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ORIGINAL ARTICLE

Comparative transmission of Bhendi yellow vein mosaic virus by two cryptic species of the whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae)

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Abstract The leaf sample from okra plants showing prominent yellow vein mosaic symptoms and healthy plant without any virus symptoms were collected from farmer's field. The presence of begomovirus in the infected sample was confirmed by polymerase chain reaction (PCR) and the amplicons were cloned and sequenced. The genome analysis showed that the isolate in the present study had 99% nucleotide identity with *Bhendi yellow vein mosaic virus* (BYVMV) revealing it as BYVMV variant. The genetic species of *Bemisia tabaci* collected from fields were identified as Asia-1 and MEAM-1 genetic species based on silver leaf assay, sequence characterized amplified region marker, and *mtCOI* gene sequence. The comparative virus–vector relationship of both genetic species of *B. tabaci* indicates a minimum of two and three *B. tabaci* in MEAM-1 and Asia-1 genetic species, respectively, per plant were required to transmit the disease. The minimum acquisition access period and inoculation access period of 15 (MEAM-1) and 20 min (Asia-1) were required to transmit the YVMD; it was further confirmed by nucleic acid hybridization using coat protein gene-specific probe of BYVMV. With respect to the sex, the female *B. tabaci* were more efficient in transmitting the disease as compared to male ones in both the genetic species of *B. tabaci*. The MEAM-1 to transmit the BYVMV more efficiently than Asia-1 genetic species of *B. tabaci.*

Keywords Bhendi yellow vein mosaic virus · *Bemisia tabaci* · Genetic species · Transmission

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Introduction

The family *Geminiviridae* comprises small circular ssDNA viruses, encapsidated within virions, having a geminate morphology and known to cause diseases in crop plants across the world. Members of the largest genus, *Begomovirus* infect primarily dicotyledonous plants and are transmitted by the whitefly *Bemisia tabaci* (Morales and Anderson 2001; Varma and Malathi 2003). The genomes of begomoviruses consist of single component or two components known as monopartite (DNA-A genome component) and bipartite (DNA-A and DNA-B genome components) viruses, respectively. The DNA-A genome component encodes pre-coat protein and CP in the sense strand (Hanley-Bowdin et al. 1999) and replication-associated protein (Rep), the replication enhancer protein (REn), the transcriptional activator protein (TrAP) in the complementary strand. The DNA-B genome component encodes for proteins required for intracellular movement

(BV1) and transport of viral ssDNA in the host plant (BC1) (Saunders et al. 2000). The monopartite begomoviruses are associated with additional ssDNA molecules known as betasatellites and/or alphasatellites (DNA1) (Lefeuvre et al. 2010). These satellites depend on their helper virus for replication, movement, encapsidation, and vector transmission. (Briddon and Stanley 2006).

