. Detection of seed borne nature of YMV

3.14.1. Genomic DNA isolation

Seeds were collected from yellow mosaic disease affected plants. Seeds were surface sterilized with Teepol 1% to remove the superficial virus and imbibed for 8 h. Then seeds were dissected into different parts such as seed coat,cotyledon and embryo axes separately. The genomic DNA was isolated from seed coat, cotyledon and embryo axes and whole seed of single seed, group of two, three, five and ten seeds following GEM-CTAB method (Rouhibakhsh *et al*., 2008) as described in above section 3.3.

3.14.2. Detection of YMV in seed through dot blot hybridization

The genomic DNA from seed samples were spotted on nitrocellulose membrane and the DNA was fixed to membrane by baking the membrane at 80ºC for 2 h. The hybridization was done with the probe to DNA A of MYMV-CA1 (KC911721) as described in section 3.6.2.

3.14.3. Detection of YMV in seed through PCR

The purified DNA from seed coat, cotyledon and embryo axes and whole seed of single seed, group of two, three, five and ten seeds was amplified by polymerase chain reaction (PCR) using universal Begomovirus-specific primers PALIc1960 and PALIr772 (Rojas *et al*., 1993) as described in above section 3.6.3. PCR amplicons from embryo,

seed coat and whole seed were purified from the gel using QIAquick Gel extraction kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's protocol and sequenced.

3.14.4. Detection of YMV in seed through Southern hybridization

The purified DNA from seed coat, cotyledon and embryo axes and whole seed of single seed, group of two, three, five and ten seeds was amplified by polymerase chain reaction (PCR) using universal Begomovirus-specific primers PALIc1960 and PALIr772 (Rojas *et al*., 1993). Further to confirm that all the amplicons produced are of viral origin, the PCR amplicons were electrophoresed and transformed to nitrocellulose membrane. The southern blot analysis was done with the probe to DNA A of MYMV-CA1 (KC911721) using protocol as described in section 3.6.2.

3.14.5. Detection of YMV in seed by ELISA

The presence of virion particles in seed tissue was determined by performing the Double antibody sandwich-Enzyme linked immunosorbant assay (DAS-ELISA) using the *African cassava mosaic virus* polyclonal antibody (Catalogue number. SRA 73600/1000).

- Capture antibody was diluted in carbonate coating buffer and 100 µl of the prepared capture antibody was added into each well.
- The plate was incubated in a humid box for 4 h at room temperature or overnight at 4ºC.
- The wells were emptied and the wells washed completely with 1X PBST twice.
- Then the 100 µl of the sample extract was added to sample wells, positive control into positive wells and sample extraction buffer into buffer wells.
- The plate was incubated inside the humid box for 2 h at room temperature or overnight at 4ºC.
- The plate was washed with 1X PBST seven times, when the sample incubation is complete.
- The alkaline phosphatase enzyme conjugate was diluted in ECI buffer and 100 µl of the enzyme conjugate was dispensed into each well.

- The plate was incubated inside the humid box for 2 h at room temperature.
- The plate was washed with 1X PBST eight times. Para-Nitrophenyl phosphate (PNP) substrate (1 mg/mL) was prepared and 100 µl was dispensed into each test well.
- The plate was incubated in a humid box for 60 min. Plates should be protected from direct or intense light.
- The plate was read at ELISA plate reader at 405 nm.

3.14.6. Detection of YMV in seed through ISEM

- Carbon-formvar grids was coated by floating on drops of antiserum and incubated for 5 min at room temperature.
- Grids were washed with 20 drops 0.1 M KPO4, pH 7. 0
- Coated grids was floated on drops of sample extract and incubated for 15-30 min at room temperature.
- Coated grids were washed with 40 drops distilled water and stained with 6 drops *2%* aqueous uranyl acetate.
- Formvar-coated copper grids were made hydrophilic by exposure for 10 sec to a carbon glow discharge. The grids were coated with virus-specific r-globulins by floating film-side down on drops of either crude antiserum.
- After washing away excess antiserum with drops of phosphate buffer, the coated grids were placed film-side down on drops of the test sample. If viral antigens in the test sample are serologically related to the coating antibodies, they will be bound to the surface of the grid.
- Unbound viral antigens or host plant constituents were washed from the surface of the grid with distilled water. The washed grids were negatively stained with 2% aqueous uranyl acetate and examined for the presence of trapped virus particles with the electron microscope.

3.15. Phenotyping of RILs against MYMV

3.15.1. Phenotyping of RILs against MYMV through agroinoculation

The blackgram RILs (195 nos) derived from *Vigna mungo* × *Vigna mungo* var *silvestris* 22/2 were screened and were agroinoculated according to Mandal et al. (1997) by seed-sprout method as described in section 3.13.3.

3.15.1.1. Analysis of viral DNA in infected plants

Leaf tissue from ten susceptible RILs and ten resistant RILs of agroinoculated plants were collected and used for analysis. Total DNA was extracted from young leaves of agro inoculated plants at 21 days post inoculation. Viral DNA was isolated from 100 mg of infected leaves of blackgram plants. Total nucleic acid was extracted following Gem-CTAB method (Rouhibakhsh *et al.,* 2008) as described above in section 3.3.

Total DNA (5µg) was electrophoresed in 1.2% agarose gel and were transferred to nitrocellulose membrane (NCM, Milipore) or nylon membrane using a standard protocol (Sambrook et al., 1989). The hybridization was done with a (α^{32}P)-dCTP labelled probes specific for CP gne of MYMV-Bg (GenBank Accession no. DQ400848) and MP gene of DNA B MYMV-Bg (GenBank Accession no. DQ400849) as described in above section 3.6.2.

3.15.2. Phenotyping of RILs against MYMV under field conditions

Recombinant Inbred Lines derived from *Vigna mungo* × *Vigna mungo* var *silvestris* were screened against Mungbean Yellow Mosaic Virus (MYMV) along with both the parents under field conditions. 195 RILs along with two parents were screened against MYMV under field conditions at the Department of Pulses, TNAU, Coimbatore, farmers' field at Vamban (Pudukottai) and Panpozhi (Thenkasi) during summer 2012 and 2013. During mid of March, each test entry was sown in a row of 4 meter in length with row to row distance 30 cm and within plant distance 10 cm. One row of highly susceptible infector variety (Blackgram – Co5) was planted after every two test entries. General cultural practices were adopted to maintain the experiment except that insecticide sprays were not applied in order to encourage the population of

whitefly for natural disease spreading. Percent disease incidence was calculated by using the following formula.

$$\text{Per cent disease incidence} = \frac{\text{Number of plants infected in a row}}{\text{Total number of plants in a row}} \times 100$$

The genotypes were later grouped into different categories based on 0-9 scale (Mayee and Datar, 1986).

Scale	Description	Category
0	No plants showing any symptoms	Immune
1	1% or less plants exhibiting symptoms	Resistant
3	1-10% plants exhibiting symptoms	Moderately Resistant
5	11-20% plants exhibiting symptoms	Moderately susceptible
7	21-50% plants exhibiting symptoms	Susceptible
9	51% and more plants exhibiting symptoms	Highly susceptible

CHAPTER IV

EXPERIMENTAL RESULTS

4.1. Disease incidence, symptoms and sample collection

Leaves of blackgram showing severe yellow mosaic symptoms were collected from three hot spot areas of Tamil Nadu, *viz*., Coimbatore (Coimbatore), Vamban (Pudukottai) and Panpozhi (Tirunelveli) which represents three districts of Tamil Nadu. Yellow mosaic disease incidence was recorded and the percentage of YMD ranged from 83 - 92 per cent, 81 – 89 per cent and 85-98 per cent during summer – 2013 respectively. Out of the three season *viz*., kharif, rabi and summer, summer crop exhibited the highest incidence of YMD. The affected plants show yellow patches intermingled with green areas, which later coalesced and show complete yellowing of leaves (Fig. 1). In severe cases pods turn yellow and contain small and shrivelled seeds. The seeds are small, slightly shrunken compared to seeds from healthy plants which appeared bold and jet black. Young leaves were collected from symptomatic plants from each district. These samples were used for the present study.

4.2. Establishment of virus

The yellow mosaic virus isolate infecting blackgram was transmitted by viruliferous *B.tabaci* from naturally infected blackgram plants to healthy blackgram (Co5) plants under glasshouse conditions. There was 65 per cent transmission and typical yellow mosaic symptoms were seen 20 days after inoculation.

4.3. Electron microscopy

Yellow mosaic disease infected leaves were examined under electron microscope. The geminate particles were observed measuring approximately 18-20 nm in diameter (Fig. 2).

4.4. Preliminary diagnosis for the presence of begomovirus

The genomic DNA was isolated from the infected blackgram samples using GEM-CTAB method with 2per cent β –mercaptoethanol. The total nucleic acid was checked on 0.8 per cent agarose gel and also the quantity of genomic DNA was

determined by Nanodrop spectrophotometer. In the gel electrophoresis a single band was seen running ~20 kb size. This unsheared band represents the good quality of genomic DNA extracted. The quantity of DNA yielded by this method was determined to be ~4.6 µg/µl.

The presence of begomovirus was confirmed by dot blot and Southern blot hybridization with specific probes (MYMV (Vam: Bg) - DNA A, DQ400848). Out of 74 samples of blackgram, 35 samples (47.29 per cent) showed positive response in the hybridization whereas healthy samples showed no hybridization (Fig. 3a). In Southern hybridization, viral replicative forms, double stranded open circular, super coiled and single stranded DNA were compared in analogy with other begomoviruses (Fig. 3b). The results were further confirmed through PCR using degenerate primers PAR1v772/ PAL1c1960. The expected amplification of ~1.1 kb was observed in 22 samples (24.32 per cent) out of 74 samples tested (Fig. 4a). The expected amplicons of ~1.1 kb was observed in all the samples tested when RCA product was subjected to PCR (Fig. 4b).

4.5. Enrichment of viral DNA through Rolling Circle Amplification (RCA)

Samples which were positive for begomovirus from PCR were subjected to rolling circle amplification to enrich the viral DNA. Two samples from each district (Coimbatore, Vamban and Panpozhi) were selected and processed further. RCA was performed with 70 ng of total nucleic acid extracted from infected leaf samples using 10 units of phi 29 DNA polymerase, exo-resistant random hexamer primers and inorganic pyrophosphatase. Gel electrophoresis of RCA product was seen as smeared DNA.

4.6. Restriction digestion

RCA product was subjected to restriction digestion with different endonucleases to know the unique site for cloning of viral DNA. Restriction of RCA with different enzymes (*Bam*HI, *Hind*III, *Cla*I and *Pst*I) yielded 2.7 kb fragment which are expected to be the full length of viral genomes, either DNA A or DNA B of the begomovirus. Restriction enzymes *Bam*HI, *Hind*III, *Cla*I and *Pst*I yielded 2.7 kb fragment when RCA product of Coimbatore samples were subjected to restriction analysis. The restriction enzymes *Bam*HI, *Hind*III and *Pst*I yielded the 2.7 kb fragment from Vamban samples.

The restriction enzymes *Bam*HI and *Pst*I yielded 2.7 kb when RCA product of Panpozhi samples was subjected to restriction analysis.

4.7. Cloning of viral DNA from the Coimbatore samples

RCA product from Coimbatore samples restricted with different endonucleases, *Hind*III, *Bam*HI, *Cla*I and *Pst*I yielded 2.7 kb fragment, which were chosen for the cloning of viral DNA components. The 2.7 kb fragment was purified using PCI (phenol:chloroform:isoamylalcohol) method and ligated with linearized pUC18 vector. Recombinant colonies were screened. A total of 70 colonies of *Hind*III clone and 45 colonies of *Bam*HI clone, nearly 1000 colonies of *Pst*I clone and *Cla*I clone were obtained. Out of these colonies, 30 colonies of *Hind*III clone, 20 colonies of *Cla*I clone and 30 colonies of *Pst*I clone were randomly selected for Miniprep. Plasmid DNA was restricted with cloning site endonuclease and *Bgl*I (which restrict the vector into two fragments, 1.5 and 1.1 kb) by which the size of the insert, 2.7 kb was easily identified. From the double digestion of plasmid DNA, *Pst*I gave two positive clones (CP-Bg8, CP-Bg9) (Fig. 5a), *Hind*III clone gave three positive clones (CH-Bg4, CH-Bg6, CH-Bg7) (Fig. 5b) and *Cla*I gave three positive clones (CC-Bg3, CC-Bg4, CC-Bg5) (Fig. 5c). From *Bam*HI clone, 30 colonies were randomly selected for Miniprep and plasmid DNA was digested with cloning site and *Sca*I. *Bam*HI clone gave five positive clones (CB-Bg5, CB-Bg7, CB-Bg8, CB-Bg11, CB-Bg12) (Fig. 5d). All the recombinant clones were grouped based on the restriction pattern. Group one consisted of *Hind*III clones and *Pst*I clones which had two *Eco*RI sites, but did not have *Bgl*I site. The second group included *Bam*HI clones and *Cla*I clones with 2.7 kb inserts with one *Bgl*I site, two *Xba*I sites, but did not have *Eco*RI site, thereby indicating that two types of DNA components have been cloned (Fig. 6a and 6b). Further, the clones were identified and authenticated by sequencing.

4.8. Cloning of viral DNA from the Vamban samples

RCA product from the Vamban samples restricted with different endonucleases, *Hind*III, *Bam*HI and *Pst*I yielded 2.7 kb fragment, were chosen for the cloning of viral DNA components. The 2.7 kb fragment was purified using PCI method and ligated with linearized pUC18 vector. A total of 150 colonies from *Hind*III clone and 35 colonies

from *Bam*HI clone, 100 colonies from *Pst*I clone were obtained. Out of which 20 colonies from *Hind*III clone and 10 colonies from *Pst*I clone were randomly selected for Miniprep. Plasmid DNA was restricted with cloning site enzyme and *Bgl*I by which the size of the insert, 2.7 kb was easily identified. From the double digestion of plasmid DNA, *Hind*III clone gave five positive clones (VH-Bg5, VH-Bg6, VH-Bg7, VH-Bg9, VH-Bg10) (Fig. 7a) and *Pst*I gave one positive clone (VP-Bg8) (Fig. 7b). From *Bam*HI clone, 20 colonies were randomly selected for Miniprep and plasmid DNA was digested with cloning site enzyme and *Sca*I. *Bam*HI clone gave seven positive clones (VB-Bg9, VB-Bg10, VB-Bg11, VB-Bg12, VB-Bg13, VB-Bg14, VB-Bg15) (Fig. 7c). All the recombinant clones were grouped based on the restriction pattern. Group one consisted of *Hind*III and *Pst*I clones which had two *Eco*RI sites, but did not have *Bgl*I site. The second group included *Bam*HI with 2.7 kb inserts with one or two *Bgl*I site, two *Xba*I sites, but did not have *Eco*RI sites thereby differentiating two population of DNA. Further, the clones were identified by sequencing.

4.9. Cloning of viral DNA from the Panpozhi samples

RCA product from Panpozhi isolate (PAN1) was digested with different endonucleases, from that *Pst*I and *Bam*HI yielded 2.7 kb fragment. The 2.7 kb fragment was purified and ligated with linearized pUC18 vector with cloning site. About 1000 colonies were obtained from *Pst*I clone and *Bam*HI clone. Eight colonies from *Pst*I clone were randomly selected for Miniprep and plasmid DNA was double digested with cloning site enzyme and *Bgl*I. From the double digestion of plasmid DNA, one true transformant was obtained from *Pst*I clone and named as clone PP-Bg8 (Fig. 8a). From *Bam*HI clone, 15 colonies were randomly selected for plasmid isolation and plasmid DNA was digested with cloning site enzyme and *Sca*I. *Bam*HI clone gave two true transformant named as clone PB-Bg7, PB-Bg9 (Fig. 8b). Clone PP-Bg8 had two *Eco*RI site and no *Bgl*I site and clone PB-Bg7, PB-Bg9 had *Xba*I site and *Bgl*I site. Selected clones were sequenced for further confirmation.

4.10. Sequence analysis

The clones CP-Bg8, CH-Bg4, CC-Bg3, CB-Bg5 and CB-Bg7 from Coimbatore samples, clones VH-Bg9, VP-Bg8, VB-Bg9, VB-Bg10, VB-Bg14 and VB-Bg15 from

Vamban samples and the clones, PP-Bg8 and PB-Bg7 from Panpozhi samples were selected and sequenced. The sequences were assembled and edited. To identify the DNA A and DNA B components, initially the sequences were individually tested in BLAST search programme. Of 13 clones, five clones CP-Bg8, CH-Bg4, VH-Bg9, VP-Bg8 and PP-Bg8 were DNA A components and eight clones, CC-Bg3, CB-Bg5, CB-Bg7, VB-Bg9, VB-Bg10, VB-Bg14, VB-Bg15 and PB-Bg7 were DNA B components. The DNA A components, CP-Bg8, CH-Bg4, VH-Bg9, VP-Bg8 and PP-Bg8 were designated as CA1, CA2, VA1, VA2 and PA1 respectively whereas DNA B components, CC-Bg3, CB-Bg5, CB-Bg7, VB-Bg9, VB-Bg10, VB-Bg14, VB-Bg15 and PB-Bg7 were designated as CB1, CB2, CB3, VB1, VB2, VB3, VB4 and PB1 respectively for the understandable discussion.

The sequence analysis clearly showed that the DNA A clones, CA1, CA2 from Coimbatore samples, VA1 and VA2 clones from the Vamban samples and PA1 clone from the Panpozhi samples showed 97-98 per cent identity with *Mungbean yellow mosaic virus*-Vam (Blackgram) (MYMV-(Vam:Bg). On the basis of threshold value of 89 per cent set for the begomovirus species demarcation by the geminivirus taxonomy study group (Kings *et al*., 2011), the virus isolates are identified to belong to the species, *Mungbean Yellow Mosaic Virus* (MYMV). For the benefit of smooth discussion of the results, the clones are designated as MYMV-CA1, MYMV-CA2 from Coimbatore, MYMV-VA1, MYMV-VA2 from Vamban and MYMV-PA1 from Panpozhi. The details of clone ID and accession numbers are provided in Table 4.

On the basis of complete nucleotide sequence, eight DNA B clones were identified, three DNA Bs, CB1, CB2 and CB3 obtained from Coimbatore, four DNA Bs, VB1, VB2, VB3, VB4 from Vamban and one DNA B, PB1 from Panpozhi. The nucleotide sequence of the DNA B components of isolates from all the three locations exhibited 95-98 per cent identity with the DNA B components of *Mungbean yellow mosaic virus* (MYMV-Vam; DQ400849). In the presentation of results the DNA Bs are designated as MYMV CB1, MYMV CB2, MYMV CB3, MYMV VB1, MYMV VB2, MYMV VB3, MYMV VB4 and MYMV PB1. Wherever the cognate DNA A and DNA B components were cloned from the same sample they are given the same clone ID no, prefixing with A or B to denote the genome.

4.11. Genome organization

Complete nucleotide sequences of the isolates MYMV DNA A were determined to be 2724 to 2738 bp in length. The Genbank accession number and the details of coding region are provided in the Table 4. The analysis of the sequences revealed that the genome organization to be typical of OW bipartite begomovirus with two open reading frames (ORFs) on viral sense strand; the ORF AV1 (30 kDa) encodes coat protein (CP) which overlaps with the small ORF AV2 (14 kDa) present only in the Old World begomoviruses. The five ORFs seen on the complementary sense strand are ORF AC1 (Replication initiator protein, Rep ~40 kDa), ORF AC2 (Transcription activation protein, TrAP ~17 kDa), ORF AC3 (Replication enhancer protein, REn ~15 kDa), ORF AC4 (PTGS suppressor, ~11.5 kDa) and ORF AC5 (~11.1 kDa) (Fig. 9a).

The DNA B components were determined to be having the genome length of 2648 to 2678 bp. All the eight DNA B components encode ORF BV1 (Nuclear shuttle protein (NSP), ~29 kDa) on the viral sense strand and ORF BC1 (Movement protein (MP), ~32 kDa) on the complementary sense strand (Fig. 9b).

4.12. Common region (CR)

In DNA A the non coding intergenic region between ORFs AV1 and AC1 was found to be ~260 bp length. In the case of DNA B component the length of non coding region between the ORF BV1 and BC1 varied from ~890 to 960 bp. The intergenic region of the cognate DNA A and DNA B were analysed in pair-wise alignment to identify the common region highly conserved between them. The length of the CR ranged from 140 to 145 nt length between the cognate DNA A and DNA B (For eg. MYMV CA1 and MYMV CB1). In begomoviruses CR is highly specific for a virus and it is expected to be near identical between DNA A and DNA B component of the same virus. However in MYMV isolates studied, the identity was only 81 per cent in CA1 and CB1. Between MYMV CA2 and MYMV CB2 it was 81 per cent and in both the VBN clones it was 87-88 per cent. The lowest identity was seen between MYMV PA1 and MYMV PB1 (78 per cent). Divergence included ~10 nt deletions and ~ 22 nt mismatches in DNA B with reference to DNA A (Fig. 10).

In the CR putative stem and loop structure with the conserved nonanucleotide sequence TAATATTAC representing the origin of replication were seen. The putative rep binding iteron sequence which are seven nucleotide repeats were identified to be ATCGGTGT which occurs as one copy in the 5' of the CR and as two tandem repeats upstream of the TATA box. In the CR TATA motif are seen at nucleotide coordinate 2631-2639 (Fig. 11).

4.13. Identity at amino acid level in the coding region

Predicted amino acid sequence of coat protein of isolates in the present study was compared with other selected MYMV and MYMIV isolates. It clearly showed that MYMV isolates, CA1, CA2, VA1 and PA1 shared maximum identity (99-100 per cent) with MYMV-Vam and 83-86 per cent identity with MYMIV. They share 99-100 per cent identity between themselves except MYMV-VA2 which share 83 per cent identity with MYMV and 71-74 per cent identity with MYMIV (Table 5). Over all alignment clearly shows the substitution of amino acids between the MYMV and MYMIV in N- terminal region (Fig. 12).

Multiple alignment of predicted amino acid sequence of pre coat protein ORF AV2 clearly showed that CA1, VA1, VA2 and PA1 shared 96-100 per cent identity with MYMV-Vam and VA2 shared very less identity (82 per cent) and the identity was 70-72 per cent with MYMIV isolates. (Fig. 13 and Table 6).

The predicted amino acid sequence of the putative protein Rep, ORF AC1 of the isolates, CA1, CA2, VA1, VA2 and PA1 were compared with that of selected YMVs. MYMV isolates of the present study shared >90 per cent identity with MYMV-Vam except CA2 and VA2 (73 per cent) and they shared very less identity with MYMIV isolates (Table 7 and Fig. 14). The iteron related sequence, through which the Rep is expected to bind with iterons in CR are ATCGGTGT similar to MYMV.

Comparison of predicted amino acid sequence of TrAP protein, ORF/AC2 of the MYMV isolates with other selected YMV isoaltes clearly showed that MYMV isolate shared 98 per cent identity with MYMV (KH-PP-03) from Cambodia and they shared <73 per cent identity with MYMIV isolates (Table 8). Overall alignment clearly shows that some amino acid deletions are there in N-terminal region in all MYMV isolates when

compared to MYMIV. The zinc finger motif (C-(1)-C-(7)-C-(6)-H-(4)-HC) in nucleic acid binding activity was located at coordinates 51-74 (Fig. 15).

The predicted amino acid sequence of REn protein, ORF AC3 of MYMV and MYMIV isolates were compared with other selected YMVs. MYMV isolates, CA1, CA2, VA2 and PA1 shared 94-99 per cent identity with MYMV-Vam and VA1 which is having C-terminal truncation shared 66 per cent identity. They shared very less identity with MYMIV isolates (Table 9 and Fig. 16).

Multiple alignment of amino acid sequences of ORF AC4 of MYMV of present isolates clearly shows that the MYMV isolates shared 98 per cent identity with MYMV-Vig (Mad) except CA2 and VA2 which are having amino acid substitution and addition in the C-terminal region. They shared very less identity (56-76 per cent) with MYMIV isolates (Table 10 and Fig. 17).

The predicted amino acid sequence of coding region, ORF AC5 of MYMV isolates were compared with other YMVs. MYMV isolates, CA1, CA2, VA1 and PA1 shared 96-99 per cent identity with MYMV-Sb (Mad) and VA2 which is having C-terminal truncation and shared 73 per cent identity. (Table 11 and Fig. 18).