Okra (*Abelmoschus esculentus* L.) is commonly known as bhendi or lady's finger, belongs to family *Malvaceae,* and is an important vegetable crop growing in India throughout the year. Yellow vein mosaic disease (YVMD), okra leaf curl disease (OLCD), and okra enation leaf curl disease (OELCD) caused by distinct mono- and bipartitie begomoviruses along with their satellites are becoming major threat for cultivation of okra in India (Venkataravanappa et al. 2011, 2012, 2013, 2014, 2015a, b). High populations of mixed genetic species of *B. tabaci* vector in the vegetable crops are present, especially during a long and hot dry season (Singh et al. 2012). Recent studies revealed that *B. tabaci* is a complex species having more than 30 morphologically indistinguishable cryptic species (De Barro et al. 2011; Boykin et al. 2013; Lee et al. 2013). Among them, the two most invasive and destructive species are the Middle East-Asia Minor I species (MEAM-1), and the Mediterranean species (MED) formerly referred to as biotype B and Q, respectively (De Barro et al. 2011; Pan et al. 2012). The *B. tabaci* MEAM-I has always been regarded most serious pest on large number of vegetables and fiber crops and are capable of transmitting the different types of begomoviruses (Polston and Anderson 1997). Transmission of begomoviruses by genetic species of *B. tabaci* in many crops has been well documented (Muniyappa and Reddy 1976; Rathi and Nene1974; Murugesan and Chellaiah 1977; Maruthi et al. 2007; Chowda-Reddy et al. 2012). The co-existence genetic species (Asia-I and MEAM-1) complexes in begomovirus-infected fields are common in southern India, especially in Karnataka (Chowda-Reddy et al. 2012). However, the MEAM-1 displaced many indigenous genetic species, because of its broader host range, higher fecundity, dispersal capacity, virus-transmission efficiency and resistance to insecticides used against *B. tabaci* (Brown et al. 1995). The annual loss of 500 million USD was estimated due to MEAM-1 transmitted begomovirus diseases in the southern states of the USA in the early 1990s on winter vegetable crops (Perring et al. 1993). In India the first the time, severe epidemics of tomato leaf curl disease were correlated with occurrence of MEAM-1 on tomato (Banks et al. 2001) and since then increased incidences of MEAM-1 transmitted begomoviruses in agricultural and horticultural crops in different locations of southern India (Narayana et al. 2006, 2007; Maruthi et al. 2007; Mahesh et al. 2010; Chowda-Reddy et al. 2012).

The relationships of genetic species of *B. tabaci* with the appearance of begomoviruses were well documented in crops like tomato, pumpkin, etc. (Maruthi et al. 2007; Chowda-Reddy et al. 2012). However, the insect–host–virus relationships of epidemiological significance are yet to known with respect to the yellow vein mosaic disease caused by *Bhendi yellow vein mosaic virus* (BYVMV). Therefore, the present study was conducted to assess AAP, IAP, number and sex of *B. tabaci,* and age of host plant required for transmission of BYVMV by genetic species (Asia-I and MEAM-1) of *B. tabaci* in okra.

Materials and method

Virus source and maintenance

The leaf sample from okra plants showing prominent yellow vein mosaic symptoms was collected from farmer field of Karnataka state, India, and the isolate was designated as OY34 (Fig. 1). Two samples from non-symptomatic plants were also collected. In order minimize chances of mixed infections, insect transmission was repeated thrice under controlled conditions and test plants were allowed for symptom expression following each transmission to okra plants. The virus culture was maintained on susceptible genotype of okra (cultivar, 1685) in an insect-proof glasshouse by frequent transmission of virus through *B. tabaci*.

Isolation of plant DNA, PCR detection, and characterization of BYVMV

DNA isolation

Total DNA was isolated from the infected leaves collected from field and plants maintained under controlled conditions by using cetyl trimethyl ammonium bromide method (Doyle and Doyle 1990). The extracted DNA was diluted

Fig. 1 Okra plants showing the yellow vein mosaic symptom under natural condition

tenfold in sterile distilled deionised water and stored at −20 °C before being subjected to PCR amplification.

PCR amplification, cloning and sequencing

The amplification of DNA-A-like sequence was performed using total nucleic acid isolated from YVMD-infected plant by PCR with begomovirus-specific primers as described by Venkataravanappa et al. (2012), except change in primer annealing temperature of 55–58 °C for 45 s. Confirmation for the presence of DNA-B-like sequence and satellites were done using degenerate primers specific to DNA-B (Rojas et al. 1993; Venkataravanappa et al. 2012), betasatellite (Briddon et al. 2002) and alphasatellites (Kumar et al. 2010). The amplified PCR products were purified and cloned into vector pTZ57R/T (Fermentas, Germany) according to manufacturer's instructions and transformed into *Escherichia coli* DH5α competent cells (Invitrogen Bioservices India Pvt Ltd, Bengaluru, India). The complete nt sequence of three clones from each sample were determined by automated DNA sequencer ABI PRISM 3730 (Applied Biosystems) at Anshul Biotechnologies DNA Sequencing facility, Hyderabad, Andhra Pradesh, India.