The predicted amino acid sequences of NSP and MP encoded in the DNA B component when analysed revealed several interesting features which are discussed herein. The ORF BV1 encodes for a protein of MW 29 kDa which is called as Nuclear shuttle protein, involved in intra cellular movement of viral DNA. It is clear from the multiple alignment of amino acid sequence of BV1 that though there is more than 85 per cent identity at amino acid level between the virus isolates (Table 12), there are striking differences in the N-terminal region. Compared to the BV1 of MYMV CB2, all the other isolates showed N-terminal truncation lacking 7-8 amino acids. The isolate MYMV PB1 was again different from other isolates that it lacked five amino acids in the N-terminal region and sequences in the N-terminal region were totally different from others upto 40^{th} amino acid. It may be noted here that the N-terminal region of the MYMV DNA B, MYMV KA27 and MYMV-Nam are again significantly different from other isolates. The nuclear localization signal, nuclear export signal motifs and phosphorylation sites seen in BV1 and BC1 protein of DNA B are different from MYMV KA 27 DNA B. A virD2

type nuclear localization signal (PRRRHRK: coordinates 33-48) was identified in the N-terminal region of BV1 protein in all the eight DNA Bs and seven amino acid residues were identical. A nuclear export signal (LYGPL, coordinates 196-200) was found in the C terminal region of BV1 protein of all the eight DNA Bs which may facilitate their import into nucleus (Fig. 19). Overall the alignment clearly shows the substitution and deletion of amino acids in all the isolates.

The ORF BC1, encodes for movement protein which mediates the movement of viral DNA BV1 complex to periphery of the cell, and for the passage of the viral DNA through plasmodesmata. It is interesting to see that in both MYMV and MYMIV the ORF BC1 is conserved and more than 70 per cent identity is seen between them. The MYMV CB1, CB2, CB3, VB1, VB2 discussed in this work, showed only 79 per cent with MYMV KA 27 and MYMV-Nam and 86-87 per cent with MYMIV (Table 13). As in the case of NSP, multiple alignment of the predicted amino acid sequence of MP of YMVs again showed diverse features. The MYMV VB3, VB4 and PB1 showed not only N-terminal truncation but totally different amino acid sequences compared to DNA B of other MYMV isolates. However in BC1 there are lots of blocks of conserved region than found in NSP. Characteristically in a segment from 281-300, the isolates CB3, VB1 and VB2 differed in their sequences from other MYMV CB1, CB2, VB3 and VB4 (Fig. 20).

4.14. Comparison with other Yellow mosaic viruses

4.14.1. Identity in DNA A component

The analysis of percent identity of nucleotide sequence of five DNA A components in present study with other yellow mosaic viruses (YMVs) and begomoviruses of Indian origin are provided in the Table 14. From the data it is evident that the MYMV-CA1, MYMV-CA2, MYMV-VA1, MYMV-VA2 and MYMV-PA1 share the highest identity of 97-98 per cent with MYMV-vam. The identity with the other important YMV, MYMIV is only 81 per cent. MYMV isolates of present study shared 95-99 per cent identity between themselves. The relationship with HgYMV is closer (84 per cent) than with MYMIV. However, these MYMV isolates exhibited only 62 per cent identity with DoYMV from India, 69 per cent with KuMV from Thailand which are the other yellow mosaic viruses affecting dolichos and kudzu bean. MYMV isolates

exhibit only 60-61 per cent identity with other begomoviruses such as *Tomato leaf curl virus, Indian cassava mosaic virus, Cotton leaf curl virus, Papaya leaf curl virus* and *Croton yellow vein mosaic virus* from Indian subcontinent and less than 56 per cent identity with New World, *Bean golden yellow mosaic virus* (BGYMV).

4.14.2. Identity in DNA B component

The comparison of complete nucleotide sequence of eight DNA B components obtained in the research work presented here with DNA B of YMVs and selected begomoviruses revealed interesting features. All the eight DNA Bs showed 94-98 per cent with one set of MYMV DNA B (MYMV KA22, AJ132574; MYMV KA34, AJ439057 ; MYMV KA28, AJ439058 and MYMV-Vam, DQ400849). Contrastingly with another set of MYMV DNA B (MYMV KA27, MYMV-Sb (Mad), MYMY-Nam, DQ865203 and MYMV-Mg (TH) D14704), the identity is much less (64-68 per cent). Infact, the eight DNA B CB1, CB2, CB3, VB1, VB2, VB3, VB4 and PB1 are more closely related to MYMIV (87-89 per cent) than to MYMV. These DNA B variants shared 93-99 per cent identity between themselves (Table 15). It is hypothesized that different types of DNA B are generated by recombination and genetic assortment in the CR. In order to understand the extent of deviation of DNA B from MYMV KA27, the nucleotide sequence of the CR region of YMV isolates were analysed in multiple sequence alignment. From the alignment it is evident that, all the other YMV isolates have undergone deletion compared to the MYMV KA27 (AF262064) and MYMV-Nam (DQ865203). The prominent being the deletion of 18 nucleotides from the nucleotide co-ordinate 2632-2649 nt. This stretch of deletion is also seen in MYMIV isolates, that explains how DNA B variants are more closely related to MYMIV. The alignment clearly shows that the CR in these YMV isolates is under evolutionary pressure undergoing lot of changes.

4.15. Phylogenetic relationship among YMVs

4.15.1. Phylogenetic analysis of DNA A component

Phylogenetic analysis of complete DNA A nucleotide sequence of YMV isolates including the sequence data available in the database is shown in Fig. 21. Striking feature of the dendrogram is the distinct separation of YMV from other begomovirus of Indian

subcontinent and China. The YMVs are well separated suggesting that they do not share any common evolutionary events. In the phylogenetic dendrogram, DNA A sequences of YMVs segregated into eight main groups. They are, group I including DoYMV, Group II, KuMV, group III, RhYMV (Pak), group IV comprising RhYMIV and VBSMV from India and a large group V encompassing MYMIV isolates. Group VI includes HgYMV isolates, group VII includes south east Asian isolates of MYMV (Thailand, Cambodia) and the last group VIII includes the MYMV isolates from the current research work (CA1, CA2, VA1, VA2 and PA1)and the isolates from database (MYMV-Mad, MYMV-Vig, MYMV-Sb). As expected, the begomovirus from the New World, BGYMV deviates from branches of both YMVs and begomoviruses from Indian subcontinent.

The heat map (Fig. 22) generated based on the complete nucleotide sequences of the 47 DNA A components clearly showed that of 34 isolates of YMV identified to infect grain legumes and leguminous weeds in South East Asia, 12 MYMV isolates shared sequence identity of 95 to 99 per cent between themselves and they shared 79 to 82 per cent identity with MYMIV isolates. The legume infecting YMV isolates are well distinguished and showed less than 70 per cent identity with rest of the isolates.

4.15.2. Phylogenetic analysis of DNA B component

Phylogenetic analysis based on alignment of complete nucleotide sequence of DNA B components is shown in Fig. 23. The DNA B component of individual species of yellow mosaic viruses branch into separate clades very clearly. But what is surprising and unexpected is within MYMV, different groups of DNA B can be recognized. They are: group I which consists of the typical cognate DNA B of MYMV, the isolates in this group are MYMV-TH (D14704), MYMV KA27 (AF262064) and MYMV-Nam (DQ865203). It may be recalled that these MYMV isolates share only 60-61per cent identity with other groups of DNA B variants of MYMV. Group II is occupied by only one DNA B MYMV CB3, group III consists of CB2, VB1, VB2 and VB3 which again get separated from each other. Group IV consists of the MYMV KA21, MYMV KA28 described earlier. Group V is represented by MYMV isolates from databank, MYMV-vam, VB4 and PB1 of present work, group VI consists of MYMV isolates from databank MYMV KA34, MYMV KA22, MYMV-Vam and CB1 of the current research work.

It is important to recognize here that the MYMV cloned in the work communicated, CA1, CA2, VA1, VA2 and PA1 shared nearly 97-99 per cent identity with MYMV-Vig (Mad) AJ132575, MYMV-Nam DQ865201 and MYMV-Sb (Mad) AJ421642 but showed less than 75 per cent identity with their DNA B component. The sequence difference and the phylogenetic separation indicated the emergence of these DNA B variants independently and all these variants have MYMIV as basal sequences than the MYMV species MYMV-Mg (TH), MYMV KA27 and MYMV-Nam.

The heat map (Fig. 24) generated based on the complete nucleotide sequences of the 37 DNA B components clearly showed MYMV isolates shared sequence identity of 95 to 99 per cent between themselves and they shared more than 85 per cent identity with MYMIV isolates. The legume infecting YMV isolates are well distinguished and showed less than 70 per cent identity with rest of the isolates.

4.16. Analysis of Recombination breakpoints of MYMV DNA A and DNA B components using Recombination Detection Program (RDP)

Attempts were made to identify the putative recombination sites that occurred between the yellow mosaic viruses using RDP alpha. In case of DNA A components, the recombination event was detected in MYMV-VA2 and showed recombination spot of a 72 nucleotide portion of the IR of the genome (nucleotide coordinates: 6-78). The unknown virus is a major parent and *Croton yellow vein mosaic virus* –Bang-Cr2 as a minor parent and the event was detected by GENECONV and Maxchi as 4.347×10^{-04} and 1.101×10^{-02} respectively (Table 16).

In case of DNA B components, three to five potential recombination events were observed. Mostly the recombination breakpoints were observed in the regions of the genome (IR, ORF BC1 and ORF BV1).Statistically significant recombination events were detected among MYMV DNA B components. The detail of the recombination events is shown in Table 16.

4.17. Construction of partial tandem repeats

The pictorial representation of construction of partial tandem repeats of DNA A and DNA B is shown in Fig. 25 and Fig. 26 respectively. The plasmid of DNA A (CA2)

and DNA B (CB2) clones from Coimbatore isolate were restricted with *Hind*III/*Eco*RI and *Bam*HI/*Xba*I to release 2 kb and 1.5 kb fragments respectively including the origin of replication (Fig. 27). The 2 kb and 1.5 kb fragment of DNA A and DNA B were separated in 1 per cent agarose gel. The DNA fragments were extracted from the gel using Quiagen gel extraction kit and ligated with *Hind*III/*Eco*RI and *Bam*HI/*Xba*I linearized pBIN19 binary vector respectively. Transformant colonies were selected on Kanamycin (50 mg/l). In case of DNA A, 22 colonies were obtained, out of which one was true transformant which released 2 kb fragment, when plasmid DNA was restricted with *Hind*III/*Eco*RI. This true transformant was named as pBIN A 0.7 (Fig. 28a). In the case of DNA B, 20 colonies were obtained and seven were true transformants which released the 1.5 kb fragment when plasmid DNA restricted with *Bam*HI/*Xba*I. This bitmer of DNA B was named as pBIN B 0.6 (Fig. 28c). Digestion of DNA A with *Hind*III and *Bgl*I released the full length 2.7 kb fragment which was gel purified and ligated into the 0.7 mer of the DNA A in pBIN19. DNA B was digested with *Bam*HI and *Sca*I to release the 2.7 kb fragment which was ligated into the 0.6 mer of DNA B in pBIN19. Transformants were selected on Luria agar plates with kanamycin 50 mg/l and the colonies were screened for the presence of insert by rapid lysis method. In the case of DNA A, one true transformant was obtained (Fig. 28b) whereas DNA B gave three true transformants (Fig. 28d). The tandem orientation of the dimer was determined by restriction analysis.

The PTR constructs of DNA A pBIN A1.7 and pBIN B1.5 mer were mobilized into *Agrobacterium tumefaciens*. Tandem partial dimeric constructs of the virus isolate were mobilized from *E.coli* strain DH5α to *Agrobacterium tumefaciens* strain EHA 105 (Hood *et al*., 1993) using pRK2013 as helper plasmid in a triparental mating system (Ditta *et al*., 1980). The recombinants were selected on riffampicin (20 mg/l) and kanamycin (50 mg/l). Two hundred colonies were obtained from DNA A and 150 colonies were obtained from DNA B. Mobilization of the partial tandem repeat construct was confirmed by colony PCR. Full length abutting primers for DNA A and DNA B were used for checking the presence of viral components. Randomly 15 colonies were picked up and tested for colony PCR. Expected amplicon of 2.7 kb was obtained from all the colonies when the PCR product was electrophoresed on agarose gel (Fig. 29a and 29b).

4.18. Infectivity of cloned components

The infectivity of YMV isolates characterized in this study was tested for MYMV CA2 and CB2 isolate. The *Agrobacterium* cells having DNA A and DNA B PTR constructs were mixed and the agroinoculations were done on sprouting seeds on the root and hypocotyls axis region of the blackgram variety Co5. The plants were maintained in insect-proof glasshouse at 28-30ºC. Yellow mosaic symptoms appeared as small well demarkated chlorotic spots, these spots coalesced and gave rise to typical yellow mosaic symptoms in the blackgram cultivar Co5 seedlings at 21 days post inoculation (Fig. 30). The infectivity percentage was 63 per cent.

4.19. Association of betasatellites and alphasatellites

4.19.1. Detection of betasatellites

DNA was isolated from field samples of blackgram showing severe yellow mosaic along with leaf puckering symptoms from Coimbatore and Vamban. When genomic DNA was subjected to PCR using betasatellite - specific primers, the amplification was seen in a single sample from Coimbatore (Fig. 31a). The results were further confirmed through PCR using RCA product, the amplification was seen in all the samples tested (Fig. 31b). The presence of betasatellite was confirmed by Southern blot hybridization with probe of *Papaya leaf curl betasatellite*, PaLcuB - [India:Chinthapalli: 2005, DQ118862]. Positive hybridization was seen with two samples out of eight samples tested (Fig. 31c).

4.19.2. Cloning of betasatellites

For cloning of betasatellites, the RCA product of one sample from each location (Coimbatore and Vamban) was amplified with universal betasatellite-specific primers and was purified by using QIAquick Gel extraction kit (QIAGEN, Valencia, CA, USA) and ligated with the linearized pGEMT-Easy vector. The transformants selected on the IPTG, X-gal, ampicillin and nalidixic acid plates were screened for the presence of the viral DNA insert. For each ligation, large numbers of transformants were obtained. Four colonies from each sample were randomly picked and the insertion of betasatellite was analysed through colony PCR. Expected amplicon ~1.3 kb was obtained almost from all

the colonies when the PCR product was electrophoresed on agarose gel (Fig. 32). One clone from each sample was sequenced.

4.19.3. Identification of genome components

Complete nucleotide sequence analysis of two betasatellite clones (one each from blackgram from Coimbatore and blackgram from Vamban) in BLAST search revealed 95 per cent identity with *Papaya leaf curl betasatellite*, PaLcuB - [India:Chinthapalli:2005, DQ118862]. The identity observed is higher than 78 per cent identity kept as threshold value for demarcation of betasatellite species. The name *Papaya leaf curl betasatellite* - [India:CBE:BG] and *Papaya leaf curl betasatellite* - [India:VBN:BG] are proposed for the new betasatellites characterized in this study. The complete sequences for the two clones of betasatellite have been deposited in the NCBI database under the GenBank accession numbers KC959933 and KC959935 respectively.

4.19.4. Genome organization and Sequence comparison

The complete nucleotide sequence of these two betasatellite isolates was determined to be 1351 to 1359 nucleotide in length. The betasatellite encode a single gene named as βC1 (MW ~14 kDa) in the complementary strand and consists of satellite conserved region and A-rich region (Fig. 33). The complete nucleotide sequence of betasatellites of present study was compared with other PaLCuB sequences and other betasatellites available in the GenBank database. They exhibited 91 to 93 per cent identity with PaLCuB from papaya, pumpkin and Ipomoea and 88 per cent identity with PaLCuB from tomato. The comparison with betasatellites reported from cowpea and french bean showed only 52 to 58 per cent identity. The lowest percentage identity was seen with *Vernonia yellow vein betasatellite* and *Bhendi yellow vein mosaic betasatellite* (36 per cent and 39 per cent respectively) (Table 17).

4.19.5. Analysis of satellite conserved region

One of the main universal features of betasatellites is the satellite conserved region (SCR). By aligning the nucleotide of non coding region of the PaLCuB, the SCR region was computed. The SCR region was determined to be 191 nucleotide long in PaLCuB- CBE-BG and 192 in VBN-BG. From the multiple alignment shown in Fig. 34,

it is clear that within the SCR, there are blocks of highly conserved region with variable region adjoining it. The two 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 per cent identity in this region with *Papaya leaf curl betasatellite* from Cowpea, Ipomoea and Pumpkin. Repeat doublets GCTACGC were found to be present in the SCR of both the betasatellite sequences.

4.19.6. Analysis of A-rich region

A-rich region is typically between 706 to 979 nucleotide with approximately 51 to 53 per cent A sequence. The A-rich region is maintained for both the betasatellites.

4.19.7. 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 MW ~14 kDa. Comparison of amino acid sequence of βC1 protein of PaLCuB (Fig. 35a) clearly shows that, the βC1 protein of legume betasatellites of the current work are nearly identical in their amino acid composition with other PaLCuBs. They shared 93 to 98 per cent identity with cowpea isolate from Gujarat, pumpkin and papaya isolate from New Delhi and tomato isolate from Jabalpur. The identity is less than 65 per cent with betasatellites from other legumes (Table 18). From the Fig. 35b, it is evident that PaLCuBs have conserved motifs, widely differing 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.

4.19.8. Phylogenetic relationship

Complete nucleotide sequences of the betasatellites of the present study was compared with other betasatellites deposited in GenBank database and the phylogenetic tree was constructed (Fig. 36). All the eight PaLCuB betasatellites (two 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 group of betasatellites consisted of molecules originating from a diverse range of host plants such as chilli, tomato and cotton. The TbLCuB showed no particular affinity for any of the cluster.

4.20. Alphasatellites

4.20.1. Detection of alphasatellites

DNA was isolated from field samples of blackgram which showed severe yellow mosaic along with leaf puckering symptoms from Coimbatore and Vamban. When genomic DNA was subjected to PCR using alphasatellite-specific primers, there was no amplification. The amplification was seen in all the samples tested when RCA product was subjected to PCR using alphasatellite-specific primers (Fig. 37a).

4.20.2. Cloning of alphasatellites

The RCA product of one sample from each location, samples of blackgram CBE (Coimbatore) and blackgram VBN (Vamban) was utilized for cloning of alphasatellites. The RCA product amplified with universal alphasatellite primers was purified by passing through QIAquick Gel extraction kit (QIAGEN, Valencia, CA, USA) and ligated with the linearized pGEMT-Easy vector. The transformants selected on the IPTG, X-gal, ampicillin and nalidixic acid plates were screened for the presence of the viral DNA insert. For each ligation, more number of transformants were obtained. Three colonies from each sample were randomly picked and the insertion of alphasatellite was analysed through colony PCR. Expected amplicon ~1.3 kb was obtained from all the colonies tested when the PCR product was electrophoresed on agarose gel (Fig. 37b). One clone from each samples was sequenced.

4.20.3. Sequence analysis of alphasatellites

Complete nucleotide sequence of two alphasatellites each from Coimbatore and Vamban samples were analysed in BLAST search programme in the NCBI. The alphasatellites shared 98-99per cent identity with *Vernonia yellow vein Fijian alphasatellite*, VYVFA, JF733780. Since the sequence identity is more than 98 per cent, the threshold value for demarcation of alphasatellites species, the alphasatellites of the present study are named as *Vernonia yellow vein Fijian alphasatellite*. The sequences were submitted in the

GenBank database under the accessions numbers, alphasatellites CBE-BG:KC959932 and alphasatellite VBN-BG: KC959931.

Complete nucleotide sequence of alphasatellites were compared with other alphasatellites. They shared 98-99 per cent identity with *Vernonia yellow vein Fijian alphasatellite*, VYVFA, JF733780 and less identity, 51-55 per cent with other alphasatellites associated with monopartite begomoviruses such as *Okra leaf curl virus*, *Cotton leaf curl virus*, *Ageratum yellow vein virus* and *Tomato leaf curl china virus*. It shared only 41-42 per cent identity with an unusual alphasatellites (DNA 2) associated with *Ageratum yellow vein Singapore virus* (Table 19).

4.20.4. Genome organization

The complete nucleotide sequence of these two alphasatellite isolates was determined to be 1350 to 1361 nucleotide in length. Alphasatellites encode a single gene, Rep protein (MW-34.4 kDa), consisting of predicted hairpin structure and A-rich region (Fig.38).

4.20.5. Analysis of predicted hairpin structure

Alphasatellites contain a stem-loop or a predicted hairpin structure containing nonanucleotide sequence TAGTATTAC (part of forming loop) which is highly conserved among all the isolates; but little bit divergence is observed in the sequences of the stem region (Fig. 39).

4.20.6. Analysis of A-rich region

One of the most common features in the alphasatellites is A- rich region, immediately downstream of the coding region of Rep gene. The length of the A-rich region of the present isolate is 207 to 212 (nucleotide co –ordinate, 919-1130) with 45 to 46 per cent A rich content.

4.20.7. Analysis of Rep protein

The coding region on the viral sense strand is 888 bp long and encodes a rolling circle replication initiator protein, similar to the nanoviruses. The alphasatellites differ from betasatellites in having viral sense ORF encoding 34.4 KDa Rep protein. The Rep protein of the alphasatellites in the present study shares 98 per cent identity with VYVFA

and less than 60 per cent identity with Rep protein of other alphasatellites (Table 19). The multiple amino acid alignment of *Vernonia* alphasatellites with selected alphasatellites are shown in Fig. 40. The position of putative motifs involved in rolling circle DNA replication (Motifs I to III), the tyrosine of motif III, the NTP binding sites motifs A and B of the Rep protein is indicated. From the alignment it is clear that the amino acid compositions differs and vary in the length. The length of Rep protein sequence of *Vernonia* alphasatellites are 295 amino acids long and showed C terminal truncation whereas the alphasatellites from okra, cotton and *Ageratum* are longer.

4.20.8. Phylogenetic relationship

The phylogenetic relationship of alphasatellites isolated in this study with other usual and unusual alphasatellites was determined by multiple alignment of complete nucleotides. In the phylogenetic tree constructed the clusters of alphasatellites are formed according to the crop from which they were isolated. Alphasatellites in the present study form grouping with *Vernonia yellow vein Fijian alphasatellite*, VYVFA, JF733780 and is distinct from others and basal to the other clusters. The alphasatellites from cotton and okra formed the separate cluster. An unusual alphasatellites formed one cluster distinct from all other alphasatellites (Fig. 41).

4.21. Seed borne nature of the YMV

The yellow mosaic disease affected plants not only affect the yield but also infect the seeds. The seeds are (1) ill-filled, (2) reduced in size and seed weight is less when compared to healthy, (3) misshapen with improper ellipsoidal shape and (4) the yellow discolouration of pods. From the above observation, it was hypothesized that the yellow mosaic virus may enter into the seed and may be present in seed tissue. To answer these questions, experiments were conducted in three batches with the seeds harvested in the summer crop 2011-2012. Seeds were harvested separately from severely infected and symptom free blackgram plants (Co5) (Fig. 42), labelled and stored separately. PCR, ELISA, ISEM and grown out tests were done with these seeds.

4.21.1. Detection of YMV by PCR

One hundred seeds each from healthy and YMD affected plants were used for the experiments. Fifty seeds each from diseased and healthy plants were sown in 6" pots separately and maintained in insect proof cages at insect free glass house. These plants served as grow-out tests control.

Total DNA was extracted from whole seed, seed coat, cotyledon and embryo axes from the remaining fifty seeds. The PCR was performed with single seed, and group of two seeds, three, four, five and ten seeds using the degenerate primers, PAR1v772/ PAL1c1960 (Rojas et al., 1993). In the agarose gel electrophoresis of PCR products' anticipated amplicons of ~1.1 kb were seen in whole seed extracts, seed coat, cotyledons and embryo axes of DNA extracted from ten seeds. PCR amplicons were seen in five such samples (each sample derived from ten seeds). PCR amplicon was not detectable in the DNA extracted from single seed, two seeds or three, five seeds. There was no amplification with DNA extracted from healthy seeds either singly or in group of ten. The amplicons were very clear for all templates, however high concentration of amplicons were seen with seed coat and embryo axes (Fig. 43a).

The PCR was again performed using the Rep antisense primer to amplify the MYMV. The amplicons of ~500 bp were clear for all the samples such as seed coat, cotyledon, embryo axes and whole seed from group of ten seeds (Fig. 43b) to confirm the presence of MYMV.

PCR detection of the YMV in group of 10 seeds were repeated six times, each time care was taken to keep equivalent grow-out tests of seeds from healthy and diseased plants. In all the replication, amplicons were seen in whole seed, seed coat, cotyledon and embryo axes.

The PCR amplicons produced from seed DNA are of viral origin was further confirmed through Southern blot analysis. The PCR amplicons were electorphoresed and transformed to nitrocellulose membrane. Southern blot analysis was performed with the probe to DNA A of MYMV-CA1 (*Mungbean yellow mosaic virus*-[India:Coimbatore: Blackgram], KC911721). In the autoradiograms, positive hybridization was observed

between the PCR amplicons and MYMV DNA A probe confirming the presence of the virus (Fig. 43c).

PCR products from three samples (Embryo axes, seed coat and whole seed) were sequenced to confirm the viral origin of the amplicons,. The 1.1kb sequence data were analyzed in BLAST programme in the NCBI (www.ncbi.nlm.nih) and the sequence analysis showed maximum identity of 99 per cent with present isolate (MYMV-CA1), MYMV-Vam, DQ400648 and MYMV-Vig (Mad), AJ132575. The sequence analysis further confirmed the presence of MYMV in seed tissues (Fig. 44).

From the perusal of the literature, it was speculated that, the amplification may also result from the integrated geminivirus genome. The DNA template was subjected to rolling circle amplification using φ29DNA polymerase to confirm whether the amplicons are produced from the genome integrated segments of the virus or from native viral replicative forms. Gel electrophoresis of unrestricted RCA product revealed, a smeared DNA along the lanes indicating that RCA has occurred efficiently on the template used. No such DNA smear was seen with template from seeds from healthy plants. In restriction with *Hind*III, *Eco*RI and *Bam*HI, 2.7 kb band, the unit genome length of begomovirus was seen. The restriction pattern was comparable to that of DNA A and DNA B component of MYMV (For eg. *Bam*HI has two sites in DNA B of MYMV). Thus the results clearly reveal that, full length 2.7kb autonomously replicative circular DNA is present in seed tissues from infected plants (Fig. 45).

4.21.2. Detection of YMV in seeds by ELISA

DAS-ELISA was peformed to investigate whether the virion particles are present in the seed tissues or only viral replicative DNA is present. In DAS-ELISA, the A_{405} value was significantly 2.5 times higher than the value of seeds from healthy plant (Table 20) and the range of A_{405} was from 0.315 to 1.125. Positive reactions were observed in seed extract from a group of three, five and ten seeds. Contrasting to seeds, the extract from primary and trifoliate leaves did not shown any colour development. From the results it can be inferred that virion particles are present in low concentration.

4.21.3. Detection of YMV in seeds by ISEM

Attempts were made to visualize the particles in Transmission electron microscope. In order to concentrate the virions on to the grids, ISEM was performed using polyclonal antibody to ToLCNDV. The particles were expected to get trapped and appear as geminate particles with halo of electron dense margin. The electron micrographs (Fig. 46) are provided for embryo, cotyledon, seed coat and leaf tissue.

4.21.4. Grow-out test

Fifty seeds each from diseased plant and healthy plants were sown in 6" pots separately and maintained in insect proof cages at insect free glass house. These plants served as grow-out tests control. There was no symptom observed from seedling from infected seeds, the presence of virus and virion particles are not detectable in young seedlings.

4.22. Phenotyping of RILs against MYMV

4.22.1. Phenotyping of RILs using agro inoculation technique

Recombinant Inbred Lines (RILs) 195 nos derived from *Vigna mungo* (VBN (Bg) 4) × *Vigna mungo* var *silvestris* 22/2 were screened against MYMV disease, along with both the parents and infector variety Bg-Co5 through agro inoculation as per the protocol described in materials and methods. The symptom expression was observed periodically. The observation was taken after 21 days post inoculation. The parent, VBN(Bg) 4 was completely resistant, whereas *Vigna mungo* var. *silvestris* 22/2 and infector variety blackgram (Co (Bg) 5) were highly susceptible (Fig. 47a). The different kinds of symptoms such as necrotic reaction and also asymptomatic was observed in resistant RILs (Fig 47b). A total of 93 RILs (Immune-65 RILs; Resistant-28 RILs) were found to be resistant and 102 RILs (Susceptible-21 RILs; Highly susceptible-81 RILs) were susceptible (Fig. 48).

4.22.2. Analysis of presence of viral DNA from agroinoculated plants

The trifoliate leaves from agro inoculated plants (ten samples each from susceptible and resistant RILs) were collected 21 days post inoculation (DPI) for PCR and Southern blot analysis. Total nucleic acid was extracted from inoculated and

uninoculated plants following the GEM-CTAB method. The total DNA was subjected to PCR using universal abutting primers DNA A and DNA B, the results revealed that the presence of viral DNA in suscebtible RILs whereas resistant RILS showed no amplification (Fig. 49 a and b).

Total DNA (5 µg) was electrophoresed in 1.2 per cent agarose gel and were transferred to nitrocellulose membrane (NCM, Milipore) or nylon membrane using a standard protocol. The hybridization was done with a (α^{32}P)-dCTP labelled probes specific for CP gene of MYMV-CA1 and MP gene of DNA B MYMV-CB1. The probes were prepared by random primer labelling method. Hybridization signal detection was carried out using a Storage Phosphor System Cyclone® Plus (Perkin Elmer, Shelton, CT, USA). The hybridization signal intensity was analyzed through densitometric analysis using Opti Quant Version 5.0 (Perkin Elmer, Shelton, CT, USA). The results showed that the presence of viral DNA forms of DNA A and DNA B (Fig. 50a and b) in susceptible RILs and there was no such form of viral DNA in resistant RILs.

4.22.3. Phenotyping of RILs under field conditions

Phenotyping of 195 RILs along with two parents against MYMV under different locations revealed that VBN(Bg) 4 was completely resistant, whereas *Vigna mungo* var. *silvestris* 22/2 was highly susceptible in all the three hot spot locations (Coimbatore, Vamban and Panpozhi). The disease incidence was scored after the 100 percent incidence exhibited in infector row. The disease spread was uniform over the field. The scoring was done based on percentage of infection. The phenotyping of RILs under field condition at Coimbatore resulted that a total of 24 RILs were immune, 15 RILS were resistant, 62 RILs were moderately resistant, four RILs were found to be moderately susceptible. Ten RILs were found to be susceptible and 80 RILs were found to be highly susceptible. The RILs coming under immune, resistant and moderately resistant were considered as resistant category. Hence a total of 101 RILS were resistant and 94 RILs were susceptible under the field condition at Coimbatore (Fig. 51 and Table 21, 22).

The phenotyping of RILs under field conditions at Vamban resulted that a total of 27 RILs were immune, 13 RILS were resistant, 55 RILs were moderately resistant, three RILs were found to be moderately susceptible. Twelve RILs were found to be susceptible

and 85 RILs were found to be highly susceptible. Finally a total of 95 RILS were considered as resistant and 100 RILs were susceptible under the field condition at Vamban (Fig. 52 and Table 21, 22).

The phenotyping of RILs under field conditions at Panpozhi revealed that a total of 53 RILs were immune, six RILs were resistant, 36 RILs were moderately resistant, three RILs were found to be moderately susceptible. Fourteen RILs were found to be susceptible and 83 RILs were found to be highly susceptible. Finally a total of 95 RILS were considered as resistant and 100 RILs were susceptible under the field condition at Panpozhi (Fig. 53 and Table 21, 22).

CHAPTER V

DISCUSSION

Yellow mosaic disease caused by MYMV is the biggest challenge in improving the productivity of grain legumes in India. The yellow mosaic disease was reported in Tamil Nadu in the year 1970s (Murugesan *et al*., 1977) and soon was found to spread and cause severe infection throughout the state. The yield loss due to the disease was reported to be 10-100% (Marimuthu *et al*., 1981) demanding necessary steps to understand the pathogen and develop strategy for the management of the disease. Vanitharani *et al*. (1996) first established the begomovirus etiology of the disease in Southern India and further Karthikeyan *et al*. (2004), Balaji *et al*. (2004), Girish and Usha (2005) characterized the yellow mosaic viruses infecting blackgram and soybean. On the basis of sequence identity of 89% with *Mungbean yellow mosaic virus* characterized from Thailand, the blackgram isolate from Vamban, Pudukottai district and soybean isolate from Madurai, Madurai district, Tamil Nadu were identified as *Mungbean yellow mosaic virus*. Subsequently there has been no study on the viruses causing yellow mosaic disease in South India. The work presented here was an attempt to identify the viruses causing yellow mosaic disease in Tamil Nadu. The results on characterization of yellow mosaic virus from three locations which are hot spot areas for yellow mosaic disease clearly showed that MYMV is present in Tamil Nadu. Among a lot of DNA B derivatives, a new set of DNA Bs were found associated with MYMV, which are distinct from DNA B and DNA B derivatives described earlier. During characterization of genomic components associated with yellow mosaic disease, the satellites DNA, alpha and betasatellite were found associated with MYMV, which constitutes the first report for MYMV. Since, seed transmission of YMV was suspected to occur for a long time, attempts were made to study the seed borne nature of the virus. Preliminary results revealed that MYMV is seed borne but not seed transmitted. Importance of these results on the viral pathogenicity and disease scenario is discussed briefly.

5.1. Survey and disease incidence

The preliminary survey conducted in three different locations of Tamil Nadu (Coimbatore, Vamban and Panpozhi) confirmed the areas to be hot spot for the yellow mosaic disease, as the disease incidence ranged from 80-100% in the susceptible blackgram var Co5. The disease incidence is severe in summer months (March-May), just prior to monsoon rain. The symptoms of the disease exhibited in the susceptible blackgram in all the three locations were somewhat different due to environmental conditions and may be due to association of satellite DNA. It is inferred that the environmental conditions play a major role in disease expression and severity. In addition to yellow mosaic symptom, crumpling, leaf distortion were seen in some blackgram plants and plants showed extreme chlorosis at the top. The affected plants produce less flowers, pods and size of the seeds were reduced considerably. The seeds were misshapen with improper ellipsoidal shape and the pods exhibited yellow discolouration.

5.2. Detection of begomovirus

In order to identify the viruses occurring in three locations, leaves samples from symptomatic plants were collected. Although symptoms were severe, very young leaves from the symptomatic plants were subjected to PCR, only 19 samples out of 74 samples tested gave PCR amplicons. The failure to get PCR amplicons may be due to low concentration of the viral DNA and the interference due to polyphenol and tannin affecting quality of DNA, the problems experienced by many research workers. Swanson *et al*. (1992) reported that the biggest hurdle in the extraction of high quality DNA from legume hosts was due to their rich phenols content and polysaccharides and also very low concentration of virus. Varma *et al*. (1992) reported that the detection of viral genome was very difficult in legume host like mungbean even in dot blot hybridization tests. Rouhibakhsh *et al*. (2008) suggested a modified CTAB method for extraction of the DNA involving high concentration of β-mercaptoethanol. To some extent this method yielded DNA which could be readily used to amplify viral DNA.

5.3. Cloning of viral DNA

The molecular cloning of geminiviral DNA was earlier achieved by cesium chloride density gradient centrifugation (Stanley and Townsend, 1985). The procedure was laborious and did not give results in host species where the concentration of viral DNA is low. Later PCR technique was applied to clone the highly conserved region, coat protein. On the basis of sequence generated, abutting primers were designed and PCR done to get a full length clone (Qazi *et al*., 2007a). In this method, chances of getting all the components of viral DNA are not possible and if the infections is due to new virus, PCR often failed to get the full length genome. In this context, when Rolling circle amplification was discovered to amplify the circular DNA, cloning of geminiviral, nanoviral DNA got accelerated (Gronenborn, 2004). Application of Rolling circle amplification to enrich the circular viral DNA is very useful. Ever since this technology has been introduced (Inoue-Nagata *et al*., 2004) for the cloning of geminivirus components, there is tremendous impetus in characterization of begomoviruses especially from tough plants like mungbean, cotton, bhendi (Haible *et al*., 2006; Ilyas *et al*., 2010). This technology was used in the present work which resulted in characterization of DNA A, DNA B, associated alpha and betasatellites.

5.4. Characterization of genomic components

The cloning of yellow mosaic virus genomic components resulted in five pairs of cognate DNA A and DNA B clones. The comparison of complete nucleotide sequences of DNA A and DNA B clearly established the presence of MYMV in Tamil Nadu. On the basis of analysis of sequence data available so far on YMVs infecting soybean and grain legumes, it was believed that the disease in northern India, Nepal, Bangladesh, Central and Eastern India is caused by MYMIV and the disease in western and Southern India is caused by MYMV isolates (Usharani, *et al*., 2004; Girish and Usha, 2004; Fauquet *et al*., 2008). In Pakistan MYMIV was isolated (Ilyas *et al*., 2010) from all the cultivated legumes and MYMV was identified only in a weed. In our study, we identified MYMV in severely infected blackgram samples from all the three locations.

5.5. Phylogenetic relationship

The phylogenetic dendrogram and the heat map generated clearly showed how these yellow mosaic viruses are distinct from other begomoviruses of Indian subcontinent and from viruses affecting bean in New World. Normally the begomoviruses of one geographical origin cluster together due to high degree of conservation in coat protein gene, which is required for recognition and transmission of the virus by a whitefly genotype prevalent in that region. In the case of *Legumovirus* this thumb rule is not followed and though they are actively transmitted by the same whitefly genotypes, they do not group together with other begomoviruses. These legume viruses along with *Cowpea golden mosaic virus* from Nigeria and *Soybean crinkle virus* from Thailand constitute a distinct group "*Legumovirus*" (Fauquet *et al*., 2005). They are not related to NW viruses and are basal to all the OW begomoviruses, but do not cluster with begomoviruses of Indian and Asian origin. The viruses are considered to be genetically isolated, as they are not transmitted to non leguminous hosts. Begomoviruses from other crop species also do not infect grain legumes. Thus there is limited possibility of genetic cross over with begomoviruses infecting other hosts. The genetic reassortment is possible only between the legumoviruses and it can occur only when they are present together in mixed infection.

The most interesting feature of the MYMV is the multiple DNA B components associated with one component of MYMV DNA A. This kind of diversity in DNA B is not seen with MYMIV. In India majority of the DNA B components characterized for MYMIV are DNA B of MYMIV with 61% identity with one set of MYMV DNA B (MYMV KA27 AF262064). The only one exception is observed with DNA B of MYMIV (IN:An:Cp25:04) from Gujarat which shared >92% nucleotide identity to sequences of DNA B associated with MYMV (IN:Vam: Vig KA34). Karthikeyan *et al*., 2004 and Balaji *et al*., 2004 earlier reported multiple DNA B components associated with MYMV-Vig (MYMV KA21, KA22, KA 27, KA28 and KA34). The eight DNA B components of MYMV characterized in this work could be differentiated into five groups. Group I, MYMV-VB4 and MYMV PB1 are closely related to MYMV-Vam DQ400849, group II, MYMV-CB3 which is distinct; group III comprising MYMV-CB2,

group IV consisting MYMV-VB1, MYMV-VB2, MYMV-VB3 and group V having MYMV-CB1. However, they shared 93-99% nucleotide identity between them.

5.6. Analysis of recombination events

Michel and Ehrlich (1986) reported that *ori* was a hot spot for recombination during replication of the circular single-stranded DNA of phage M13 and Stanley (1995) reported that *ori* was a site of forced recombination in *African cassava mosaic virus*. Recombination events were detected in the IR, Rep gene of MYMIV isolate and such recombination may facilitate the virus to recognize and replicate DNA B molecules with divergent common regions (Girish and Usha, 2005). In the present case, statistically significant recombination events were detected in the IR, MP gene and NSP gene.

5.7. Identity at amino acid level

The changes in DNA B derivatives compared to DNA B KA27 seen were in the form of substitution, insertion and deletion in nucleotides both in BV1 and BC1. The movement protein (ORF BC1) of bipartite begomoviruses is considered to be the major symptom determinant. Transient or constitutive expression of ORF BC1 has induced disease like symptoms in several hosts (Pascal *et al*., 1993; Ingham *et al*., 1995, Hou *et al*., 2000). Mahajan *et al*. (2011) showed that the interaction between the KA27 DNA B encoded MP and NSP and plant proteins negatively interfere with normal plant development and cause severe stunting. But coinoculation of MYMV DNA A with DNA B KA22 and DNA B KA27 rescue the plants from symptoms because of competition for binding between viral proteins encoded by both DNA B components with plant proteins which are responsible for movement of macromolecules. More than the movement protein, the changes in NSP is also important as NSP is shown to play a major role in symptom development (Hussain *et al*., 2005). Mahajan *et al*. (2011) showed that swapping MYMV KA27 DNA B with the NSP gene of MYMV KA22 abolished severe stunting and caused yellow mosaic suggesting that NSP is the symptom determinant in DNA B. In the present study comparison of amino acid sequence of MYMV DNA B, indicates the various changes in the NSP and MP protein. Mostly the differences are observed in the N terminal region. However in the present research work these changes did not affect the functional domain of these proteins and did not reflect on the viral

pathogenicity. Even in the field screening studies, the performance of RILs from the three hot spot locations were compared, there was no striking difference either in symptom expression or in the pathogenecity which was clear from phenotyping observations. It is inferred that whatever changes observed in these isolates have not contributed significantly to the changes in the pathogenecity. But the different kinds of symptoms were observed from the yellow mosaic disease affected plants like leaf distortion and leaf puckering and this may be due to association of alpha and betasatellites.

5.8. Infectivity of cloned components

It is necessary to prove the infectivity of the cloned components to establish the Koch's postulates. Normally for begomoviruses viral constructs are delivered either through agroinoculation or through biolistic inoculation. In this study, *Agrobacterium. tumefaciens* strain EHA 105 with a recombinant of Ti plasmid pTiB0542 (Hood *et al*., 1993) was used for inoculation. A reasonably good percentage of infectivity was seen, confirming the infectivity of the clones. Infectivity of isolates of MYMIV and MYMV on grain legumes had been shown earlier by several workers (Mandal *et al*., 1997; Balaji *et al*., 2004; Karthikeyan *et al*., 2004; Girish and Usha, 2005; Malathi *et al*., 2005; Usharani *et al*., 2005). Agroinoculation of five DNA B components (MYMV KA21, KA22, KA 27, KA28 and KA34) revealed that all the DNA B components exhibited symptom expression (Karthikeyan *et al*., 2004). While MYMV DNA B KA22 caused yellow mosaic symptom in blackgram, MYMV DNA B KA27 caused severe stunting along with the delay in flowering in greengram (Balaji *et al*., 2004). In addition Mahajan *et al*. (2011) demonstrated alleviation of symptoms of MYMV KA27 DNA B type when MYMV KA22 DNA B type was provided in co-infection or post infection. In the present study, agroclones of MYMV DNA A and DNA B from Coimbatore isolates have been developed and used for phenotyping of RILs. Hence this can be used for screening the resistance source against MYMV and the protocol of agroinoculation can be applied in resistance breeding programme.

5.9. Association of betasatellites

In recent years, yellow mosaic is associated with leaf curl and leaf distortion symptoms in blackgram 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 begomovirus causes the crinkling symptoms and any betasatellite components are involved. The results revealed that the *Papaya leaf curl betasatellite* was associated with MYMV.

The betasatellites associated with bipartite begomovirus was reported by Rouhibakhsh and Malathi (2005) who investigated the cowpea plants showing severe leaf curl symptoms in northern India and reported the presence of *Tomato leaf curl betasatellite* species. Subsequently Sivalingam *et al*. (2010) and Jyothsna *et al*. (2013b) demonstrated their association with the bipartite begomovirus *Tomato leaf curl New Delhi virus* (ToLCNDV). Qazi *et al*. (2007b) suggested that severe leaf curl and crumpling symptoms in cowpea plants 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 (Sivalingam and Varma, 2012).

The begomovirus-betasatellite complex have been reported in legumes in recent years. They are *Tomato leaf curl betasatellite* 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), *Mungbean yellow mosaic India betasatellite* associated with MYMIV in cowpea from India (JX443646, www.ncbi.nlm.nih.gov), the *Papaya leaf curl betasatellite* from cowpea have been reported (DQ118862, www.ncbi.nlm.nih.gov). A new monopartite begomovirus, *French bean leaf curl virus* (Kamaal *et al*., 2012) has been identified in French bean and it is associated with *French bean leaf curl betasatellite* (FbLCB). In the present study, the bipartite MYMV are associated with *Papaya leaf curl betasatellite*. Whether this betasatellite association in the yellow mosaic viruses will continue to be maintained or as the time progresses is an issue which needs to be investigated. The association of betasatellite with MYMV was observed from the two locations, Coimbatore and Vamban. Due to heavy rainfall

there was a less incidence and severity of YMD at Panpozhi, hence the presence of betasatellites showed negative results or escaped from detection.

Betasatellites belong to PaLCuB showed high degree of conservation in amino acid sequences 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 DNA accumulation (Guo *et al*., 2008) and symptoms severity in the host plant (Jyothsna *et al*., 2013a). All the betasatellites molecules under study showed several characteristic features in common with other betasatellite 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 region. 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 elements needed for the binding of Rep-protien (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 a conducive 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 are 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 occurrence of betasatellite/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, www.ncbi.nlm.nih.gov). It is very difficult to hypothesize the mechanism by which MYMIV and MYMV could have picked up *Papaya leaf curl*

betasatellite, as legume infecting begomoviruses do not infect the other hosts enlisted above and chances of they occurring in mixed infection with any other monopartite begomovirus are very less. However there was a recent report of *Tomatoleaf curl Karnataka virus* in soybean (Raj *et al*., 2006). It is possible that monopartite begomoviruses of the other host 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 MYMV will affect its host range, virulence and transmission to contain the YMD spread and mitigate the yield loss.

5.10. Association of alphasatellites

In the present research, the alphasatellites have been cloned and characterized from the YMD infected blackgram samples using RCA products for the first time in Southern India. The satellites were identified as alphasatellites because of their genome organization with a hairpin structure, an A-rich region and a viral sense ORF coding for replication initiation protein (Briddon *et al*. 2004). The satellites from Coimbatore and Vamban shared 97% identity in nucleotide sequence and 96.2 per cent of amino acid sequence of Rep protein between themselves. Though they have been isolated from different places, separated from each other by 300km, they both belong to the same alphasatellite species, *Vernonia yellow vein Fijian alphasatellite*, VYVFA.

It is relevant to note here that *Vernonia* is affected by *Vernonia* yellow vein mosaic disease caused by a monopartite begomovirus, *Vernonia yellow vein virus* and a betasatellite in Southern India (Packialakshmi *et al*., 2010). Association of alphasatellite with the monopartite begomovirus and betasatellite is well known in many hosts plants (Briddon *et al*. 2004). From this it is inferred that *Vernonia* present in the legume field showing yellow vein mosaic symptoms, might have been associated with DNA A, beta and alphasatellite, from which whitefly might have transmitted the alphasatellite into blackgram plants infected by MYMV. Until now, in India, alphasatellites have not yet been found in *Vernonia*, but chances are high as alphasatellites are ubiquitously associated with monopartite begomovirus-betasatellite complex. Mansoor *et al*. (1999), Saunders and Stanley (1999) and Briddon *et al*. (2004), demonstrated that the DNA1 or

alphasatellite molecule can be maintained by *African cassava mosaic virus* (ACMV). From this it can be hypothesized that, there is adaptation between alphasatellites and helper begomovirus, they will co-exist together. Since MYMV is similar to ACMV in its Old World bipartite genome organization properties, it is predicted that some degree of adaptability between the *Vernonia yellow vein Fijian alphasatellite* and MYMV may occur.

By comparing the Rep protein sequences, it is clear that, the alphasatellites are very distinct, and if a representative from each recognized species (Kings *et al*. 2011) are compared, the identity at amino acid sequence is very low, almost less than 60% identity is observed between them. This is quite contrasting to observation made by Briddon *et al*., 2004. They analysed alphasatellites associated with cotton leaf curl, Ageratum, okra and tomato and suggested that they are conserved and there is nearly 86% amino acid sequence identity except the alphasatellite, AYVD201-Sin. Reports on alphasatellites occurring in New world Brazil, (Paprotka *et al*. 2010) also suggest the identity to be less. We infer that, when the alphasatellites described by Briddon *et al*. (2004), showed high degree of conservation in Rep gene, the newly identified emerging alphasatellites tend to exhibit divergence.