Sequence analysis

The sequences obtained were initially analyzed using the Vector NTI Advance TM 9 software (Invitrogen, Foster City, CA, USA) to remove vector sequences. Then the ORF Finder program (http://www.ncbi.nlm.nih.Gov/projects/gorf/) was used to identify putative genes. Sequence nucleotide identity searches were performed by comparing sequence to all available sequences in the GenBank database (Supplementary Table 1) using BlastN (Altschul et al. 1990). Sequences showing the highest nucleotide identity scores with the present isolate were aligned using the Muscle method in SDT version 1.0 (Muhire et al. 2014) and percent pairwise identity of the identified sequences and the representative sequences from the database were generated. A phylogenetic tree was generated using MEGA 6.01 software (Tamura et al. 2013) and the Neighbor-Joining method with 1000 bootstrap replications to estimate evolutionary distances between all pairs of sequences simultaneously.

Source, maintenance and identification of *B. tabaci* genetic species

The *B. tabaci* adults were collected from two different source plants, i.e. horsegram and cabbage, which are known to be source plants for MEAM-1 and Asia-I genetic species, respectively (Rekha et al. 2005; Shankarappa et al. 2007) from Kolar, Karnataka state of India. The collected *B. tabaci* from cabbage and horsegram were brought to the Indian Institute of Horticultural Research laboratory and released on healthy cotton (*Gossypium hirsutum* L. cv. DCH2) plants and eggplant (*Solanum melongena* L.), respectively. After that the eggs of *B. tabaci* cultures were collected and tagged separately into new cotton and eggplant grown in separate cages. Later, genetic species of *B. tabaci* were identified. To ensure genetic species of *B. tabaci* free from virus, PCR was carried out using begomovirus-specific primers (Venkataravanappa et al. 2012). The virus-free stocks of *B. tabaci* were reared separately on healthy cotton and eggplant in large wooden cages (45 × 45 × 30 cm), covered with 40-mesh size nylon net and kept in controlled glasshouse.

Identification of *B. tabaci* genetic species (Asia-I and MEAM-1) by biological and molecular assays

Squash silver leaf assay

Squash (*Cucurbita pepo* cv. Ambassador) seeds were sown in 4 × 6 inches polythene bags filled with compost mixture and were kept in insect-proof cage. The adult genetic species of *B. tabaci,* about 50 numbers each, were collected in plastic containers from cotton and eggplant maintained in the glasshouse and allowed separately to feed on 2-week-old squash seedlings for 48 h. Plants were maintained for 20–25 days in an insect-proof cage for expression of silver leaf symptoms.

SCAR marker analysis of two genetic species

Totally ten adult *B. tabaci* each maintained on cabbage and eggplant were collected separately from glasshouse using aspirator and transferred into a 1.5-ml micro centrifuge tube containing DNA extraction buffer. The total nucleic acid from the *B. tabaci* collected from cabbage and eggplant was isolated separately the method described by De Barro and Driver (1997). The DNA was amplified through PCR using MEAM-1 specific sequence characterized amplified region (SCAR) marker primers (forward primer—5′ ACCCGTTGAATCCTATAGAC 3′ and reverse primer—5′ TGTTATGCTTACCCGGAAC 3′) (Shankarappa et al. 2007) to differentiate MEAM-1 from Asia-I genetic species. The DNA amplification was performed with 35 cycles of denaturation for 1 min at 94 °C, primer annealing for 45 s at 55 °C and primer extension for 1 min 30 s at 72 °C, with an initial denaturation at 94 °C for 3 min, and final extension for 15 min at 72 °C. The optimized PCR was carried out in a Gene Amp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler. The total volume of 25 µL PCR mix containing

2 µL DNA template, 1.5 U *Taq* polymerase, 25 mM $MgCl_2$, 2 mM dNTPs, and 25 pmol of each primer was taken in a PCR tube. PCR products were electrophoresed (1 h at 80 V) in 0.8% agarose gels in Tris–borate-EDTA buffer, pH 8. Gels were stained with ethidium bromide (10 mg/mL) and viewed in a Gel documentation system (Alpha Innotech, USA).