Though alphasatellites have been found associated with monopartite begomovirus and betasatellite complex, exactly their contribution to viral pathogenicity is not yet clear. One role suggested by Saunders *et al*. (2002) is that they may modulate the symptoms by competing for resources inside the cell. The role may be similar to defective DNA molecules, derived from DNA B which are half the length of DNA B, but interfere with accumulation of DNA A and DNA B component. Recently, Idris *et al*. (2011) showed that an unusual alphasatellite (DNA 2) associated with *Tomato yellow leaf curl Oman virus* ameliorated symptoms and significantly reduced betasatellite DNA accumulation. In the case of yellow mosaic symptoms, lot of variations are seen in symptom severity ranging from well restricted yellow or chlorotic area to completely bleached yellow leaf, green and yellow patches distributed randomly; a very severe necrotic spots on the leaf, etc. Whether such irregular pattern of disease symptom development arises due to presence of alphastellite needs to be looked into. Future work with infectious clones should clarify these issues.

The satellite Rep encoding component (SRC) of dicot infecting nanoviruses do not show any affinity to their associated virus or host (Briddon *et al*., 2004; Paprotka *et al*., 2010). The alphasatellites too do not show any affinity to host or geography and they are promiscuous to an extent that the same alphasatellite may be associated with different begomoviruses. The appropriate example is the research work presented here, the presence of a weed infecting alphasatellite in cultivated grain legume crop, blackgram associated with MYMV was demonstrated.

5.11. Seed transmission/ seed borne nature of MYMV

The plants get infected with YMD at early stage, the yield loss is computed to be 100%, causing huge deficit in the availability of the pulses for consumption. The following observation recorded in yellow mosaic disease infected seeds led to the speculation that the viruses may be seed borne, (1) the seeds are misshapen, shrunk contrasting to fully filled black seeds from healthy plants, (2) the disease symptoms are seen when the first trifoliate leaves are just unfurling. These observations led to the question whether seeds do have viruses and whether they give rise to seedling infection? Attempts have been made to address these questions in the research work submitted.

The results on various tests conducted to detect the yellow mosaic disease in blackgram seed tissue revealed that the MYMV is present in seed coat, cotyledon and embryo axes; the virus concentration in seed coat is higher than embryo. In the study on detection of MYMV in the seed, the tests were performed using seeds from infected plants by extracting DNA from single seed, two seeds, four seeds, five seeds and ten seeds. The positive results were seen in DNA from whole seeds, seed coat, cotyledonary tissue and embryo axes of ten seeds. Absence of amplicons in DNA extracted from less than ten seeds, clearly indicates that, the viral DNA concentration is less, or the frequency of viruses presence is very low. This work was supported by the statement of Pahalawatta *et al*. (2007) that the presence of *Dahlia mosaic virus* was detected primarily in cotyledon and rarely in seed coat from group of ten seeds through PCR.

From the literature, it is speculated, the amplification may also be result from the integrated geminivirus genome (Jakowitsch *et al*., 1999; Schoelz *et al*., 2005). Whether the amplicons are produced from the integrated segments of the virus or from native viral

replicative forms, the DNA template was subjected to rolling circle amplification followed by restriction. In restriction with *Hind*III, *Eco*RI and *Bam*HI, 2.7 kb band, the unit genome length of begomovirus was seen. The restriction pattern was comparable to that of DNA A and DNA B component of MYMV. As the presence of full length genome is further confirmed through RCA restriction, it is independent of integrative events predicted for some geminiviruses.

The results obtained from PCR are contradictory to results of ELISA. In ELISA, even the single seed extract gave A_{405} value of 406 in five replicates tested. The contrasting results obtained here is believed to be due to low concentration and the elaborate process of DNA preparation which might have led to the loss of DNA. In the PCR reaction both replicative forms (double stranded) and encapsidated form of DNA will serve as template. In ELISA, the detection of only virion particles are possible. Inspite of the low concentration, detection of virus in single seed was possible in ELISA. From the preliminary results on detection of MYMV, it is deduced that both viral replicative DNA forms and virion particles are present in the seed. It is distributed evenly in seed coat, cotyledonary tissue and embryo. These observation need to be confirmed by analyzing more number of seeds, different genotypes of blackgram and different legume hosts.

The major question that is difficult to answer is how does the begomovirus gain entry into embryo. It is very difficult for the phloem bound viruses through long distance transport to reach floral meristem and it is easy to conceive the direct embryo invasion. For the virus to enter immature embryo, the vascular tissue is present only upto funiculus and hence virus speculated to have entry through the micropylar region. To address all these events, a detailed investigation from distribution in floral tissue onwards, is required.

The most interesting feature of the present study, is absence of virus detection in the grow –out test seedlings. Not only the seedlings fail to show symptoms, the virion particles and viral DNA were not at all detectable, both in ELISA and PCR. It is possible that the virus concentration is very low and escapes detection. It is very well known that the seed coat and embryo infection need not always lead to symptomatic seedlings. This was supported by the work of Tomlinson and Walker (1973) that the *Cauliflower mosaic*

virus has to be seed borne due to the occurrence of virus in the seed coat, although there was no subsequent transmission to seedlings. The presence of virus in a seed, even in the embryo (Nolan and Campbell, 1984), does not always lead to seedling infection. It is hypothesized that, the vigorous metabolic environment of growing seedling may not be conducive to rapid build up of virus, their multiplication and interference with metabolic pathways to lead to symptom expression.

The most important aspect of detection of MYMV in seeds is whether it will serve as a potential inoculums source, in which case it will be of great concern in plant quarantine and trade related movement. Whether, the presence in seed will contribute as initial inoculums within the field, which can get actively transmitted once whitefly population pick it up is a key question which should be addressed immediately. There are so many questions on seed transmission of YMV which remain unanswered. Future work should be focussed on the mechanism and movement of the virus in seed in detail by analyzing more number of seeds, different genotypes of black gram and different legume hosts.

5.12. Phenotyping of RILs

The incidence of yellow mosaic disease has been increased due to high frequency of whitefly outbreaks (Ahmad *et al*., 2010) resulting demands for resistant cultivars. Resistance has been identified on the basis of field screening using alternating rows of highly susceptible varieties under natural hot spot areas. In the present study, 195 RILs derived from cross between VBN (Bg)4 × *Vigna mungo* var *silvestris* 22/2 were screened against MYMV under natural field conditions at three different locatons of Tamil Nadu, Coimbatore, Vamban and Panpozhi which representing the hot spot areas for YMD. The phenotyping of RILs under field conditions resulted that the 101 RILS were resistant and 94 RILs were susceptible at Coimbatore whereas 95 RILS were resistant and 100 RILs were susceptible at Vamban. In case of Panpozhi, 95 RILS were resistant and 100 RILs were susceptible.

The term ‘resistant’ has been variably used to identify the lines developing asymptomatic (Gurha *et al*., 1982) or necrotic mottle (Nene *et al*., 1972), having less than 2 per cent yellow mosaic incidence (Chaudhary *et al*., 1981) or less than 10 per cent yellow mosaic incidence (Singh *et al*.,1979). In the present work, the RILs showed less

than 10 per cent disease incidence has been categorized into moderately resistant. The RILs under moderately resistant category recorded disease severity index of less than 1 per cent. Hence the RILs under moderately resistant category were considered as resistant as per the disease scoring index given by Akhtar *et al*. (2011). The RILs having more than 10 per cent disease incidence has been categorized into susceptible.

Phenotyping of RILs based on field screening in the hot spot areas does not give consistent results and 20 per cent deviation was observed among three locations. This deviation was due to single factor or a combination of factors *viz*., environmental conditions (temperature, relative humidity, wind direction), vector host preference, duration of the crop and soil condition (Hoogstraten, 1992; Akhtar *et al*., 2010a). To overcome these problems, the RILs were further screened under artificial condition through agroinoculation technique. It is an effective method by which infectious viral DNA can be introduced into plants using *Agrobacterium tumefaciens*. Karthikeyan *et al*. (2011) identified 30 F_2 individual based on field screening and they were further screened under artificial condition using agroinoculation technique. The results showed that among the thirty individuals only five individuals are resistant. In the present study, phenotyping of RILs through agroinoculation resulted in 93 RILs resistant (Immune – 65 RILs; Resistant – 28 RILs) and 102 RILs susceptible (Susceptible – 21 RILs; Highly susceptible – 81 RILs). There was no RILs observed under the category of moderately resistant and moderately susceptible. Phenotyping of RILs under artificial condition through agroinoculation technique has given clear cut result and there was no confusion to categorize the disease response of the RILs when compared to field screening. However, phenotyping of RILs under natural hot spot areas have given a basic idea about disease reaction of RILs. Since the artificial screening through agroinoculation has given clear cut and consistent result, this technique may be applied in the future resistance screening programme and to develop the resistant varieties in blackgram.

CHAPTER VI

SUMMARY

Characterization of the yellow mosaic virus in blackgram, development of diagnostics and screening for resistance source were the prime objective of the present study. The key findings are presented below.

- Leaves of blackgram showing severe yellow mosaic symptoms were collected during summer from three hot spot areas of Tamil Nadu, *viz*., Coimbatore (Coimbatore), Vamban (Pudukottai) and Panpozhi (Tirunelveli) which represents three districts of Tamil Nadu.
- The aetiological role of begomovirus in yellow mosaic disease of blackgram was confirmed by electron microscopy and whitefly transmission. Detection of begomovirus was performed through PCR using PAR1v772/ PAL1c1960 and MYMV through dot blot and Southern blot hybridization.
- Five DNA A and eight DNA B clones were cloned and sequenced from all the three locations. The sequences of MYMV isolates of present study have been deposited in the NCBI database (Appendix II).
- The sequence analysis clearly showed that the DNA A clones showed 97-98 per cent identity with *Mungbean yellow mosaic virus*-Vam-Bg (MYMV-Vam;DQ400848) and DNA B clones exhibited 95-98 per cent identity with the DNA B components of *Mungbean yellow mosaic virus* (MYMV-Vam; DQ400849). On the basis of threshold value of 89 per cent set for the begomovirus species demarcation by the geminivirus taxonomy study group, the virus isolates are identified to belong to the species, *Mungbean yellow mosaic virus* (MYMV).
- The genome organization of MYMV revealed to be typical of OW bipartite begomovirus. DNA A components encode two open reading frames (ORFs) on viral sense strand and the five ORFs seen on the complementary sense strand. DNA B components encode ORF BV1 on the viral sense strand and ORF BC1 on the complementary sense strand.

- In DNA A component, among various open reading frames, ORF AV1 and AC1 was highly conserved when all MYMV isolates were compared. In DNA B component, both ORFs were conserved amongst MYMV isolates.
- The most interesting feature is that more than 15 per cent divergence was observed between the common region (CR) of DNA A and DNA B compared to other begomoviruses in which CR is highly specific for a virus and it is expected to be near identical between DNA A and DNA B component of the same virus.
- The analysis of percent identity of nucleotide sequence of five DNA A components in the present study with other yellow mosaic virus isolates and begomoviruses of Indian origin revealed that they shared the highest identity of 97-98 per cent with MYMV and less identity with MYMIV (81 per cent). MYMV isolates of present study shared 95-99 per cent identity between themselves and less than 56 per cent identity with New World, *Bean golden yellow mosaic virus* (BGYMV).
- The comparison of complete nucleotide sequence of eight DNA B components obtained in the present work with other yellow mosaic virus isolates and selected begomoviruses revealed interesting features. All the eight DNA Bs showed 95-98 per cent with MYMV-Vam (DQ400849) and they shared maximum identity with MYMIV (87-89 per cent). These DNA B variants shared 93-99 per cent identity between themselves.
- Phylogenetic analysis of complete DNA A nucleotide sequence of YMV isolates including the sequence data available in the database revealed that the distinct separation of YMV from other begomovirus of Indian subcontinent and China. The YMVs are well separated suggesting that they do not share any common evolutionary events. As expected, the begomovirus from the New World, BGYMV deviates from branches of both YMVs and begomoviruses from Indian subcontinent.
- Phylogenetic analysis based on alignment of complete nucleotide sequence of DNA B components revealed that the DNA B component of individual species of yellow mosaic viruses branched into separate clades very clearly. From the

analysis of recombination breakpoints using recombination detection programme, statistically significant recombination events were detected among MYMV DNA B components in the intergenic region.

- Partial tandem dimers of DNA A and DNA B of MYMV isolate of black gram (Coimbatore) were constructed by directional cloning of a bitmer followed by further cloning of full length of DNA A or DNA B appropriately. Then the dimeric constructs were mobilized into *Agrobacterium tumefaciens* EHA105*via* triparental mating using pRK2013 as a helper. The agroclones were highly infectious on blackgram and infectivity percentage was 63 per cent in blackgram Co5.

- The MYMV DNA A and DNA B specific probes have been developed from the Coimbatore isolate, MYMV-CA1 and MYMV-CB1 to detect the MYMV in many crops.

- The betasatellites DNA associated with yellow mosaic virus affected blackgram plants were cloned and sequenced from Coimbatore and Vamban. Complete nucleotide sequence analysis of two betasatellite clones (one each from Coimbatore and Vamban) in BLAST search revealed 95 per cent identity with *Papaya leaf curl betasatellite*, PaLcuB - [India:Chinthapalli:2005, DQ118862].

- The identity observed is higher than 78 per cent identity kept as threshold value for demarcation of betasatellite species, the name *Papaya leaf curl betasatellite* - [India:CBE:BG] and *Papaya leaf curl betasatellite* - [India:VBN:BG] are proposed for the new betasatellites characterized in this study and the sequences have been deposited in the NCBI database under the GenBank accession numbers KC959933 and KC959935 respectively.

- The alphasatellites DNA associated with yellow mosaic virus infected blackgram plants were cloned and sequenced from Coimbatore and Vamban. Complete nucleotide sequence of two alphasatellites each from Coimbatore and Vamban samples were analysed in BLAST search programme in the NCBI. The alphasatellites shared 98-99 per cent identity with *Vernonia yellow vein Fijian alphasatellite*, VYVFA, JF733780. Since the sequence identity is more than

98 per cent, the threshold value for demarcation of alphasatellites species, the alphasatellites of the present study are named as *Vernonia yellow vein Fijian alphasatellite* and the sequences have been deposited in the NCBI database under the accession numbers, KC959932 and KC959931.

- The satellite DNA, alpha and betasatellites were found associated with MYMV, which constitutes the first report for MYMV.
- Attempts were made to study the seed borne nature of the yellow mosaic virus. The viral genome and virion particles were seen in whole seed extracts, seed coat, cotyledons and embryo axes from ten seeds through PCR, ELISA and ISEM and Southern blot analysis.
- The presence of MYMV in seed was further confirmed through sequencing. The sequence analysis showed maximum identity of 99 per cent with present isolate, MYMV-CA1, MYMV-Vam, DQ400648 and MYMV-Vig (Mad), AJ132575. However the seedlings failed to show symptoms and also the virion particles and viral DNA were not at all detectable in seedlings, both in ELISA and PCR. Preliminary results revealed that MYMV is seed borne but not seed transmitted.
- Phenotyping of Recombinant Inbred Lines (RILs) (195 nos) derived from *Vigna mungo* (VBN (Bg) 4) × *Vigna mungo* var *silvestris* 22/2 were screened against MYMV under natural hot spot areas (Coimbatore, Vamban and Panpozhi). VBN(Bg) 4 was completely resistant, whereas *Vigna mungo* var. *silvestris* 22/2 was highly susceptible in all the three hot spot locations. A total of 101 RILS were resistant and 94 RILs were susceptible under the field condition at Coimbatore whereas 95 RILS were resistant and 100 RILs were susceptible at Vamban. In case of Panpozhi, 95 RILS were resistant and 100 RILs were susceptible under field condition.
- For further confirmation, phenotyping of RILs were done under artificial condition using agroinculation technique. The results revealed that a total of 93 RILs were found to be resistant and 102 RILs were susceptible.

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APPENDIX - I

REAGENTS AND BUFFERS

Antibiotics:

Name of the antibiotic	Stock solution (mg/mL)	Solvent	Working concentration (mg/L)
Ampicillin	50	Water	50
Kanamycin	50	Water	50
Rifampicin	30	Methanol	30
Nalidixic acid	30	Potassium hydroxide	30

All antibiotics, which are water-soluble, were sterilized by filtration (0.22 μm pore size) and then stored as frozen stock solution at - 20^{o}C.

Gel Electrophoresis

TAE (Tris-Acetate EDTA Buffer, pH 8.0; 50X)

Tris- base	242 g
Glacial Acetic Acid	58.8 mL (of 96.6 %)
EDTA (0.5M, pH 8.0)	20 mL
dd H2O	up to 1000 mL

6X Loading dye (MBI fermentas)

1 % Bromophenol blue	200 mL
Glycerol	200 mL
10%SDS	60 mL
0.5M EDTA	50 mL
10X TAE	60 mL
ddH_2O	30 mL

Southern Hybridization

Denaturing solution

NaCl	1.5 M
NaOH	0.5 M

Neutralisation solution

NaCl	1.5 M
Tris-HCl (pH-7.4)	1.0 M

Denhardt's solution (50X)

Ficoll (type 400)	1%	5.0 gm
PVP (Sigma)	1%	5.0 gm
BSA	1%	5.0 gm
Distilled water		up to 500 mL

Pre-hybridization Solution

Denhardt's	5X
SSC	6X
SDS	0.2 %
Yeast RNA / Salmon sperm	100 µg/mL

Washing solution

SDS	0.1 %
SSC	2X

Culture Media

Luria Agar Medium

Bactotryptone	10.0 g
Bactoyeast extract	5.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	up to 1000 mL

Luria Broth Medium

Bactotryptone	10.0 g
Bactoyeast extract	5.0 g
NaCl	5.0 g
Distilled water	up to 1000 mL

Commercially available LA and LB medium was used from Himedia company.

Plasmid isolation buffers (modified alkaline lysis method)

Solution I (Resuspension buffer - at 4°C)

1M Tris-HCl (25mM) (pH 8.0)	2.5 mL
20% Glucose (50mM)	4.5 mL
0.5M EDTA (10mM)	2.0 mL
Sterile distilled water	up to 100 mL

Solution II (Lysis buffer)

10N NaOH (0.2N)	2.0 mL
1% SDS	5.0 mL
Distilled water	up to 100 mL
(autoclaving not required)	

Solution III (pH 4.8/5.2) (Neutralization buffer)

3 M Sodium acetate

Preparation of Commonly used stock solutions

Solution	Method of preparation
0.1M $CaCl_2$	73.5 gm of $CaCl_2.H_2O$ was dissolved in 450 mL of distilled water, the volume was make up to 500 mL and sterilized by autoclaving and stored at 4^oC
0.5M EDTA	186.1 gm of EDTA was added to 200 mL of distilled water and stirred vigorously on a magnetic stirrer and pH was adjusted to 8.0 with NaOH and sterilized by autoclaving
Ethidium Bromide	100 gm of Ethidium bromide was added to (10 mg/mL) 100 mL of distilled water and stirred on magnetic stirrer for several hours to ensure that dye has dissolved. The solution was transferred to a dark colored bottle and stored at room temperature
0.1M IPTG	1.19 g IPTG (isopropyl-β-D-1-thiogalactopyranoside) powder dissolved in 40 mL distilled water and volume was made up to 50 mL by distilled water sterilized by filtration by using disposable filter. The solution was dispensed into aliquot and stored at -20^oC
0.1M $MgCl_2$	101.5 gm $MgCl_2.6H_2O$ was dissolved into 450 mL of distilled water, the volume was make up to 500 mL and sterilized by autoclaving and stored at 4^oC

3M Sodium acetate

pH 4.8 & 5.2 408.1 gm of $CH_3COOH.3H_2O$ was dissolved in 800 mL of distilled water and pH was adjusted to 4.8 and 5.2 with glacial acetic acid volume was made up to 1 liter with sterile water and sterilized by autoclaving

5M NaCl 233.8 gm of NaCl was dissolved in 800 mL of distilled water and volume was made up to 1liter and sterilized by autoclaving

Phenol:Chloroform:Isoamylalcohol (PCI)

Buffer saturated phenol, chloroform and isomyl alcohol were mixed in the ratio of 25:24:1. The equilibrated mixture was stored under a layer of 0.01M Tris-HCl (pH 7.6) at 4^0C in dark glass bottle

Chloroform:Isoamylalcohol (CI)

Buffer saturated chloroform and isomyl alcohol were mixed in the ratio of 24:1. The equilibrated mixture was stored at 4^0C in dark glass bottle

10% SDS 100 gm of SDS (Electrophoresis grade) was dissolved in 900 mL of distilled water and heated to 68^{o}C to dissolve it and made up to 1L.

20X SSC 175.3 gm of NaCl and 88.2gm Sodium citrate was dissolved in 800 mL of distilled water and the pH was adjusted with NaOH up to 7.4. Volume made up to 1 liter and sterilized by autoclaving

1M Tris-HCl 121.2 gm Tris base was dissolved in 800 mL of distilled water and pH was adjusted to desired value by adding HCl and volume was made up to 1liter and sterilized by autoclaving.

X-gal (5-Bromo-4-Chloro-3-Indolyl-β-D-glactopyranoside). The stock solution was made by dissolving X-gal in diethylformamide to make a 20 mg/mL solution and stored at -20^{o}C in dark bottle and covered with aluminium foil

0.1% DEPC treated water

Add 1 mL of DEPC to 1 liter of sterile distilled water. Leave it for overnight and autoclave

DNA Molecular Weight Marker

One kilobase (1 kb) DNA ladder of MBI Fermentas was used as marker. The ladder is formed by fourteen DNA fragments of 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3.5 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 0.75 kb, 0.5 kb and 0.25 kb

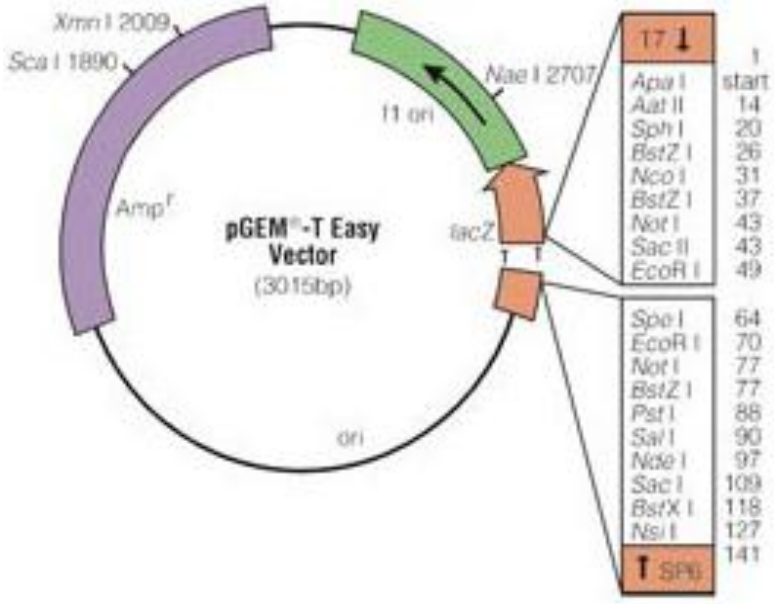
APPENDIX II

List of sequences of MYMV isolates and their associated alphasatellites and betasatellites of present study with their accession numbers

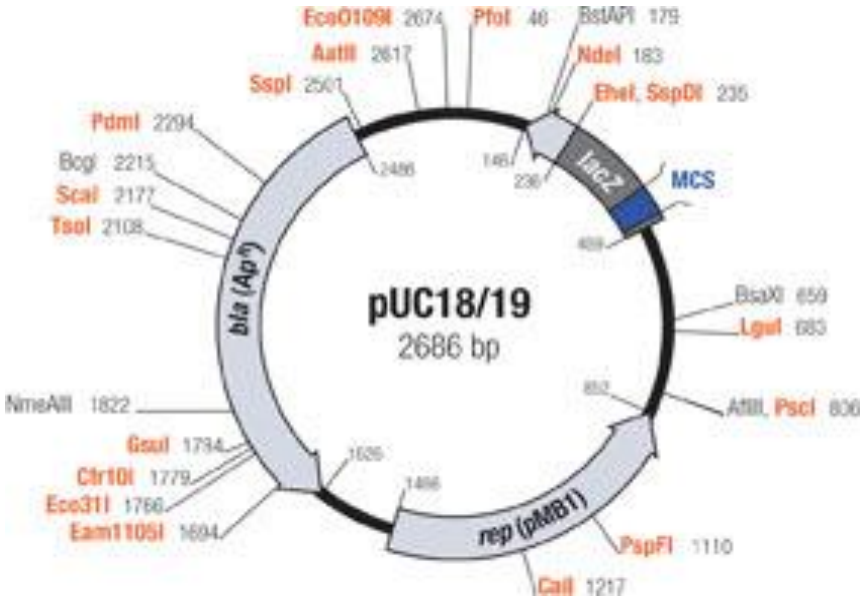
Virus	Abbreviation	Accession number
DNA A component	MYMV-CA1	KC911721
	MYMV-CA2	KC911718
	MYMV-VA1	KC911722
	MYMV-VA2	KC911723
	MYMV-PA1	KC911717
DNA B component	MYMV-CB1	KC911724
	MYMV-CB2	KC911730
	MYMV-CB3	KC911729
	MYMV-VB1	KC911728
	MYMV-VB2	KC911727
	MYMV-VB3	KC911726
	MYMV-VB4	KC911725
	MYMV-PB1	KC911731
Betasatellites	CBE-Bg	KC959933
	VBN-Bg	KC959935
Alphasatellites	CBE-Bg	KC959932
	VBN-Bg	KC959931

APPENDIX -III

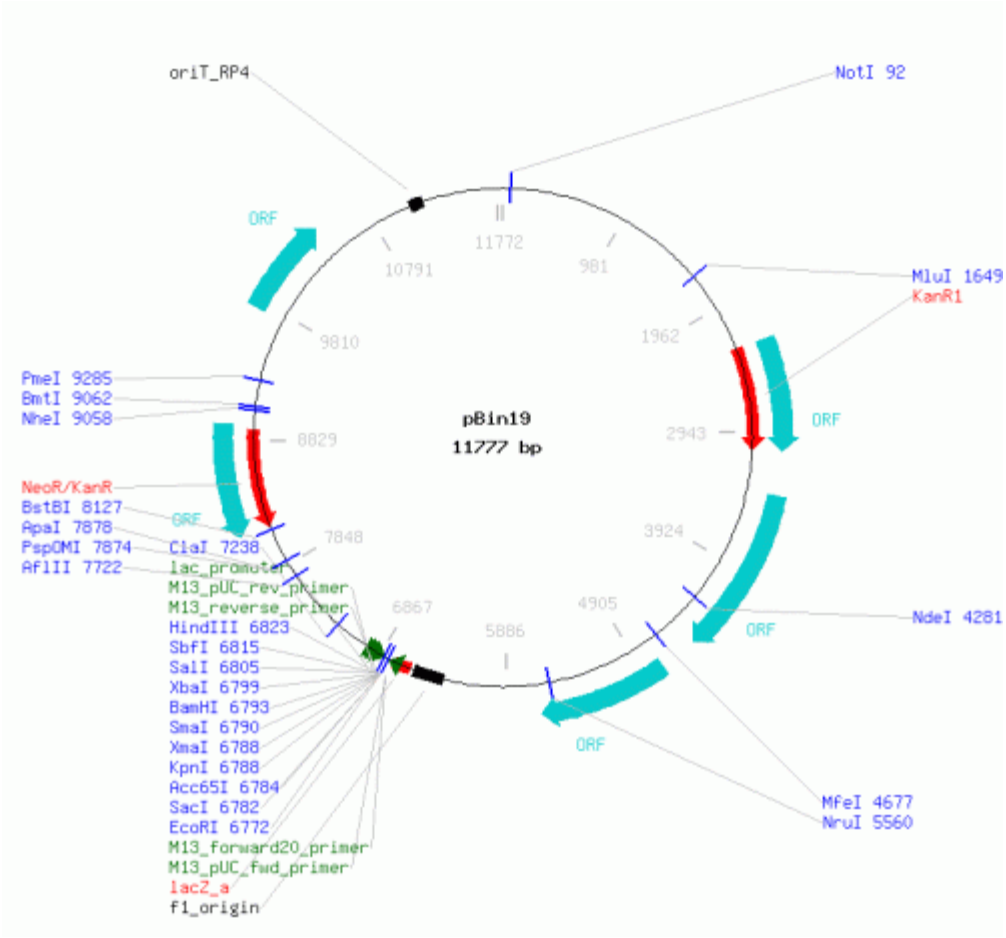
VECTOR MAPS



Physical map of pGEM-T Easy vector showing multiple cloning sites (MCS) with restriction sites for cloning of viral gene



Physical map of pUC18 vector showing MCS for cloning of viral gene



Physical map of pBIN19 binary vector showing MCS for cloning of viral gene

APPENDIX IV

List of sequences used for sequence analysis and phylogenetic comparison

List of begomoviruses DNA A sequences

Viruses	Abbreviation	Accession number
Mungbean yellow mosaic virus Vamban Blackgram	MYMV-Vam	DQ400848
Mungbean yellow mosaic virus-Vigna (Maduari)	MYMV-Vig (Mad)	AJ132575
Mungbean yellow mosaic virus-Soybean (Madurai)	MYMV-Sb (Mad)	AJ421642
Mungbean yellow mosaic virus -Namakkal	MYMV-Nam	DQ865201
Mungbean yellow mosaic virus-Mungbean (Thailand)	MYMV-Mg (TH)	D14703
Mungbean yellow mosaic virus-Thailand	MYMV-Th	AY271892
Mungbean yellow mosaic virus-Cambodia	MYMV (KH PP-03)	AY271892
Mungbean yellow mosaic India virus - Mungbean (Bangladesh)	MYMIV-Mg (BD)	AF314145
Mungbean yellow mosaic India virus - Mungbean (Nepal)	MYMIV-Mg (Np)	AY271895
Mungbean yellow mosaic India virus - Mungbean (Pakistan)	MYMIV-Mg (Pak)	AJ512495
Mungbean yellow mosaic India virus - Blackgram (New Delhi)	MYMIV-Bg3	AF126406
Mungbean yellow mosaic India virus - Cowpea	MYMIV-Cp7	AF481865
Mungbean yellow mosaic India virus - Soybean	MYMIV-Sb2	AY049772
Mungbean yellow mosaic India virus - Soybean (Pakistan)	MYMIV-PK-Sb	AM992618
Mungbean yellow mosaic India virus - Vigna (Pakistan)	MYMIV-PK-Vig	FM955600
Mungbean yellow mosaic India virus – Cowpea (Gujarat)	MYMIV-CpMBKA25	AY937195
Horsegram yellow mosaic virus – Coimbatore-India	HgYMV-[IN Coi]	AJ627904
Horsegram yellow mosaic virus – Banglore	HgYMV-Ban	AM932427
Horsegram yellow mosaic virus – French bean	HgYMV-Fb	AM932425
Horsegram yellow mosaic virus – Lima bean	HgYMV-Lb	AM932429
Rhynchosia yellow mosaic India virus	RhYMIV	HM777509
Rhynchosia yellow mosaic virus	RhYMV	AM999981

Viruses	Abbreviation	Accession number
Velvet bean severe mosaic virus	VBSMV	FN543425
Kudzu yellow mosaic virus	KuMV-Yg3	FJ539014
Kudzu yellow mosaic virus-Vietnam	KuMV-VN	DQ641690
Dolichos yellow mosaic virus-Banglore	DoYMV-Ban	AM157412
Dolichos yellow mosaic virus-New Delhi	DoYMV-ND	AY309241
Indian cassava mosaic virus-Maharashtra	ICMV-Mah	AY730035
Sri Lankan cassava mosaic virus-Colombia	SLCMV-Col	AJ314737
Tomato leafcurl New Delhi virus-Srinagar	ToLCNDV-Svr	U15015
Tomato leafcurl Bangalore virus	ToLCBV-Ban5	AF295401
Cotton leafcurl Multan virus-Rajasthan	CLCuMV-Raj	AF363011
Cotton leafcurl Multan virus-Pakistan	CLCuMV-[PK:Mul]	AJ496461
Bean golden yellow mosaic virus-Rajasthan	BGYMV	D0201
Papaya leaf curl China virus-Ageratum	PaLCuCNV-Age	AJ876548
Papaya leaf curl China virus-Tomato	PaLCuCNV-Tom	DQ641700
Bhendi yellow vein mosaic virus-Madurai	BYVMV-Mad	AF241479
Croton yellow vein mosaic virus-New Delhi	CYVMV-Del-Cr	JN817516
Croton yellow vein mosaic virus-Bangalore	CYVMV-Bang-Cr2	JN831446

List of begomoviruses DNA B sequences

Viruses	Abbreviation	Accession number
Mungbean yellow mosaic virus-Vigna (Maduari)	MYMV [KA22]	AJ132574
Mungbean yellow mosaic virus-Vigna (Maduari)	MYMV-[KA21]	AJ439059
Mungbean yellow mosaic virus-Vigna (Maduari)	MYMV-[KA34]	AJ439057
Mungbean yellow mosaic virus-Vigna (Maduari)	MYMV [KA28]	AJ439058
Mungbean yellow mosaic virus-Blackgram (Vamban)	MYMV-Vam	DQ400849
Mungbean yellow mosaic virus-Soybean (Maduari)	MYMV-Sb (Mad)	AJ867554
Mungbean yellow mosaic virus-Vigna (Maduari)	MYMV [KA27]	AF262064
Mungbean yellow mosaic virus-Thailand	MYMV-Mg (TH)	D14704
Mungbean yellow mosaic virus-Namakkal	MYMV-Nam	DQ865203
Mungbean yellow mosaic India virus – Blackgram (New Delhi)	MYMIV-ND-Bg3	AF142440
Mungbean yellow mosaic India virus - Cowpea (New Delhi)	MYMIV-ND-Cp7	AF503580
Mungbean yellow mosaic India virus - Soybean (New Delhi)	MYMIV-ND-Sb2-99	AY049771
Mungbean yellow mosaic India virus - Cowpea (Gujarat)	MYMIV AnaCp	AY937196
Mungbean yellow mosaic India virus - Pakistan	MYMIV (Pak)	AM992617
Mungbean yellow mosaic India virus - Mungbean (Nepal)	MYMIV-Mg (Np)	JN543396
Horsegram yellow mosaic virus – Coimbatore-India	HgYMV-[IN Coi]	AJ627905
Horsegram yellow mosaic virus – Banglore	HgYMV-Ban	AM932428
Horsegram yellow mosaic virus – French bean	HgYMV-Fb	AM932426
Horsegram yellow mosaic virus – Lima bean	HgYMV-Lb	AM932430
Rhynchosia yellow mosaic virus	RhYMV	AM999982
Rhynchosia yellow mosaic virus	RhYMV	FM208848
Rhynchosia yellow mosaic India virus	RhYMIV	HM777510
Kudzu yellow mosaic virus-Vietnam	KuMV-VN	DQ641691
Kudzu yellow mosaic virus	KuMV-Yg3	FJ539015
Velvet bean severe mosaic virus	VBSMV	FN543426
Bean golden yellow mosaic virus	BGYMV	D00200
Tomato leafcurl New Delhi virus-Srinagar	ToLCNDV-Svr	U15017
Indian cassava mosaic virus-Maharashtra	ICMV-Mah	AY730336
Sri Lankan cassava mosaic virus-Colombia	SLCMV-Col	AJ314738

List of betasatellites

Betasatellites	Abbreviation	Accession number
Papaya leaf curl betasatellite- Chinthapalli	PaLCuB-[IN:Chi:05]	DQ118862
Papaya leaf curl betasatellite- New Delhi-Ipomea	PaLCuB-[IN:ND:Ipo:09]	JX050199
Papaya leaf curl betasatellite- New Delhi-Pumpkin	PaLCuB-[IN:ND:Pumpkin:10]	JX040472
Papaya leaf curl betasatellite- New Delhi-Papaya	PaLCuB-[IN:ND:Papaya:03]	AY244706
Papaya leaf curl betasatellite- Jabalpur	PaLCuB [IN:Jab:03]	AY230138
Papaya leaf curl betasatellite- New Delhi-Chilli	PaLCuB-[IN:ND:Chi:10]	JN663869
Papaya leaf curl betasatellite- New Delhi-Tomato	PaLCuB-[IN:ND:Tom:09]	GU370715
Papaya leaf curl betasatellite- Cowpea	PaLCuB-[IN:Cp:04]	AY728263
Papaya leaf curl betasatellite- New Delhi-Papaya	PaLCuB-[IN:ND:Papaya:07]	EU126825
Potato apical leaf curl betasatellite- Meerut	PoALCuB-[IN:MRT:05]	EF043234
Cotton leaf curl betasatellite- Lucknow	CLCuB [IN:Luc:10]	HM143916
Chilli leaf curl betasatellite- Pakistan	ChLCB-[Pk:Fai62:04]	AM279672
Chilli leaf curl betasatellite- Pakistan	ChLCB-[PK:Si:04]	AM279662
Tobacco leaf curl betasatellite- Pakistan	TbLCB-[PK:Bah:99]	AJ316034
Tomato leaf curl betasatellite- Pune	ToLCMaB-[IN:Pun:04]	AY838894
Cotton leaf curl betasatellite- Lucknow	CLCuB-[IN:Luc:10]	GU440581
Cotton leaf curl betasatellite- Srinagar	CLCuB-[IN:Sri:08]	GQ370388
Mungbean yellow mosaic India betasatellite	MYMIVB-[IN:Fai:Cp:12]	JX443646
Bhendi yellow vein mosaic betasatellite-Coimbatore	BYVMB-[IN:Coi:OYCO1:05]	GU111975
Bhendi yellow vein mosaic betasatellite-Thadagam	BYVMB-[IN:Tha:OY158:06]	GU111971
Bhendi yellow vein mosaic betasatellite-Trichy	BYVMB-[IN:Tri:OY118:06]	GU111970
Tomato leaf curl betasatellite- Bhuvanesvar	ToLCB-[IN:Bhu:13]	JN663851
Tomato leaf curl betasatellite- Bihar	ToLCB-[IN:Bih3:10]	GU732205
Tomato leaf curl betasatellite- New Delhi	ToLCB-[IN:ND:Papaya:09]	HM143911
French bean leaf curl betasatellite- Kanpur	FbLCB-[IN:Kan:11]	JQ866298
Ageratum yellow leaf curl betasatellite- Lucknow	AYLCB-[IN:Luk:11]	JQ408218
Bhendi yellow vein mosaic betasatellite- Westbengal	BYVMB-[IN:WB:07]	EF417919
Vernonia yellow vein betasatellite-Madurai	VYVB-[IN:Mad:10]	FN435836
Ageratum yellow vein betasatellite-Madurai	AYVB-[IN:Mad:03]	AJ557441
Bhendi yellow vein mosaic betasatellite- Madurai	BYVMB-[IN:Mad:03]	AJ308425

List of alphasatellites

Alphasatellites	Abbreviation	Accession number
Vernonia yellow vein Fijian alphasatellite	VYVFA	JF733780
Okra leafcurl Multan alphasatellite	OLCuMA	EU589450
Okra leafcurl Sudan alphasatellite	OLCuSA	FJ868830
Papaya leafcurl alphasatellite	PaLCuA	JQ322970
Tomato leafcurl China alphasatellite	ToLCCNA	AM749493
Tomato yellow leafcurl China alphasatellite	TYLCCNA	AM749494
Sida yellow vein Vietnam alphasatellite	SiYVVA	DQ641718
Cotton leafcurl Multan alphasatellite	CLCuMuA	GQ374450
Cotton leafcurl Lucknow alphasatellite	CLCuLA	HQ343234
Cotton leafcurl alphasatellite	CLCuDaA	AJ512957
Cyamopsis tetragonoloba leaf curl alphasatellite	CTLCuIA	GU385877
Verbesina encelioides leaf curl alphasatellite	VLCuRA	HQ631431
Ageratum yellow vein India alphasatellite	AYVIA	AJ512958
Ageratum yellow vein alphasatellite	AYVA	AJ512959
Ageratum yellow vein Singapore alphasatellite	AYVSGA	FJ956707
Ageratum yellow vein Singapore alphasatellite	AYVSGA	AJ416153

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) belongs to the genus *Begomovirus* (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 identify 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, association of betasatellite was found with MYMV and 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.

Key words: Yellow mosaic disease, grain legumes, leaf puckering, betasatellite, begomovirus.

Abbreviations: YMD= yellow mosaic disease; MYMV=*Mungbean yellow mosaic virus*; MYMIV=*Mungbean yellow mosaic India virus*; CR=Common region;SCR= satellite conserved region; RCA=Rolling circle amplification

Introduction

Yellow mosaic disease (YMD) is a major threat to the cultivation of grain legumes in India. The disease affects the four major crops *viz*., 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, *Mungbean yellow mosaic virus* (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 consists 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, 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 represent 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 nucleotide in length). 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; Briddon *et al*., 2003; Jose and Usha, 2003; Mansoor *et al*., 2003; Briddon and Stanley, 2006). Wherever the begomovirus is associated with betasatellites, one more satellite is also found, which 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) of ~150 nt length encompassing a hairpin structure with the loop containing the nonanucleotide sequence TAATATTAC and a positionally conserved open reading frame is also present on the complementary strand, coding for a ~13 KDa protein called as 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 understood fully. 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 associated with a many members of monopartite begomoviruses. In recent years, betasatellites have increasingly been identified in association 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 association was seen with *Tomato leaf curl New Delhi virus*. In both the cases the presence of betasatellite led to more severe symptoms than inoculation of DNA A and DNA B components. The survey was conducted on yellow mosaic disease of urdbean and mungbean in different districts of Tamil Nadu during the year 2011-12, the plants exhibited yellow mosaic, leaf distortion, enation and puckering symptoms. Research carried 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 2011-12.

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

polymerase chain reaction (PCR) was performed to detect the presence of begomovirus and betasatellites using begomovirus-specific degenerate primers, PALIc1960 (5'-TGGACTGCAGACNGGNAARACNATGTGGGC-3') and PALIr772 (5'-ATATCTG CAGGGNAARATHTGGATGGA-3') that flank from nucleotide co-ordinate 772 to 1960 in the DNA A component (Rojas *et al*., 1993) and universal betasatellite-specific primer β01 (5'-GGTACCACTACGCTACGCAGCAGCC-3') and β02 (5'-GGTACCTA CCCTCCCAGGGGTACAC-3') (Briddon *et al*., 2002). Reactions were performed in 25µl mixture containing approximately 50 ng of genomic DNA, 5 mM each dNTPs, 20 pmol of each forward and reverse primer and 0.5 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The reaction was carried out in Eppendorf epgradient S Master cycler (Eppendorf, Hamburg, 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 gels in TAE buffer and visualized by staining with ethidium bromide and recorded with an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). The sizes of the PCR products were determined by comparison with standard 1 kb DNA ladder (Fermentas).

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, 70ng of total nucleic acid was subjected to rolling circle amplification (RCA) with 10 units of Ø29 DNA polymerase, 500 µM of exo-resistant random hexamer primers and 0.1 unit of pyrophosphatase (Haible *et al*., 2006).

Cloning of viral genome

RCA product (about 500ng to 1µg) was subjected to restriction with different endonucleases, *Hind*III, *Bam*HI, *Pst*I and *Xba*I to identify the unique sites for cloning of viral genome. The restricted product of ~2.7 kb fragment from *Hind*III and *Bam*HI was purified and cloned into pUC18 vector and sequenced. For cloning of betasatellites, the RCA product was subjected to PCR using universal betasatellite-specific primers β01 and β02. PCR amplicons of ~ 1.3kb was electrophoresed on 1% agarose gel and were purified

from the gel using QIAquick Gel extraction kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's protocol. The purified PCR fragments were cloned into the pGEM-T easy vector (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol and the recombinant plasmids were used to transform *Escherichia coli* strain DH5α. The insertion of betasatellite was analysed through colony PCR. The selected clones were sequenced.

Sequence analysis

Sequence of the selected full length clones was done at Scigenom labs, Kerala, India using primer walking method. Nucleotide similarity searches were performed by BLAST at NCBI (www.ncbi.nlm.nih). Complete nucleotide sequences of the full length genomes were aligned and percentage pair-wise identity matrix was generated in BioEdit programme. 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 are 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 (Figure 1). Twenty two samples out of 74 samples tested gave expected amplicon of ~1.1 kb with Rojas 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 extreme low concentration of virus. Therefore the viral DNA components in the samples were enriched by RCA. The expected amplicons ~1.3kb was obtained in all the samples when RCA product was subjected to PCR (Figure 2).

Cloning of viral genome

The RCA product 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, three *Bam*HI clone and three betasatellite clones were analyzed in BLAST search programme. 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 *Mungbean yellow mosaic virus* (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 *Mungbean yellow mosaic virus*.

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 GenBank accession numbers 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 nucleotide in length. The complete nucleotide sequence of

betasatellites of 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 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 nucleotide of non coding region of the PaLCuB, the SCR was computed. The SCR was determined to be 191 nucleotide long in PaLCuB- CBE-BG, 193 in CBE-GG and 192 in VBN-BG. From the multiple alignment shown in Figure 3, it is clear that within the SCR, there are blocks of highly conserved region with variable region 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 *Papaya leaf curl betasatellite* 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 nucleotide with approximately 51 to 53 per cent A sequence. The A-rich region is maintained for 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 MW ~14 KDa. Comparison of amino acid sequence of βC1 protein of PaLCuB (Figure 4) clearly shows that, the βC1 protein of legume betasatellites of 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 Figure 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 of the present study was compared with other betasatellites deposited in GenBank database and the phylogenetic tree was constructed (Figure 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 group of betasatellites consisted of molecules originating from a diverse range of host plants such as chilli, tomato and cotton. The TbLCuB showed no particular affinity for any of the cluster.

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 begomovirus causes the crinkling symptoms and any betasatellite components are involved. The results revealed that the *Papaya leaf curl betasatellite* was associated with MYMV.

The betasatellites associated with bipartite begomovirus was reported by Rouhibakhsh and Malathi (2005) who investigated the cowpea plants showing severe leaf curl symptoms in northern India and reported the presence of *Tomato leaf curl betasatellite* species. Subsequently Sivalingam *et al*. (2010), Jyothsna *et al*. (2013b) demonstrated their association with the bipartite begomovirus *Tomato leaf curl New Delhi virus* (ToLCNDV). Qazi *et al*. (2007a) suggested that severe leaf curl and crumpling symptoms in cowpea plants 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 have been reported in legumes. They are *Tomato leaf curl betasatellite* 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), *Mungbean yellow mosaic India betasatellite* associated with MYMIV in cowpea from India (JX443646, www.ncbi.nlm.nih.gov), the *Papaya leaf curl betasatellite* from cowpea have been reported (DQ118862, www.ncbi.nlm.nih.gov). A new monopartite begomovirus, *French bean leaf curl virus* (Kamaal *et al*., 2012) has been identified in French bean and it is associated with *French bean leaf curl betasatellite* (FbLCB). In the present study, the bipartite MYMV are associated with *Papaya leaf curl betasatellite*. Whether this tripartite association in the yellow mosaic viruses will continue to be maintained or as the time progresses is an issue which needs to be investigated.

Betasatellites belong 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 DNA accumulation (Guo *et al*., 2008) and symptoms severity in the host plant (Jyothsna *et al*., 2013a). All the betasatellites molecules under study showed several characteristic features in common with other betasatellite 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 elements needed for binding of Rep-protien (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 a conducive 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 are 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 betasatellite/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, www.ncbi.nlm.nih.gov). It is very difficult to hypothesize the mechanism by which MYMIV and MYMV could have picked up *Papaya leaf curl betasatellite*, as legume infecting begomoviruses do not infect the other hosts enlisted above and chances of they occurring in mixed infection with any other monopartite begomovirus are very less. However there was a recent report of *Tomatoleaf curl Karnataka 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.

Acknowledgements

The authors are grateful for the facilities provided by The Director (CPPS), Professor and Head, Department of Plant Pathology,TNAU for conducting the study. The financial assistance by the Department of Biotechnology, Government of India is duly acknowledged. The senior author (VKS) is thankful to The Dean, SPGS, Tamil Nadu Agricultural University.

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Figure legends

Figure 1. Yellow mosaic disease affected plants showing yellow mosaic and leaf puckering symptoms

Figure 2. Agarose gel electrophoresis showing detection of betasatellites from yellow mosaic disease affected plants. PCR amplicons of ~1.3 kb representing the presence of betasatellites. Legends; Lane M: 1 kb ladder (Fermentas); Lane 1-10: YMD infected samples showing the presence of betasatellites.

Figure 3. Multiple alignment of satellite conserved region of Papaya leaf curl betasatellites and betasatellites associated with legumes

Figure 4. Multiple alignment of predicted amino acid sequences of βC1 protein of papaya leaf curl betasatellites and betasatellites associated with legumes

Figure 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.

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.