PCR amplification and sequence analysis of mtCOI gene from the B. tabaci genetic species

For identification of *B. tabaci* genetic species (Asia-I and MEAM-1), the mitochondrial cytochrome oxidase I (*mtCOI*) gene sequence was used as marker in *B. tabaci* (Frohlich et al. 1999; Dinsdale et al. 2010). Fifty nanograms of total *B. tabaci* genomic DNA was amplified by PCR using *mtCOI* gene-specific forward primer (C1-J-2195-TTGATTTTTTGGTC ATCCAGAAGT) and reverse primer (L2-N-3014-TCCAATGC ACTAATCTGCCA-TATTA) (Simon et al. 1994). Negative control without DNA was included in each amplification. PCR amplification was performed as described above. The amplified products were cloned into the pTZ57R/T vector (Fermentas, Germany) and transformed into *Escherichia coli* DH5α competent cells (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Three clones from each transformation colonies were purified and automated sequencing was carried out with ABI PRISM 3730 (Applied Biosystems) automated sequencer at DNA Sequencing facility, Eurofins Genomics India Pvt. Ltd (Bangalore, Karnataka, India).

The nucleotide sequence identity of *mtCOI* gene was initially analyzed using the BLAST program available at the NCBI (National Center for Biotechnology Information, San Diego, CA, USA). Further, the *mtCOI* gene sequences of *B. tabaci* genetic species were compared with the consensus sequences generated by Dinsdale et al. (2010) to determine whether they diverged or not by more than 3.5%, the value these authors have reported to identify putative species genetic boundaries. The multiple alignments were produced using Clustal-X (Thompson et al. 1994) and phylogenetic trees were generated using the neighbor-joining method in MEGA 6.01 (Tamura et al. 2013) with 1000 bootstrap replications.

Nucleic acid hybridization and colorimetric staining

Preparation of DNA probe, nucleic acid extraction, pre-hybridization, hybridization, and detection procedures were carried out as described by Venkataravanappa et al. (2013). The most conserved CP gene of the BYVMV (Acc.number GU112034) was selected for DNA probe for detection BYVMV in *B. tabaci*.

Virus–vector–host relationship transmission assays

Number of B. tabaci required for transmission of BYVMV

To determine the number of *B. tabaci* required for successful transmission of BYVMV, newly emerged (0–24 h) non-viruliferous adults of *B. tabaci* of both (Asia-I and MEAM-1) genetic species without differentiation the sex were given an AAP of 24 h on BYVMV-infected plant (*cv.* 1685) separately. The viruliferous *B. tabaci* were then transferred to 7-day-old healthy okra seedlings at the rate of 0, 1, 2, 4, 6, 8, 10, 12, 14, and 20 per seedling separately, and ten plants were inoculated in each treatment (experiment was repeated thrice). After an IAP of 24 h, *B. tabaci* were killed by spraying imidacloprid at 0.05%, (Bayer Crop Science, Ltd., Mumbai, India), plants were kept in insect-proof cages for symptom expression and percent transmission was recorded. Further to detect the virus in genetic species of *B. tabaci*, the dot blot assay was carried out by dotting of DNA on nitrocellulose membrane (15 × 9.2 cm, 0.45 mm) in different numbers (1, 2, 4, 6, 8, 10, 12, 14, 16, and 20) in each spot. The pre-hybridization, hybridization, and detection procedures were carried out according to the protocol given in DIG High Prime DNA labeling and detection starter kit II (Roche diagnostics, Germany).

Viral acquisition assay

The effect of different AAP on the rate of transmission of BYVMV was tested for newly emerged (0–24 h) adults of both genetic species of *B. tabaci* by allowing to feed for 0, 5, 10, 15, 20, 30 min, and 1, 4, 6, 8, 12, 16, and 24 h on YVMD-infected plant separately. After the prescribed AAP, the *B. tabaci* were collected and transferred on 7-day- old healthy okra seedlings (cv. 1685) at the rate of 10 insects per plant. For each treatment, 10 okra seedlings were inoculated (experiment was repeated thrice). After 24 h of IAP, insects were killed as described above. Inoculated plants were kept in the glasshouse for symptom development. Further the virus in genetic species of *B. tabaci* was detected by dot blot assay using CP gene probe of BYVMV.