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

Figure 1

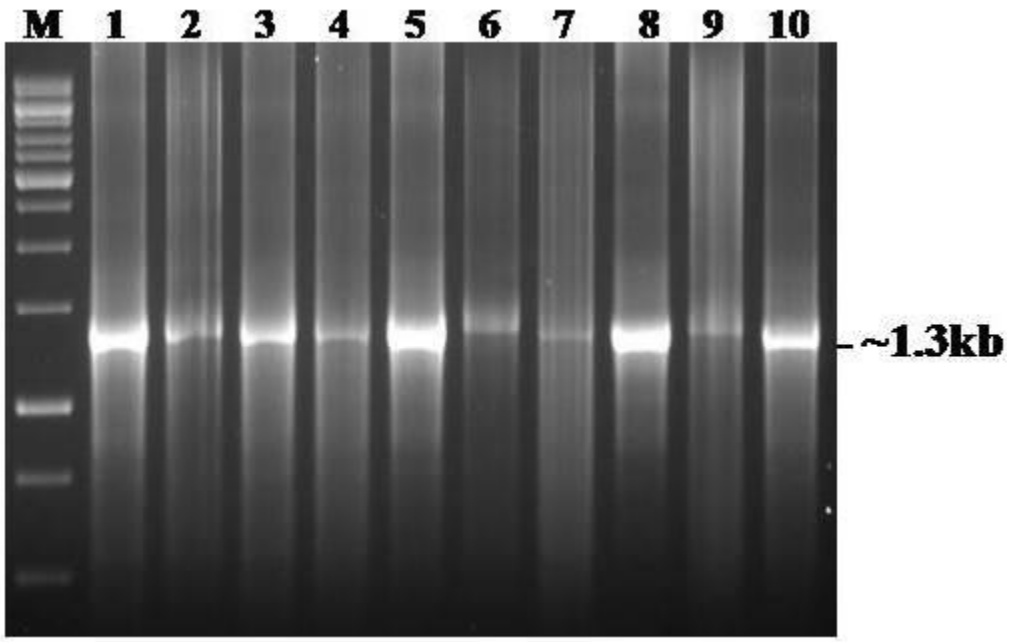
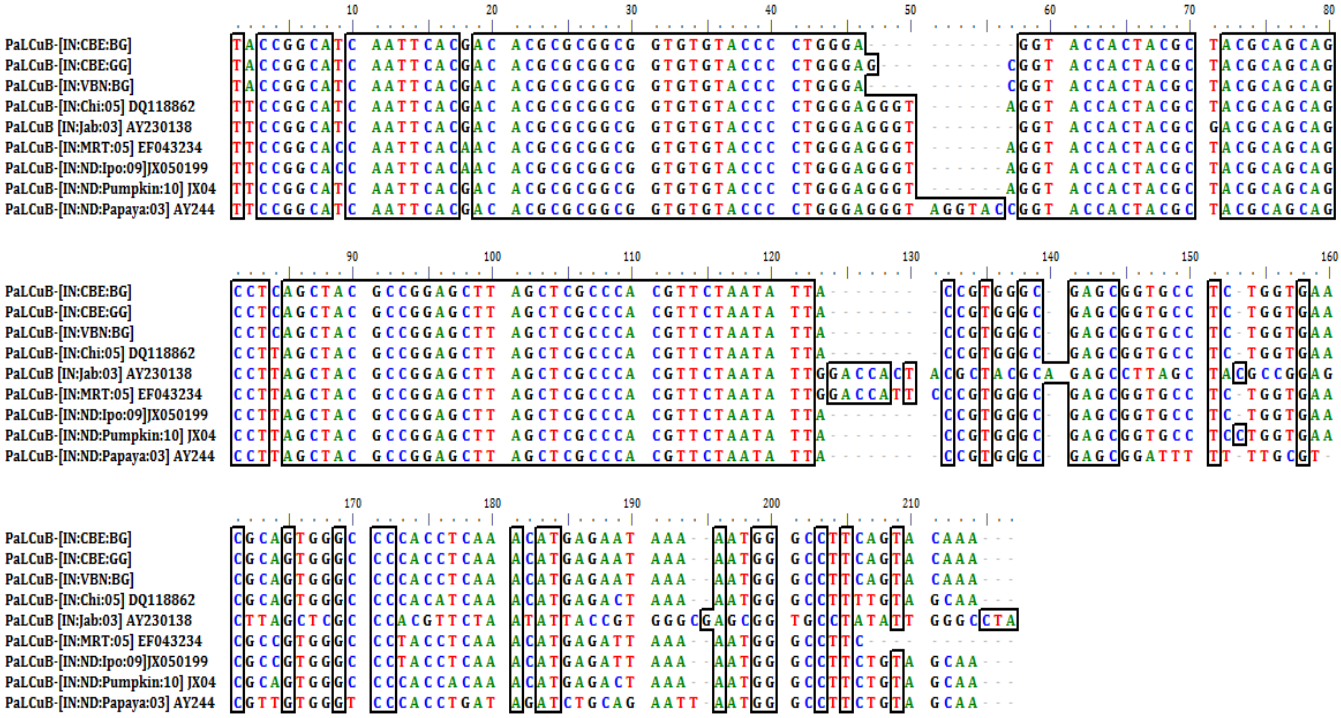


Figure 3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PaLCuB-[IN:CBE:GG]
PaLCuB-[IN:VBN:BG]
PaLCuB-[IN:Chi:05] DQ118862
PaLCuB [IN:Jab:03] AY230138
PaLCuB-[IN:MRT:05] EF043234
PaLCuB-[IN:ND:Ipo:09]JX050199
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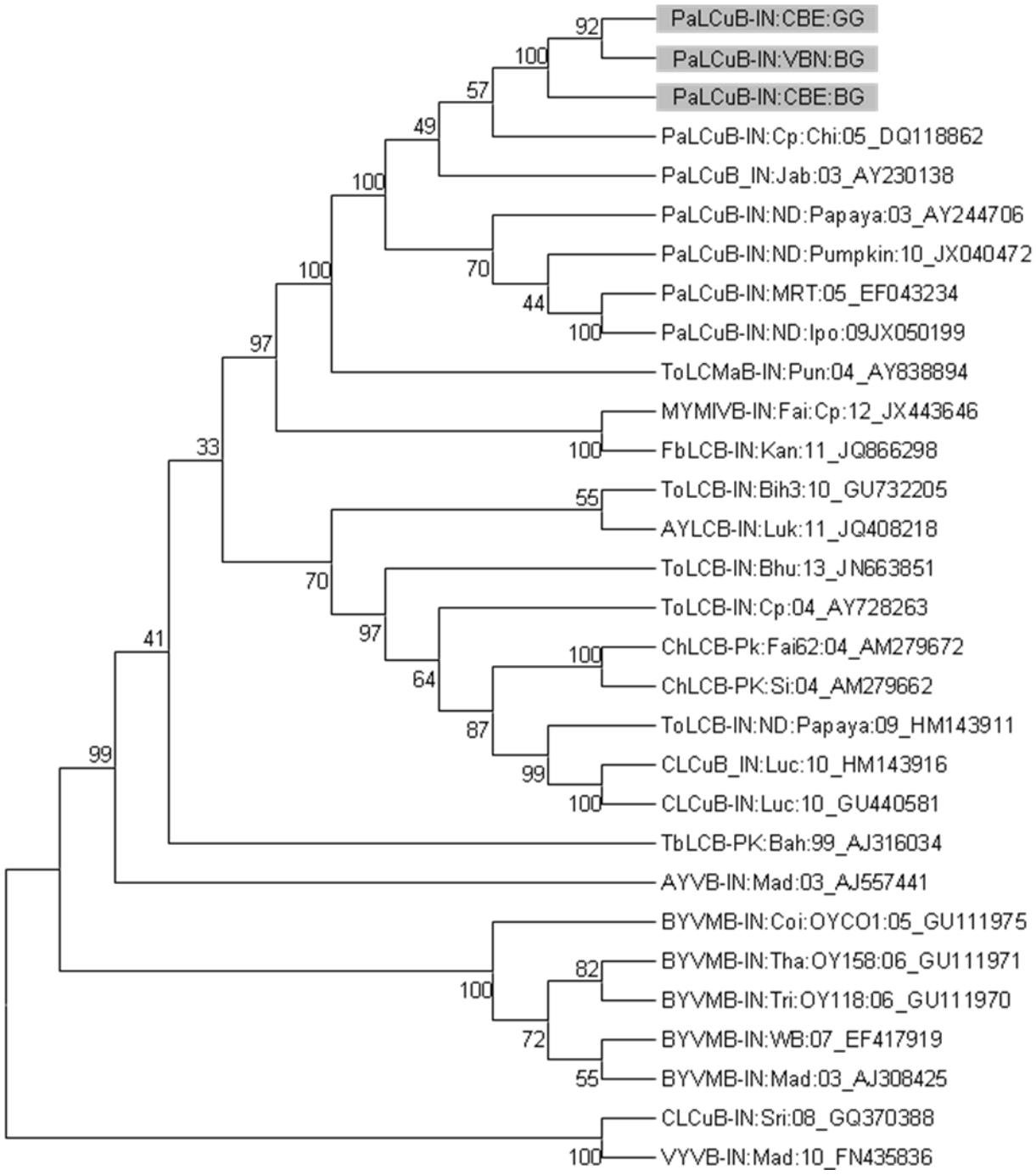
PaLCuB-[IN:ND:Papaya:03] AY244

Figure 5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PaLCuB_IN:Jab:03_AY230138
PaLCuB-IN:ND:Papaya:03_AY244706
PaLCuB-IN:ND:Pumpkin:10_JX040472
PaLCuB-IN:MRT:05_EF043234
PaLCuB-IN:ND:Ipo:09JX050199
ToLCMaB-IN:Pun:04_AY838894
MYMIVB-IN:Fai:Cp:12_JX443646
FbLCB-IN:Kan:11_JQ866298
ToLCB-IN:Bih3:10_GU732205
AYLCB-IN:Luk:11_JQ408218
ToLCB-IN:Bhu:13_JN663851
ToLCB-IN:Cp:04_AY728263
ChLCB-Pk:Fai62:04_AM279672
ChLCB-PK:Si:04_AM279662
ToLCB-IN:ND:Papaya:09_HM143911
CLCuB_IN:Luc:10_HM143916
CLCuB-IN:Luc:10_GU440581
TbLCB-PK:Bah:99_AJ316034
AYVB-IN:Mad:03_AJ557441
BYVMB-IN:Coi:OYCO1:05_GU111975
BYVMB-IN:Tha:OY158:06_GU111971
BYVMB-IN:Tri:OY118:06_GU111970
BYVMB-IN:WB:07_EF417919
BYVMB-IN:Mad:03_AJ308425
CLCuB-IN:Sri:08_GQ370388
VYVB-IN:Mad:10_FN435836
92
100
57
49
100
100
70
44
100
97
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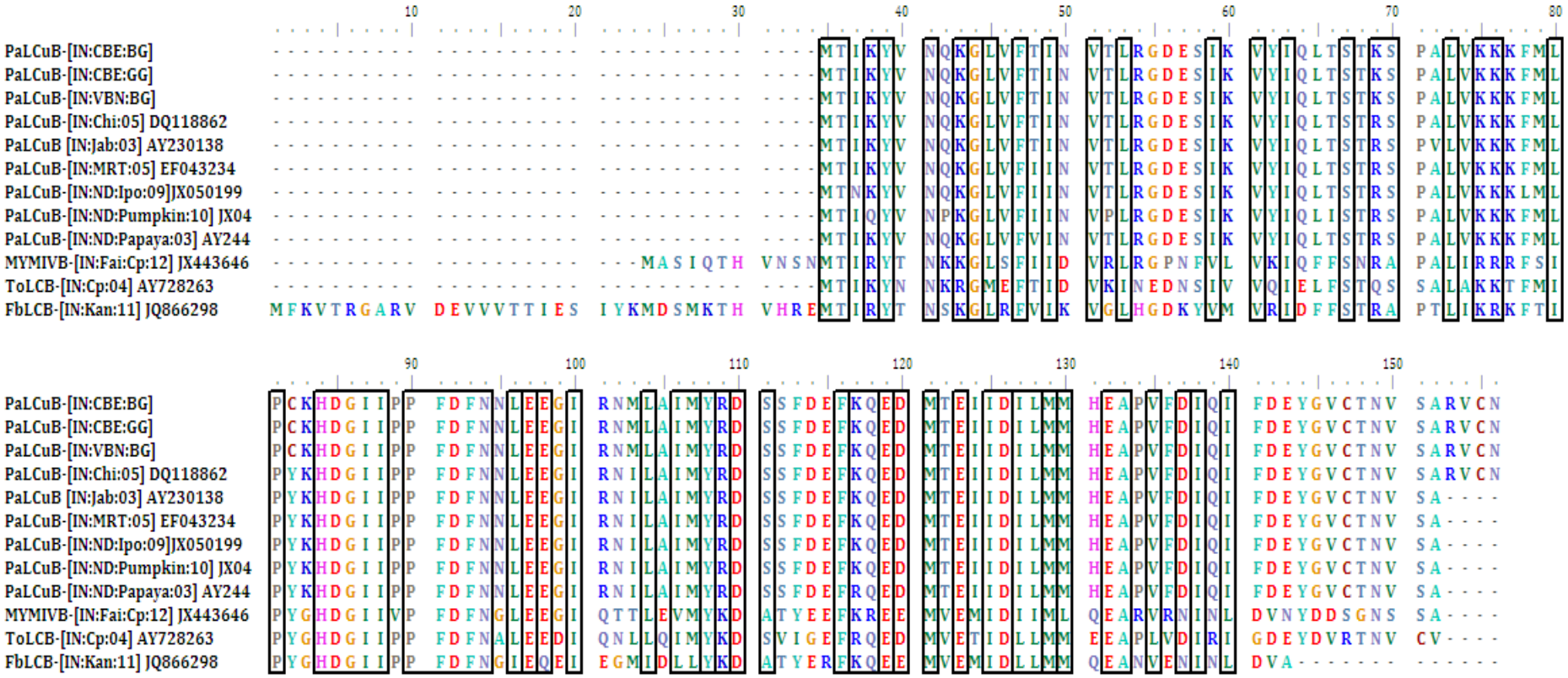
33
55
70
97
64
100
87
99
99
100
41
82
100
72
55
100

Figure 4


PaLCuB-[IN:CBE:BG]
PaLCuB-[IN:CBE:GG]
PaLCuB-[IN:VBN:BG]
PaLCuB-[IN:Chi:05] DQ118862
PaLCuB [IN:Jab:03] AY230138
PaLCuB-[IN:MRT:05] EF043234
PaLCuB-[IN:ND:Ipo:09]JX050199
PaLCuB-[IN:ND:Pumpkin:10] JX04
PaLCuB-[IN:ND:Papaya:03] AY244
MYMIVB-[IN:Fai:Cp:12] JX443646
ToLCB-[IN:Cp:04] AY728263
FbLCB-[IN:Kan:11] JQ866298

Molecular detection of *Mungbean yellow mosaic virus* and their associated satellite DNA from field samples of Tamil Nadu

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Abstract

Pulses are the important source of protein in vegetarian diet and they are affected by several several viral diseases. Among the virus diseases, yellow mosaic disease (YMD) caused by *Mungbean yellow mosaic virus* (MYMV) belonging to the genus *Begomovirus* (family: *Geminiviridae*) is a serious malady in cultivation of grain legumes in India and cause severe yield loss. YMD has very narrow host range within legumes and cause biologically indistinguishable symptoms, so the identification of virus is difficult. The molecular detection of MYMV from infected blackgram plants showing yellow mosaic symptoms was carried out. PCR with begomovirus specific primers detected the presence of begomovirus from the infected samples with amplicons of ~1100bp. The PCR with MYMV-specific coat protein primers detected the presence of MYMV from the samples collected from three districts of Tamil Nadu with the amplicons of ~1000bp. The dot blot hybridization showed positive response with the infected samples and negative response with healthy samples. The associated satellite DNA components were detected using universal abutting primers. Hence, molecular detection of MYMV seems simple and very sensitive method.

Key words: *Mungbean yellow mosaic virus*, polymerase chain reaction, Dot blot hybridization

Introduction

Pulse crops also known as grain legumes are the second most important group of crops grown essentially in South Asia. In India, they form the important constituent in the vegetarian diet and are cultivated under different agroclimatic conditions. They are cultivated as a short duration crop either as sole crop or intercropping system round the year in all the four crop seasons. India holds the first rank in pulses production and consumption in the world. India grows the largest varieties of pulses in the world accounting for about 32 per cent of the area and 23 per cent of the world production. It is grown in an area of 10.84 million hectares in India with a production of 17.09 million tonnes (productivity – 699 kg/ha) (Anonymous, 2012). Among pulses, black gram (*Vigna mungo* L. Hepper) occupies a prominent place in India, covering an area about 3.29 million hactares with the production of 1.83 million tonnes and productivity is 555 kg/ha (AICRP report, 2012-13).

Viruses belonging to *Como-*, *Gemini-*, *Ilar-*, *Poty-* and *Tospovirus* groups are known to infect pulses under natural conditions in various parts of the world. Major damage is due to the yellow mosaic disease, caused by *Mungbean yellow mosaic virus* (MYMV) belonging to the genus *Begomovirus* of the family *Geminiviridae*. This disease is a serious malady of the pulses and is the major threat to cultivation of pulses in India (Malathi, 2007). The yellow mosaic disease was reported in mungbean by Nariani (1960) and it causes severe yield loss. Detection and diagnosis of MYMV and their associated satellite DNA components are essential for managing the disease caused by them and in studying the epidemiology of the viruses. Most of the yellow mosaic viruses infecting grain legumes in India are not sap – transmissible, share a very narrow host range within legumes and cause biologically indistinguishable symptoms, making specific identification of the viruses difficult.

In India, there has been shift from identification of the virus based on biological characteristics like symptoms, host range and transmission characters to serological and nucleic acid based techniques. Because of the difficulty in purifying begomovirus virion particles, nucleic acid based approaches like PCR and hybridization with radiolabelled probes are being widely preferred for their diagnosis in the place of serological

techniques. Roy and Malathi (2004) developed nucleic acid hybridization technique to detect the *Cowpea Golden Mosaic Virus* (CPGMV) in many crops and weed species. This study showed that CPGMV DNA A probe detected all the begomoviruses where as CPGMV DNA B probe is specific to CPGMV. Deng *et al*. (1994) designed the degenerate primers to amplify the virus belonging to the family *Geminiviridae*. Rojas *et al*. (1993) designed the degenerate primers from the highly conserved region of DNA A component to amplify the virus belonging the genus *Begomovirus*. Naimuddin *et al*. (2011a) designed the specific primers to amplify both the DNA components of MYMV and MYMIV and the primers were designed from the coat protein region DNA A component, whereas for DNA B, the primers were designed from the movement protein region.

Keeping the importance of accurate identification of virus and their associated DNA components in view, the present study was carried out to identify the viruses associated with yellow mosaic disease of blackgram at Tamil Nadu.

Materials and Methods

Sample collection

Blackgram plants affected with severe yellow mosaic disease showing severe yellow mosaic symptoms were collected from widely distributed districts of Tamil Nadu *viz*., experimental fields at TNAU, Coimbatore (Coimbatore), farmers field at Vamban (Pudukottai) and Panpozhi (Tirunelveli) (Table 1) where the disease incidence was 90-100 per cent. Young leaf samples showing yellow mosaic symptoms were collected and used for this research work.

Extraction of genomic DNA from infected samples

Total DNA was extracted from symptomatic young leaves of blackgram and greengram showing yellow mosaic symptoms collected from Coimbatore, Vamban and Panpozhi by GEM-CTAB method (Rouhibakhsh *et al.,* 2008) using 2 % β - mercapto ethanol. The symptomatic fresh young leaves were collected from field in the early morning. 100mg of leaves were ground to fine powder using liquid nitrogen. Prewarmed DNA extraction buffer (N-cetyl-N,N,N trimethyl ammonium bromide (2 %), 100 mM Tris

Hcl (pH: 8.0), 1.4 M NaCl, 20 mM EDTA (pH: 8.0) and β - mercaptoethanol (2%)) was added to the ground leaves and incubated at 65°C for 30 min followed by the addition of 0.7-0.8 vol. of chloroform and isoamylalcohol (24:1 v/v). The contents were gently mixed by inverting the tube for 10 min and then centrifuged at 10,000 rpm for 10 min. The upper aqueous phase containing DNA was transferred to a new 1.5 ml micro centrifuge tube and added with equal volume of isopropanol and mixed well by inverting the tube to precipitate the nucleic acid and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and the DNA pellet was washed with 70% ethanol and air dried. The DNA pellet was resuspended in 50 µl of sterile double distilled water. The genomic DNA was checked by 0.8% agarose gel electrophoresis and stored at -20°C for further use.

Detection of YMV through dot blot hybridization

The presence of begomovirus from the infected samples was verified by dot blot hybridization. The total genomic DNA was spotted into nitrocellulose membrane and was fixed onto membrane by baking the membrane at 80ºC for 1-2 h using a standard protocol (Sambrook et al., 1989). The hybridization was done with a (α^{32}P)-dCTP labelled probes specific for CP gene of MYMV-Bg (GenBank Accession no.DQ400848). The probes were prepared by random primer labelling method (Feinberg and Vogelstein, 1983). Hybridization signal detection was carried out using a Storage Phosphor System Cyclone® Plus (Perkin Elmer, Shelton, CT, USA). The hybridization signal intensity was analyzed through densitometric analysis using Opti Quant Version 5.0 (Perkin Elmer, Shelton, CT, USA).

Detection of begomovirus through PCR

The purified genomic DNA was amplified by polymerase chain reaction (PCR) using universal Begomovirus-specific primers PALIc1960 (5'-ACNGGNAARA CNATGTGGGC-3') and PALIr772 (5'-GGNAARATHTGGATGGA-3') (Rojas *et al*., 1993). Reactions were performed in 20 µl mixture containing approximately 50 ng of genomic DNA, 5 mM each dNTPs, 20 pmol of each PALIc1960 forward primer and PALIr772 reverse primer (Table 3) and 0.5 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The reaction was carried out in Eppendorf epgradient S Master cycler (Eppendorf, Hamburg, 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 gels in TAE buffer and visualized by staining with ethidium bromide and recorded with an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). The sizes of the PCR products were determined by comparison with standard 1 kb molecular marker (Fermentas).

Detection of MYMV through PCR

To detect the presence of MYMV, the PCR was performed using MYMV-specific coat protein primers (forward primer, 5'-ATGGG(T/G)TCCGTTGTATGCTTG-3' and reverse primer, 5'-GGCGTCATTAGCATAGGCAAT-3') (Naimuddin *et al*., 2011).

Detection of associated satellite DNA component

Detection of alpha and betasatellite

The purified genomic DNA was amplified by polymerase chain reaction (PCR) using universal betasatellites specific primers β01 (5'- GTAGGTACCACTACGCTACG CAGCAGCC-3')and β02 (5'-AGTGGTACCTACCTACCCTCCCAGGGGTA CAC-3') (Briddon *et al*., 2002) and alphasatellites were amplified using universal abutting primers UN101 (5'-AAGCTTGCGACTATTGTATGAAAGAGG-3') / UN102(5'- AAGCT TCGTC TGTCTTACGAGCTCGCTG -3') (Bull et al. 2003).

Results and Discussion

Disease incidence, symptoms and sample collection

Leaves of blackgram showing severe yellow mosaic symptoms (Fig 1) were collected from three hot spot areas of Tamil Nadu, *viz*., Coimbatore (Coimbatore), Vamban (Pudukottai) and Panpozhi (Tirunelveli) which represents three districts of Tamil Nadu. The affected plants show yellow patches intermingled with green areas, which later coalesced and show complete yellowing of leaves (Fig. 1). In severe cases pods turn yellow and contain small and shrivelled seeds. The seeds are yellow coloured, small, slightly shrunken compared to seeds from healthy plants which appeared shiny and jet black. Young leaves samples were collected from symptomatic plants from each district. These samples were used for the present study.

Preliminary diagnosis for the presence of MYMV through dot blot hybridization

The genomic DNA was isolated from the infected samples using GEM-CTAB method (Rouhibakhsh et al., 2008) with 2% β –mercaptoethanol. The presence of MYMV was confirmed by dot blot hybridization with specific probes of coat protein gene of MYMV- DNA A (DQ400848). Out of 74 samples, 47 samples (63.51%) showed positive response in the hybridization whereas healthy samples showed no hybridization (Fig 2). Mandal *et al*. (1997) detected the MYMV using dot blot hybridization. Roy and Malathi (2004) developed nucleic acid hybridization technique to detect the *Cowpea Golden Mosaic Virus* (CPGMV) in many crops and weed species.

Detection of MYMV through PCR

The results were further confirmed through PCR using degenerate primers PAR1v772/ PAL1c1960 (Rojas *et al*., 1993). The expected amplification of 1.1 kb was observed in 22 samples (29.72%) out of 74 samples tested (Fig 3) confirming the presence of begomovirus. In order to detect the MYMV, the PCR was performed using the MYMV-specific coat protein primers and the results revealed that the expected amplicons of ~1000 bp (Fig 4) indicating the presence of MYMV. The failure to get PCR amplicons may be due to low concentration of the viral DNA and the interference due to polyphenol and tannin affecting quality of DNA, the problems experienced by many research workers. Swanson *et al*. (1992) reported the biggest hurdle in the extraction of high quality DNA from legume hosts, which are rich in phenols and polysaccharides and also with very low concentration of virus. Varma *et al*. (1992) reported that the detection of viral genome in the legume host like mungbean, is very difficult even in dot blot hybridization tests. The association of a begomovirus was confirmed by PCR using primer pairs specific to MYMIV and MYMV that commonly infect cultivated species of *Vigna* in different parts of India (Karthikeyan *et al* ., 2004; Usharani *et al.,* 2004; Naimuddin *et al*., 2011b).

Detection of associated satellite DNA component

DNA was isolated from field samples of blackgram showing severe yellow mosaic along with leaf puckering symptoms. When genomic DNA was subjected to PCR using betasatellite - specific primers, the amplification was seen in the YMD affected

plant samples (Fig. 5). In recent years, leaf curl and leaf distortion symptoms are observed in blackgram and greengram plants in farmers' field in Tamil Nadu. The yellow mosaic symptoms are severe and trifoliate leaves exhibit asymmetry and leaflets look distorted. The results revealed the association of betasatellites with MYMV. In recent years, the begomovirus-betasatellite complex have been reported in legumes. They are *Tomato leaf curl betasatellite* 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), *Mungbean yellow mosaic India betasatellite* associated with MYMIV in cowpea from India (JX443646, www.ncbi.nlm. nih.gov), the *Papaya leaf curl betasatellite* from cowpea (DQ118862, www.ncbi.nlm. nih.gov). A new monopartite begomovirus, *French bean leaf curl virus* (Kamaal *et al*., 2012) has been identified in French bean and it is associated with *French bean leaf curl betasatellite* (FbLCB).

DNA was isolated from field samples of blackgram which showed severe yellow mosaic along with leaf puckering and leaf distortion symptoms. When genomic DNA was subjected to PCR using alphasatellite-specific primers, the amplification was seen in all the samples tested (Fig. 6). Association of alphasatellite with the monopartite begomovirus and betasatellite is well known in many hosts plants (Briddon *et al*., 2004). Recently, Idris *et al.* (2011) showed that an unusual alphasatellite (DNA 2) associated with *Tomato yellow leaf curl Oman virus* ameliorated symptoms and significantly reduced betasatellite DNA accumulation. In the case of yellow mosaic symptoms, lot of variations are seen in symptom severity ranging from well restricted yellow or chlorotic area to, completely bleached yellow leaf, green and yellow patches distributed either randomly or closely; and a very severe necrotic spots on the leaf.

Acknowledgement: The authors are grateful for the facilities provided by Professor and Head, Department of Plant Pathology for conducting the study. The financial assistance by the DBT, Government of India is duly acknowledged. The senior author (VKS) is thankful to the The Dean, SPGS, TNAU, Coimbatore, Dr. V.G. Malathi,CSIR, Emeritus scientist, Dr. R. Velazhahan, Professor (Plant Pathology) and Dr. P. Jayamani, Professor (PBG).

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Table 1. Sample collected from different location in the year 2011-2013

Crop	**Location**	**No. of samples**	**Symptoms**
Black gram	Coimbatore, Coimbatore Vamban, Pudukottai Panpozhi, Tirunelveli	45 27 4	Yellow mosaic, complete yellowing, bleached appearance of leaf, necrosis, leaf puckering
Total number of samples		76	

Fig 1. Yellow mosaic disease affected blackgram plants showing severe yellow mosaic symptoms

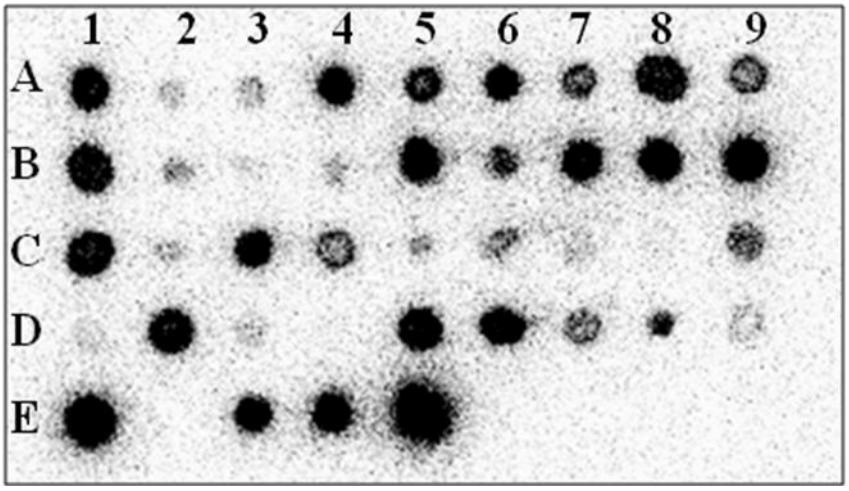

Fig 2. Preliminary diagnosis of *Mungbean Yellow mosaic virus* from yellow mosaic disease affected samples through dot blot hybridization using the probe to MYMV-Vam, DQ400848. Row A,B,C,D and E1-E5- Infected field samples; Row E6-9: Helathy samples

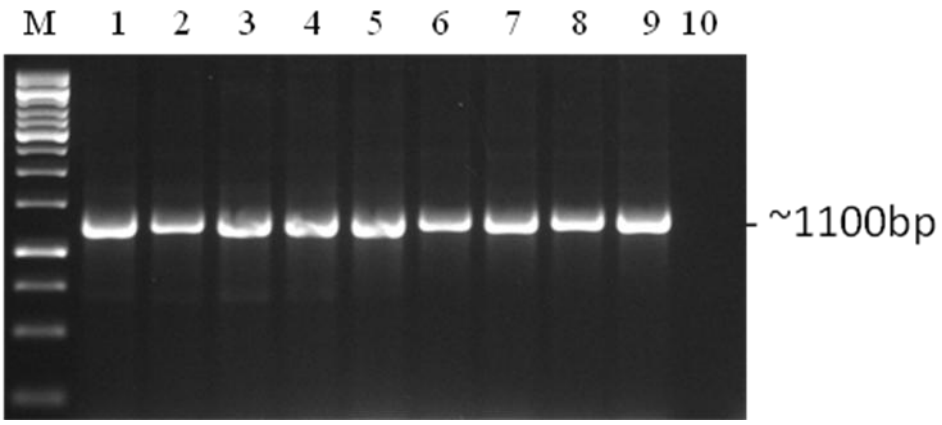

Fig 3. Agarose gel electrophoresis showing presence of begomovirus from yellow mosaic disease affected samples. The amplicons ~1100bp representing the presence of begomovirus.Lane M- 1Kb ladder, Lane 1-9: YMD affected plant samples; Lane 10: Healthy samples.

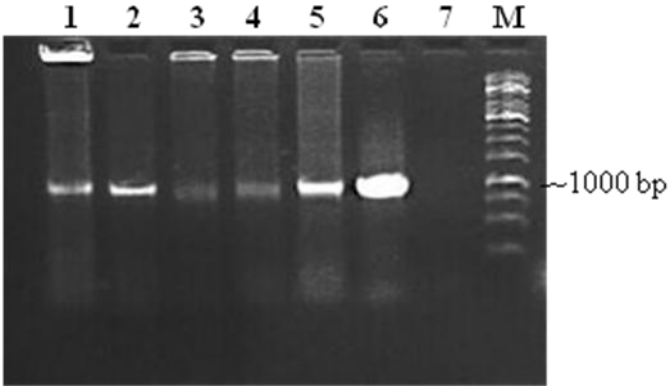

Fig 4. Agarose gel electrophoresis showing presence of MYMV from yellow mosaic disease affected samples through PCR using MYMV-specific primers. The amplicons ~1100bp representing the presence of begomovirus.Lane M- 1Kb ladder, Lane 1-6: YMD affected plant samples; Lane 7: Healthy samples.

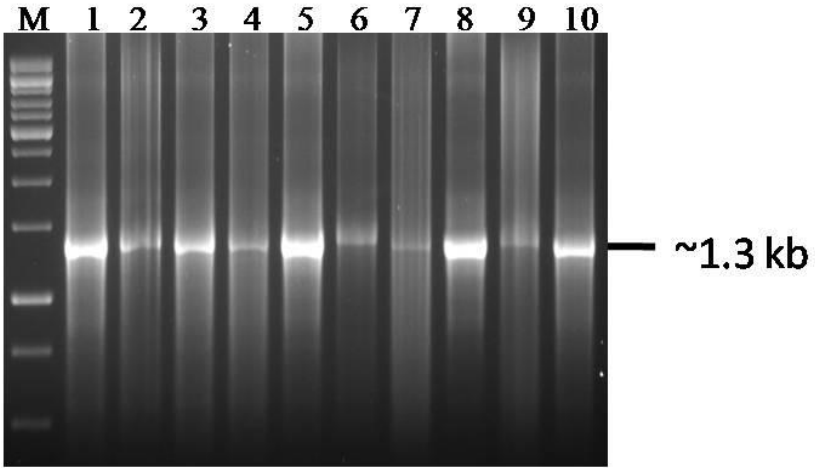

Fig. 5. Detection of betasatellites through PCR using betasatellite specific primers.

Lane M- 1Kb ladder, Lane 1-10: YMD affected plant samples

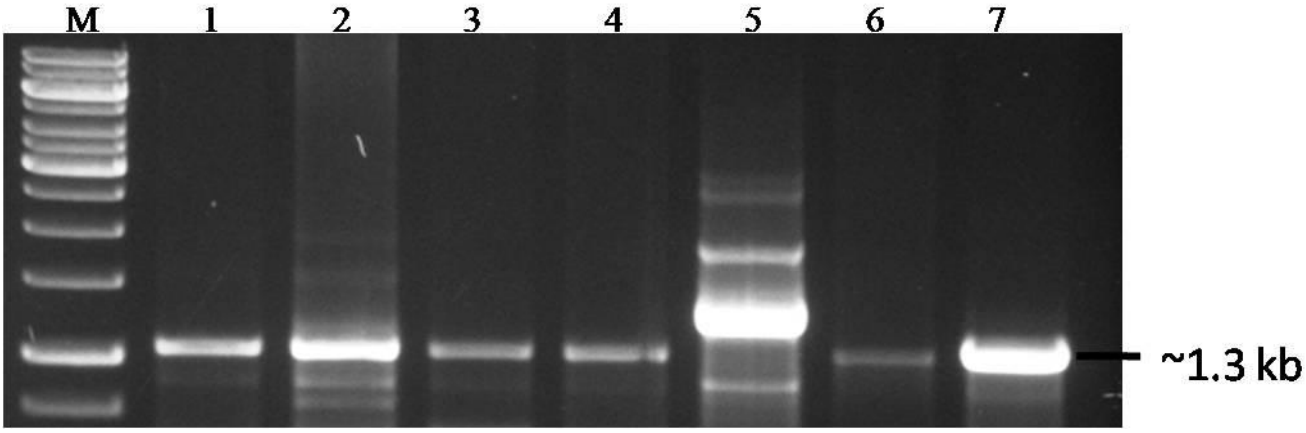

Fig. 6. Detection of betasatellites through PCR using alphasatellite specific primers.

Lane M- 1Kb ladder, Lane 1-7: YMD affected plant samples

ABSTRACT

CHARACTERIZATION OF YELLOW MOSAIC VIRUS (YMV) INFECTING GRAIN LEGUMES AND DEVELOPMENT OF DIAGNOSTICS

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Yellow mosaic disease is a major threat to the cultivation of legumes in Tamil Nadu and causes yield loss upto 100 per cent. The prime focus of this study is to characterize the yellow mosaic virus in blackgram, development of diagnostics and screening for resistance source. Leaves of blackgram showing severe yellow mosaic symptoms were collected during summer from three hot spot areas of Tamil Nadu, *viz*., Coimbatore (Coimbatore), Vamban (Pudukottai) and Panpozhi (Tirunelveli) which represents three districts of Tamil Nadu. Five DNA A and eight DNA B clones were cloned and sequenced from all the three locations. The virus isolates are identified to belong to the species, *Mungbean yellow mosaic virus* (MYMV). The genome organization of MYMV revealed to be typical of Old World bipartite begomoviruses. DNA A components encode two open reading frames (ORFs) on viral sense strand and five ORFs seen on the complementary sense strand. DNA B components encode ORF BV1 (Nuclear shuttle protein on the viral sense strand and ORF BC1 on the complementary sense strand. In DNA A component, among various open reading frames, ORF AV1 and AC1 was highly conserved when all MYMV isolates were compared. In DNA B component, both ORFs were conserved amongst MYMV isolates The most interesting feature is that more than 15 per cent divergence was observed between the common region (CR) of DNA A and DNA B. Phylogenetic analysis of complete nucleotide sequence of YMV isolates including the sequence data available in the database revealed that the distinct separation of YMV from other begomovirus of Indian subcontinent and China. The satellite DNA, alpha and betasatellites were found associated with MYMV, which constitutes the first report for MYMV. Seed borne nature of MYMV has been studied for the first time but it is not seed transmitted. MYMV DNA A and DNA B specific probes have been developed to detect MYMV in many crops. Phenotyping of RILs was done under natural hot spot areas and artificial condition using agroinoculation technique. The protocol for agroinoculation technique has been developed to screen the varieties against MYMV.

Table 1. Seed-transmitted viruses of major importance in some legume crops

Virus	Host	Transmission (%)	Reference
Cowpea mild mottle virus	Soybean	<91.7	Allen (1983)
Soybean mosaic virus	Soybean	0.05-10.2	Goodman and Oard (1980)
Tobacco ring spot virus	Soybean	100	Lizuka (1973)
Cowpea severe mosaic virus	Cowpea	10	Singh and Allen (1980)
Cowpea aphid-borne mosaic virus	Cowpea	0-40	Singh and Allen (1980)
Cowpea mottle virus	Cowpea	3-10	Singh and Allen (1980)
Cowpea banding mosaic virus	Cowpea	9-34	Prakash and Joshi(1980)
Cowpea chlorotic spot	Cowpea	4-20	Singh and Allen (1980)
Blackeye cowpea mosaic virus	Cowpea	13	Zettler and Evans (1973)
Cowpea yellow mosaic virus	Cowpea	1-5	Allen (1983)
Bean common mosaic virus	Green gram	8-32	Kaiser and Mossahebi (1974)
Urd bean leaf curl virus	Green gram	21.13	Kanimozhi *et al*. (2009)
	Blackgram	26.25	Kanimozhi *et al*. (2009)

Table 2. Sample collected from different location in the year 2011-2013

Crop	Location	No. of samples	Symptoms
Black gram	Coimbatore, Coimbatore Vamban, Pudukottai Panpozhi, Tirunelveli	45 27 2	Yellow mosaic, complete yellowing, bleached appearance of leaf, necrosis, leaf puckering
Total number of samples		**74**	

Table 3. Primers used in the present study

Primer ID	Primer Sequence (5' - 3')	Region amplified	Component detected	Source of the primer sequence
PAR1v772	GGNAARATHTGGATGGA	772 – 1960 nt (1.1 kb)	DNA A of begomoviruses	(Rojas *et al*., 1993)
PAL1c1960	ACNGGNAARACNATGTGGGC			
Beta 01 (β01)	GTAGGTACCACTACGCTACGCAGCAGCC	Full length genome (1.3kb)	DNA betasatellites	Briddon *et al*., 2002
Beta 02 (β02)	AGTGGTACCTACCTACCCTCCCAGGGGTACAC			
UN 101	AAGCTTGCGACTATTGTATGAAAGAGG	Full length genome (1.3kb)	DNA alphasatellites	Bull *et al*. 2003
UN 102	AAGCTTCGTCTGTCTTACGAGCTCGCTG			
DNA 101	CTGCAGATAATGTAGCTTACCAG	Full length genome (1.3kb)	DNA alphasatellites	Bull *et al*. 2003
DNA 102	CTGCAGATCCTCCACGTGTATAG			
DNA A - AV	AAAGCTTACATCCTCCAC	Full length genome (2.7 kb)	DNA A component	Qazi *et al*. (2007) Abutting primers
DNA A-AC	GTAAAGCTTTACGCATAATG			
DNA B-BV	CCAGGATCCAATGATGCCT	Full length genome (2.7 kb)	DNA B component	Qazi *et al*. (2007) Abutting primers
DNA B-BC	ATTGGATCCTGGAGATTCA			

Table 4. Details of clones of the present study and their features

DNA A									DNA B			
Clone ID	Genbank Accession no.	Coding Region Nucleotide co ordinates							Coding Region Nucleotide co ordinates			
		ORF AV1 (CP)	ORF AV2 (Pre-CP)	ORF AC1 (Rep)	ORF AC2 (TrAP)	ORF AC3 (REn)	ORF AC4 (PTGS)	ORF AC5	Clone ID	Genbank Accession no.	ORF BV1 (NSP)	ORF BC1 (MP)
CA1	KC911721	307-1080	147-497	2617-1529	1629-1222	1481-1077	2466-2167	975-724	CB1	KC911724	419-1189	2117-1221
CA2	KC911718	303-1076	143-493	2607-1522	1622-1215	1474-1073	2456-2112	971-720	CB2	KC911730	392-1183	2129-1215
VA1	KC911722	302-1075	142-492	2611-1523	1623-1216	1475-1194	2460-2161	970-719	CB3	KC911729	429-1199	2136-1231
VA2	KC911723	302-1090	142-492	2621-1536	1636-1229	1488-1087	2470-2126	985-779	VB1	KC911728	418-1188	2128-1220
PA1	KC911717	301-1074	141-491	2611-1523	1623-1216	1475-1071	2460-2161	969-718	VB2	KC911727	419-1189	2108-1221
									VB3	KC911726	420-1190	2049-1222
									VB4	KC911725	419-1189	2096-1221
									PB1	KC911731	419-1189	2033-1221

Table 5. Percentage identity of predicted amino acid sequence of ORF AV1 of YMV isolates of present study with other selected YMV isolates.

Virus isolates	% Identity				
	MYMV CA1	**MYMV CA2**	**MYMV VA1**	**MYMV VA2**	**MYMV PA1**
MYMV-CA1	100				
MYMV-CA2	100	100			
MYMV-VA1	99	99	100		
MYMV-VA2	84	84	83	100	
MYMV-PA1	99	99	99	83	100
MYMV Vam	100	100	99	83	99
MYMV-Vig (Mad)	99	99	99	83	100
MYMV-Sb (Mad)	99	99	98	82	99
MYMV Nam	99	99	99	82	100
MYMV (TH)	97	97	97	82	98
MYMV (KH-PP-03)	97	97	97	82	98
MYMIV-Mg (BD)	84	84	84	73	84
MYMIV-Mg (NP)	85	85	85	73	85
MYMIV-Mg (Pak)	86	86	86	74	86
MYMIV Cp7	86	86	86	74	86
MYMIV Sb2	86	86	86	74	86
MYMIV PK-Sb	86	86	86	74	86
MYMIV PK-Vig	83	83	83	71	83
MYMIV MBKA25	85	85	85	73	85

Table 6. Percentage identity of predicted amino acid sequence of ORF AV2 of YMV isolates of present study with other selected YMV isolates.

Virus isolates	% Identity				
	MYMV CA1	**MYMV CA2**	**MYMV VA1**	**MYMV VA2**	**MYMV PA1**
MYMV-CA1	100				
MYMV-CA2	80	100			
MYMV-VA1	96	82	100		
MYMV-VA2	96	82	97	100	
MYMV-PA1	94	80	96	95	100
MYMV-Vam	96	82	97	100	95
MYMV-Vig (Mad)	95	80	96	96	99
MYMV-Sb (Mad)	94	79	95	95	97
MYMV-Nam	96	81	97	97	98
MYMIV-Mg (BD)	72	58	72	72	70
MYMIV-Mg (NP)	71	56	71	71	69
MYMIV-Mg (Pak)	71	58	72	71	71
MYMIV Cp7	70	56	70	70	68
MYMIV Sb2	72	59	73	72	72
MYMIV PK-Sb	72	59	72	72	71
MYMIV PK-Vig	72	59	72	72	71
MYMIV MBKA25	73	60	73	73	72

Table 7. Percentage identity of predicted amino acid sequence of ORF AC1 of YMV isolates of present study with other selected YMV isolates.

Virus isolates	% Identity				
	MYMV CA1	**MYMV CA2**	**MYMV VA1**	**MYMV VA2**	**MYMV PA1**
MYMV CA1	100				
MYMV CA2	82	100			
MYMV VA1	89	72	100		
MYMV VA2	82	100	72	100	
MYMV PA1	89	72	100	72	100
MYMV Vam	90	73	98	73	98
MYMV-Vig (Mad)	89	72	99	72	99
MYMV-Sb(Mad)	90	72	99	72	99
MYMV Nam	88	71	96	71	96
MYMV (KH-PP-03)	89	72	96	72	96
MYMIV-Mg (BD)	77	63	84	63	84
MYMIV-Mg (NP)	79	64	86	64	86
MYMIV-Mg (Pak)	79	65	86	65	86
MYMIV Bg3	78	64	85	64	85
MYMIV Cp7	78	64	86	64	86
MYMIV Sb2	78	64	85	64	85
MYMIV PK-Sb	78	64	85	64	85
MYMIV PK-Vig	79	64	86	64	86
MYMIV MBKA25	78	64	85	64	85

Table 8. Percentage identity of predicted amino acid sequence of ORF AC2 of YMV isolates of present study with other selected YMV isolates.

Virus isolates	% Identity				
	MYMV CA1	**MYMV CA2**	**MYMV VA1**	**MYMV VA2**	**MYMV PA1**
MYMV-CA1	100				
MYMV-CA2	99	100			
MYMV-VA1	97	98	100		
MYMV-VA2	99	100	98	100	
MYMV-PA1	97	98	100	98	100
MYMV-Vig (Mad)	97	98	100	98	100
MYMV-Sb(Mad)	97	97	98	97	98
MYMV Nam	94	95	94	95	94
MYMV (KH-PP-03)	98	99	98	99	98
MYMIV-Mg (BD)	74	74	73	74	73
MYMIV-Mg (NP)	77	77	76	77	76
MYMIV-Mg (Pak)	75	75	74	75	74
MYMIV-Bg3	80	80	80	80	80
MYMIV-Cp7	83	83	84	83	84
MYMIV-Sb2	84	84	83	84	83
MYMIV PK-Sb	74	74	73	74	73
MYMIV PK-vig	73	73	73	73	73
MYMIV MBKA25	75	75	74	75	74

Table 9. Percentage identity of predicted amino acid sequence of ORF AC3 of YMV isolates of present study with other selected YMV isolates.

Virus isolates	% Identity				
	MYMV CA1	**MYMV CA2**	**MYMV VA1**	**MYMV VA2**	**MYMV PA1**
MYMV-CA1	100				
MYMV-CA2	94	100			
MYMV-VA1	66	67	100		
MYMV-VA2	94	100	67	100	
MYMV-PA1	96	90	69	90	100
MYMV-Vam	99	94	66	94	96
MYMV-Vig (Mad)	96	90	69	90	99
MYMV-Sb(Mad)	97	91	67	91	96
MYMV-Nam	92	86	60	86	90
MYMV (KH-PP-03)	99	93	66	93	96
MYMIV-Mg (BD)	75	71	51	71	74
MYMIV-Mg (NP)	77	73	53	73	75
MYMIV-Mg (Pak)	77	73	53	73	76
MYMIV Bg3	63	62	53	62	62
MYMIV Cp7	76	72	54	72	76
MYMIV Sb2	67	67	57	67	67
MYMIV PK-Sb	75	71	51	71	73
MYMIV PK-Vig	75	71	51	71	74
MYMIV MBKA25	67	66	57	66	66

Table 10. Percentage identity of predicted amino acid sequence of ORF AC4 of YMV isolates of present study with other selected YMV isolates.

Virus isolates	% Identity				
	MYMV CA1	**MYMV CA2**	**MYMV VA1**	**MYMV VA2**	**MYMV PA1**
MYMV-CA1	100				
MYMV-CA2	72	100			
MYMV-VA1	96	70	100		
MYMV-VA2	72	100	70	100	
MYMV-PA1	96	70	100	70	100
MYMV-Vam	98	74	96	74	96
MYMV-Vig (Mad)	98	72	98	72	98
MYMV-Sb(Mad)	86	71	86	71	86
MYMV-Nam	99	71	95	71	95
MYMV (KK-PP-03)	95	68	91	68	91
MYMIV-Mg (NP)	74	56	73	56	73
MYMIV-Mg (Pak)	76	58	76	58	76
MYMIV Bg3	74	56	75	56	75
MYMIV Cp7	74	56	73	56	73
MYMIV Sb2	76	58	75	58	75
MYMIV PK-Sb	75	57	74	57	74
MYMIV PK-Vig	74	56	73	56	73
MYMIV MBKA25	76	57	75	57	75

Table 11. Percentage identity of predicted amino acid sequence of ORF AC5 of YMV isolates of present study with other selected YMV isolates.

Virus isolates	% Identity				
	MYMV CA1	**MYMV CA2**	**MYMV VA1**	**MYMV VA2**	**MYMV PA1**
MYMV-CA1	100				
MYMV-CA2	100	100			
MYMV-VA1	95	95	100		
MYMV-VA2	72	72	77	100	
MYMV-PA1	95	95	93	71	100
MYMV-Vam	96	96	94	72	94
MYMV-Vig (Mad)	96	96	94	72	99
MYMV-Sb(Mad)	99	99	96	73	96
MYMV-Nam	96	96	94	72	94
MYMV (KK-PP-03)	93	93	95	72	90
MYMIV-Mg (BD)	58	58	58	57	59
MYMIV-Mg (NP)	64	64	64	52	65
MYMIV-Mg (Pak)	65	65	65	51	66
MYMIV Bg3	65	65	64	49	66
MYMIV Cp7	66	66	66	53	67
MYMIV Sb2	69	69	69	54	70
MYMIV PK-Sb	64	64	64	52	65
MYMIV PK-Vig	65	65	65	52	66

Table 12. Percentage identity of predicted amino acid sequence of ORF BV1 of YMV isolates of present study and other selected YMV isolates.

Virus isolates	% Identity							
	MYMV CB1	**MYMV CB2**	**MYMV CB3**	**MYMV VB1**	**MYMV VB2**	**MYMV VB3**	**MYMV VB4**	**MYMV PB1**
MYMV-CB1	100							
MYMV-CB2	95	100						
MYMV-CB3	98	96	100					
MYMV-VB1	98	95	99	100				
MYMV-VB2	98	96	99	100	100			
MYMV-VB3	98	96	99	100	100	100		
MYMV-VB4	92	89	93	92	93	93	100	
MYMV-PB1	99	96	100	99	100	100	93	100
MYMV [KA22]	99	96	100	99	100	100	93	100
MYMV-[KA21]	99	96	100	99	100	100	93	100
MYMV-[KA34]	98	95	99	98	99	99	92	99
MYMV [KA28]	97	95	99	98	98	98	92	98
MYMV [KA27]	75	73	75	75	75	75	71	75
MYMV Nam	76	73	76	76	76	76	71	76
MYMIV Bg3	92	90	94	93	93	93	86	93
MYMIV Sb	95	93	97	96	96	96	89	96
MYMIV AnaCp	96	93	97	96	96	96	90	97

Table 13. Percentage identity of predicted amino acid sequence of ORF BC1 of YMV isolates of present study and other selected YMV isolates.

Virus isolates	% Identity							
	MYMV CB1	**MYMV CB2**	**MYMV CB3**	**MYMV VB1**	**MYMV VB2**	**MYMV VB3**	**MYMV VB4**	**MYMV PB1**
MYMV-CB1	100							
MYMV-CB2	92	100						
MYMV-CB3	90	84	100					
MYMV-VB1	95	88	90	100				
MYMV-VB2	97	90	90	95	100			
MYMV-VB3	89	89	81	86	88	100		
MYMV-VB4	86	86	79	83	85	89	100	
MYMV-PB1	90	88	80	85	88	97	88	100
MYMV [KA22]	100	92	90	95	97	89	86	90
MYMV-[KA21]	100	91	89	94	97	89	86	90
MYMV-[KA34]	100	91	89	94	97	89	86	90
MYMV [KA28]	100	92	90	95	97	89	86	90
MYMV [KA27]	88	80	80	84	85	78	76	79
MYMV Nam	89	81	81	84	86	78	76	79
MYMIV Bg3	86	79	77	82	83	79	77	80
MYMIV Sb	95	87	85	91	92	85	82	86
MYMIV AnaCp	97	89	87	92	94	86	84	87

Table 14. Percentage identity of complete nucleotide sequence of DNA A component of YMV isolates of present study and other selected YMV isolates.

Virus Isolates	% Identity				
	MYMV CA1	**MYMV CA2**	**MYMV VA1**	**MYMV VA2**	**MYMV PA1**
MYMV-CA1	100				
MYMV-CA2	97	100			
MYMV-VA1	97	96	100		
MYMV-VA2	96	97	95	100	
MYMV-PA1	97	96	99	95	100
MYMV-Vam	98	97	98	97	98
MYMV-Vig (Mad)	97	96	99	95	99
MYMV-Sb (Mad)	98	97	98	95	98
MYMV-Nam	97	95	97	94	97
MYMV-Mg (TH)	90	89	90	88	90
MYMV-Th	97	96	97	95	97
MYMV (KH PP-03)	97	96	97	95	97
MYMIV-Mg (BD)	81	79	81	79	81
MYMIV-Mg (Np)	81	80	81	79	81
MYMIV-Mg (Pak)	82	80	81	80	81
MYMIV-Bg3	81	80	81	79	81
MYMIV-Cp7	82	80	81	80	81
MYMIV-Sb2	82	81	82	80	82
MYMIV-PK-Sb	81	80	81	79	81
MYMIV-PK-Vig	81	80	81	79	81
MYMIV-CpMBKA25	81	80	81	79	81
HgYMV-[IN Coi]	84	83	84	82	84
HgYMV-Ban	85	83	84	82	84
HgYMV-Fb	85	83	84	82	84
HgYMV-Lb	84	83	84	82	84
RhYMIV	72	71	72	70	71
RhYMV	74	73	74	72	74
VBSMV	74	73	74	72	73
KuMV-Yg3	69	68	69	67	69
KuMV-VN	69	68	69	67	69
DoYMV-Ban	62	61	62	61	62
DoYMV-ND	62	62	62	61	62
ICMV-Mah	62	61	62	61	62
SLCMV-Col	61	60	61	59	61
ToLCNDV-Svr	61	61	61	60	61
ToLCBV-Ban5	63	63	63	62	63
ToLCNDV-Svr	61	61	61	60	61
CLCuMV-Raj	61	61	61	60	61
CLCuMV-His[PK:Mul]	61	60	61	60	61
BGYMV	56	56	57	56	57
PaLCuCNV-Age	65	64	65	64	65
PaLCuCNV-Tom	65	64	65	64	65
BYVMV-Mad	61	61	62	60	62
CYVMV-Del-Cr	61	61	62	60	61
CYVMV-Bang-Cr2	62	61	62	61	62

Table 15. Percentage identity of complete nucleotide sequence of DNA B component of YMV isolates of present study and other selected YMV isolates.

Virus Isolates	% Identity							
	MYMV CB1	**MYMV CB2**	**MYMV CB3**	**MYMV VB1**	**MYMV VB2**	**MYMV VB3**	**MYMV VB4**	**MYMV PB1**
MYMV-CB1	100							
MYMV-CB2	95	100						
MYMV-CB3	95	93	100					
MYMV-VB1	96	95	94	100				
MYMV-VB2	97	95	94	99	100			
MYMV-VB3	97	96	94	99	99	100		
MYMV-VB4	97	95	94	96	96	96	100	
MYMV-PB1	98	95	95	96	97	96	97	100
MYMV [KA22]	97	94	94	96	97	96	96	97
MYMV-[KA21]	97	95	94	96	97	96	96	97
MYMV-[KA34]	98	95	94	96	97	96	96	97
MYMV [KA28]	97	95	94	96	97	96	96	97
MYMV-Vam	98	95	95	96	96	97	98	98
MYMV-Sb (Mad)	68	67	67	68	68	68	67	68
MYMV [KA27]	70	68	68	69	69	69	69	70
MYMV-Mg (TH)	65	64	63	64	65	64	64	65
MYMV-Nam	69	69	68	69	69	69	69	69
MYMIV-ND-Bg3	86	85	84	86	86	86	86	87
MYMIV-ND-Cp7	89	87	86	88	88	88	88	89
MYMIV-ND-Sb2-99	89	87	87	88	88	88	88	89
MYMIV AnaCp	92	90	90	91	92	92	91	92
MYMIV (Pak)	89	87	87	88	88	88	88	89
MYMIV-Mg (Np)	89	87	86	88	88	88	88	89
HgYMV-[IN Coi]	68	67	67	68	68	68	67	68
HgYMV-Ban	67	66	66	67	67	67	67	67
HgYMV-Fb	68	67	67	68	68	68	67	68
HgYMV-Lb	68	67	67	68	68	68	67	68
RhYMV	58	57	57	57	57	57	57	58
RhYMV	58	57	57	57	57	57	57	58
RhYMIV	51	50	50	50	51	51	50	51
KuMV-VN	53	53	53	53	53	53	53	53
KuMV-Yg3	52	52	52	53	52	53	52	53
VBSMV	51	51	51	51	51	51	51	51
BGYMV	40	39	40	40	40	40	39	40
ToLCNDV-Svr	37	37	37	37	37	37	37	38
ICMV-Mah	35	35	35	35	35	35	35	35
SLCMV-Col	35	35	35	35	35	35	35	35

Table 16. Analysis of Recombination events in DNA A and DNA B

Virus	Major parent	Minor parent	nt coordinates	RDP	GENECONV	Bootscan	Maxchi	Chimaera	Siscan	3Seq
MYMV-VA2	Unknown	CYVMV			4.347×10^{-04}		1.101×10^{-02}			
MYMV-CB1	MYMIV-ND-Bg3	MYMV-VB3	1-140	8.546×10^{-39}	4.940×10^{-74}	6.119×10^{-52}	5.570×10^{-25}	6.131×10^{-15}	3.569×10^{-34}	2.906×10^{-02}
	MYMV-KA28	MYMIV-AnaCP	274-382	1.126×10^{-03}	7.400×10^{-04}	9.054×10^{-04}	6.085×10^{-08}			
	MYMV-VB4	MYMV-Nam	1352-6	5.294×10^{-78}	6.051×10^{-126}		2.907×10^{-38}	1.173×10^{-37}	1.175×10^{-50}	1.329×10^{-249}
MYMV-CB2	MYMIV-ND-Bg3	MYMV-VB3	1-194	$8.546x10^{-39}$	4.940×10^{-74}	6.119×10^{-52}	5.570×10^{-25}	6.131×10^{-16}	3.569×10^{-34}	2.906×10^{-02}
	MYMV-VB2	MYMIV-ND-Sb2-99	193-249	-	$9.661x10^{-4}$	-	$3.622x10^{-06}$	$8.352x10^{-06}$	-	-
	MYMIV (pak)	MYMV-VB1	299-347	-	$9.534x10^{-05}$	-	$7.290x10^{-07}$	-	-	$1.669x10^{-05}$
MYMV-CB3	MYMV-Vam	MYMV-VB3	45-411	1.211×10^{-68}	4.866×10^{-100}	1.811×10^{-48}	7.386×10^{-28}	1.364×10^{-18}	1.696×10^{-38}	
MYMV-VB1	MYMV-VB3	MYMIV-AnaCP	17-227	6.527×10^{-35}	9.728×10^{-55}	2.158×10^{-57}	5.279×10^{-17}	2.573×10^{-12}	1.081×10^{-20}	
	MYMV-KA21	MYMIV-AnaCP	392-1010	6.925×10^{-111}	1.013×10^{-170}		6.107×10^{-47}	1.181×10^{-40}	2.361×10^{-39}	1.370×10^{-62}
	MYMV-KA34	MYMV-Sb(Mad)	38-113	8.935×10^{-04}	7.856×10^{-04}		1.627×10^{-06}	5.534×10^{-06}	9.074×10^{-05}	2.424×10^{-02}
	MYMV-KA21	MYMV-VB4	1995-2029	4.156×10^{-03}	4.759×10^{-12}		4.661×10^{-05}	1.602×10^{-04}		
MYMV-VB2	MYMIV-ND-Bg3	MYMV-VB3	1-140	8.546×10^{-39}	4.940×10^{-74}	6.119×10^{-52}	5.570×10^{-25}	6.131×10^{-16}	3.569×10^{-34}	2.906×10^{-02}
	MYMV-KA28	MYMIN-AnaCP	274-378	1.126×10^{-03}	7.400×10^{-04}	6.085×10^{-04}				4.241×10^{-04}
	MYMV-VB4	MYMV-Nam	1351-2638	5.294×10^{-78}	6.051×10^{-126}		2.907×10^{-38}	1.173×10^{-37}	1.175×10^{-50}	1.329×10^{-249}
MYMV-VB3	MYMIV-AnaCP	MYMV-KA28	284-382	1.126×10^{-03}	7.400×10^{-04}	9.054×10^{-04}	6.085×10^{-08}			4.241×10^{-04}
MYMV-VB4	MYMIV-ND-Bg3	MYMV-VB3	1-410	8.546×10^{-39}	4.940×10^{-74}	6.119×10^{-52}	5.570×10^{-25}	6.131×10^{-16}	3.569×10^{-34}	2.906×10^{-02}
	MYMV-CB2	MYMV-Vam	411-1854	6.638×10^{-08}	1.864×10^{-64}	1.276×10^{-91}	1.088×10^{-25}		7.448×10^{-108}	1.538×10^{-12}
	MYMV-Vam	MYMIV-AnaCP	540-588		1.159×10^{-39}		6.221×10^{-09}	6.150×10^{-09}		
	MYMV-KA28	MYMIV-AnaCP	274-382	1.126×10^{-03}	7.400×10^{-04}	9.054×10^{-04}	6.085×10^{-08}			4.241×10^{-04}
	MYMIV-Pak	MYMV-Sb(Mad)	1329-1854	5.284×10^{-34}			2.466×10^{-09}	1.257×10^{-19}		
MYMV-PB1	MYMIV-ND-Bg3	MYMV-VB3	1-410	8.546×10^{-39}	4.940×10^{-74}	6.119×10^{-52}	5.570×10^{-25}	6.131×10^{-16}	3.569×10^{-34}	2.906×10^{-02}
	MYMV-KA28	MYMIV-AnaCP	274-378	1.126×10^{-03}	7.400×10^{-04}	9.054×10^{-04}	6.085×10^{-08}			4.241×10^{-04}
	MYMV-VB4	MYMV-Nam	1350-2626	5.294×10^{-78}	6.051×10^{-126}		2.907×10^{-38}	1.173×10^{-37}	1.175×10^{-50}	1.329×10^{-249}
	MYMV-Nam	MYMV-KA21	2161-2611	1.358×10^{-08}			1.865×10^{-23}	1.395×10^{-14}	8.765×10^{-67}	

Table 17. Percentage nucleotide identity of Papaya leaf curl betasatellites from legumes with other selected betasatellites

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

Betasatellites in the present study are in bold letters.

Table 18. Percentage amino acid identity of βC1 protein of Papaya leaf curl betasatellites from legumes with βC1 protein of selected betasatellites

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

Table 19. Percentage nucleotide identity and amino acid identity of Rep gene of alphasatellites

Alphasatellites	Nucleotide identity		Rep gene*	
	BG -VBN	BG -CBE	BG -VBN	BG -CBE
BG-VBN	100	97	100	96.2
BG-CBE	97	100	96.2	100
VYVFA JF733780	98	99	98.3	97.6
CLCuA (Luc) HQ343234	55	55	50.5	50.8
OLCuMA EU589450	53	53	52.9	53.6
OLCuSA FJ868830	52	52	53.3	53.9
PaLCuA JQ322970	52	53	47.3	47.9
ToLCCNA AM749493	52	51	50.7	51.7
TYLCCNA AM749494	52	51	50.7	51.7
SiYVVA DQ641718	52	53	49.5	50.4
CLCuDaA AJ512957	52	52	52.3	52.6
CLCuMuA GQ374450	52	52	46.6	46.6
CTLCuIA GU385877	53	53	52.8	53.1
AYVIA AJ512958	51	52	48.1	48.9
AYVA AJ512959	53	53	48.6	48.9
AYVSGA AJ416153	42	41	36.8	36.8
AYVSGA FJ956707	42	41	36.8	36.8

*Amino acid alignment

Table 20. Detection of virion particles in infected seeds through ELISA

Particulars	A_{405}							
	Whole seed		Embryo axes		Cotyledon		Seed coat	
	Crude	1:10	Crude	1:10	Crude	1:10	Crude	1:10
Single	0.472	*	*	*	*	*	*	*
	0.419	*	*	*	*	*	*	*
	0.322	*	*	*	*	*	*	*
	0.343	*	*	*	*	*	*	*
	0.409	*	*	*	*	*	*	*
	0.385	*	*	*	*	*	*	*
Two	0.438	0.311	*	*	*	*	*	*
	0.441	0.334	*	*	*	*	*	*
	0.365	0.315	*	*	*	*	*	*
Three	0.421	0.301	0.240	0.166	0.353	0.276	0.393	0.345
Five	1.039	0.448	0.325	0.295	0.336	0.271	0.327	0.263
Ten	1.566	0.560	0.371	0.218	0.397	0.285	0.409	0.362
Positive	0.858	0.619						
Healthy	0.243	0.219						
Buffer	0.158							

*Not done (Reason- PCR amplicons were not detectable from those sets)

Table 21. Phenotyping of RILs against MYMV

Disease category	Coimbatore	Vamban	Panpozhi	Agroinoculation
Immune (No plants showing any symptoms)	RIL1, 2, 19, 27, 28, 39, 44, 53, 87, 110, 128,130, 136, 140, 145, 146, 148, 152, 189, 190, 191, 193, 194, 195	RIL2, 19, 21, 28, 58, 63, 72, 80, 92, 101, 102, 110, 115, 136, 137, 145,147, 148, 149, 150, 166, 171,181,184,190, 191, 195	RIL1,2,3, 19, 21, 27, 28, 35, 37,38,39,53,55,58, 59, 61, 67,72, 76, 80, 81, 82,83, 92, 112, 114, 137, 138, 141, 143, 144, 145, 147, 148, 149, 154, 159, 162, 163, 166, 167,179, 180, 181, 184, 187, 188, 190, 191, 192, 193, 194, 195	RIL 27, 28, 32, 33, 34, 35, 39, 55,59, 60, 62, 64,65,71, 72, 75, 80, 81, 82, 83, 87,88, 93, 96, 98, 100, 101, 102, 107,110, 112,113,121, 122, 128, 130, 131, 136,137,138,139, 140, 141, 142, 143, 144, 148, 152, 156, 157, 166, 167, 181, 182, 183, 184, 186, 188, 189, 190, 191, 192, 193, 194, 195
Resistant (1% or less)	RIL10, 32,35, 80,81, 82,83, 100, 101,105, 107, 112,113, 144, 149	RIL1, 35, 81, 82, 83, 100, 105, 112,113, 141,144, 152,163	RIL 4, 10, 63, 75, 139, 140	RIL1,2,4,5,6,9,10, 17, 19,21, 40, 41, 42, 44,45, 48, 49, 52,53, 114,118,120, 145, 146, 147, 171, 173, 175,
Moderately Resistant (1-10% plants exhibiting symptoms)	RIL 3,4,5,6,9,17,18, 21, 24, 30, 33, 34, 40, 41, 42, 45,46,47, 48,51,52,55, 59, 61,63,71, 72,75, 76, 88,92, 93, 96, 98, 114,117, 118, 119,120, 122, 124, 131, 137,138, 139, 141, 143, 147, 156,157, 166,167, 171, 173, 175,181, 182 183, 184, 186, 188, 192	RIL3,4, 5,6,10, 17,18,24, 27, 30,32,33,34,39, 46, 51, 52, 53, 55, 59,61,71,75, 76, 87, 88, 93, 96, 107, 114, 118, 120,131, 139,138, 146, 140, 143, 154, 156, 159, 162, 165, 167, 169,175,177, 179, 180, 182, 183, 187, 188, 193,194,	RIL 5, 6, 17, 18, 24, 30,32, 33, 34, 46, 51, 52, 71, 88, 93,96, 100, 105,107,110, 113, 118, 120, 125,129, 131, 136, 165, 169, 171, 175, 177, 182, 183, 186, 189	-
Moderately Susceptible (11-20%)	RIL 12, 115, 134,170	RIL7, 8, 89,170, 172	RIL 101, 109, 158	-
Susceptible (21-50%)	RIL7,8, 31,43,49,91, ,102,111,126,127,	RIL 47,54, 56, 67, 74, 85, 155, 168, 186, 192, 185, 189	RIL16, 25, 29, 60, 62, 77, 90, 97, 111, 115, 121, 142, 155	RIL3, 7,8,11,12,13,14, 15, 16,18,20,22, 23,24, 25, 66, 67, 68, 69, 111, 115,
Highly Susceptible (51% and more plants exhibiting symptoms)	RIL11,13,14,15,16,20,22,23,25,26,29 ,36,37,38,50,54,56,57,58,60,62,64,65 ,66,67,68,69,70,73,74,77,78,79,84,85 ,86,89,90,94,95,97,99,103,104,106,1 08,109,116,121,123,125,129,132,133 ,135,142,150,151,153,154,155,158,1 59,160,161,162,163,164,165,168,169 ,172,174,176,177,178,179,180,185,1 87	RIL 9, 11, 12, 13, 14, 15, 16, 20,22, 23, 25, 26,29, 31, 36, 37, 38, 40, 41,42,43, 44, 45, 48, 49 50,57, 60, 62,64, 65, 66, 68,69,70, 73, 77,78,79, 84, 86, 90, 91, 94,95, 97,98, 99, 103, 104, 106, 108, 109, 111, 116, 117,119, 121, 122, 123, 124, 125,126, 127, 128, 129, 130, 132,133,134, 135, 142, 151, 153, 157, 158,160, 161, 164, 173, 174, 176, 178	RIL 7,8, 9, 11, 12, 13, 14, 15, 20, 22, 23, 26, 31, 36,40, 41, 42, 43, 44, 45, 47, 48, 49, 50, 54, 56, 57, 64, 65, 66, 68, 69, 70, 73, 74, 78, 79, 84, 85, 86, 87, 89, 91, 94, 95, 98, 99, 102, 103, 104, 106, 108, 116, 117, 119, 122, 123, 124, 126, 127, 128, 130, 132, 133, 134, 135, 146, 150, 151, 152, 153, 156, 157, 160, 161, 164, 168, 170, 172, 173, 174, 176, 178, 185	RIL 26, 29, 30, 31, 36, 37,38, 43, 46, 47, 50, 51, 54, 56, 57, 58, 61, 63, 70, 73, 74, 76, 77, 78, 79, 84, 85,86, 89, 90, 91, 92, 94, 95, 97, 99, 103, 104, 105, 106, 108, 109, 116, 117, 119, 123, 124, 125, 126, 127, 129, 132, 133, 134, 135, 149, 150, 151, 153, 154, 155, 158, 159, 160, 161, 162, 163, 164, 165, 168, 169, 170, 172, 174, 176, 177, 178, 179, 180, 185, 187,

Table 22. Overview of phenotyping of RILs against MYMV

Disease category	Coimbatore	Vamban	Panpozhi	Agroinoculation
Immune (No plants showing any symptoms)	24	27	53	65
Resistant (1% or less plants exhibiting symptoms)	15	13	6	28
Moderately Resistant (1-10% plants exhibiting symptoms)	62	55	36	-
Moderately Susceptible (11-20% plants exhibiting symptoms)	4	3	3	-
Susceptible (21-50% plants exhibiting symptoms)	10	12	14	21
Highly Susceptible (51% and more plants exhibiting symptoms)	80	85	83	81
Total	**195**	**195**	**195**	**195**