Viral transmission assay or viral inoculation assay

To determine the influence of different IAP on transmission of BYVMV, both genetic species of *B. tabaci* were allowed for 24 h of AAP on BYVMV-infected plant separately. The viruliferous *B. tabaci* were then transferred to 7-day-old okra seedlings with an IAP of 1, 5, 10, 15, 20, 30 min, and 1, 4, 8, 12, 16, and 24 h at the rate of 10 insects per seedling. In each treatment ten okra seedlings

were inoculated (experiment was repeated thrice). After different IAP in each treatment, the *B. tabaci* were killed as explained above and seedlings were kept in an insect-proof glasshouse for symptom development. Further the virus titer in biotypes was quantified by dot blot assay similar to the above.

Effect of age of host plant and its susceptibility to BYVMV

To determine the effect of age of okra seedlings and its susceptibility to BYVMV infection, ten viruliferous *B. tabaci* of both genetic species were enclosed on individual okra seedling of different ages; 7, 10, 15, 20, 25, and 30 days separately and in each case 10 plants were inoculated (experiment was repeated thrice). After 24 h IAP, insects were killed as described earlier and plants were kept in an insect-proof glasshouse for development of disease.

Efficiency of B. tabaci gender on viral transmission

An experiment was conducted to compare the efficiency of transmission of BYVMV by adult male and female *B. tabaci* as described by Simmons et al. (2009). Adult male and female *B. tabaci* were collected in separate aspirator bottles and released to feed separately on infected okra plants for 24 h as AAP as described above. A total of 10 adults of each sex were set up in this manner. Then *B. tabaci* (ten per test seedling) were released on healthy okra seedlings (experiment was repeated thrice). After 24 h of IAP, plants were sprayed with imidocloprid and maintained in insect-proof cages. Additional plants in each set up with non-viruliferous male female *B. tabaci*, and okra plants without *B. tabaci* as checks were maintained. After 30 days, test plants were evaluated for symptom expression and confirmation for the presence of BYVMV was done by PCR using begomovirus specific primers as described above.

Results

PCR amplification, genome organization and sequence analysis of begomovirus

The complete DNA A-like genome component of virus isolate (OY34) was amplified from the okra sample infected with YVMD collected from the field as well as glasshouse maintained culture using three sets of primers. These primers were designed to amplify fragments with approximately more than 200 bp overlapping region of DNA-A-like genome component, in order to avoid mixed infection. Amplification with nucleic acid extracts yielded no product from healthy control plants. Positive PCR amplification of betasatellite and alphasatellites (Data not shown) and no amplification to DNA-B-like genome component revealed that, the current isolate under study is monopartite begomovirus.

The DNA A-like genome component of the virus isolate was determined in both orientations and it was found to be 2743 nt in length and the sequence is available in the database with accession number of GU112064. The sequence had features, typical of old world monopartite begomoviruses, with two open reading frames (ORFs) [AV1 (CP), AV2] in virion-sense strand and five ORFs [AC1 (Rep), AC2, AC3, AC4, AC5] in complementary sense strand separated by an intergenic region (IR). In the IR region, the sequence identity of virus isolate was more than 90% with IRs of BYVMV, for which a full-length sequence is available in the databases. The length of IR is 298 nt and encompasses an absolutely conserved hairpin structure containing nonanucleotide sequence (TAATATTAC) that marks the origin of virion-strand DNA replication and with repeated sequences known as "iterons" (GGAGTC) adjacent to the TATA box which is recognition sequence for binding of the rep to the promoter (Arguello-Astorga and Ruiz-Medrano 2001).

The comparison of DNA-A-like genome component with the selected begomoviruses revealed that it shared highest nt sequence identity of 99% with BYVMV (AF241479) infecting okra in India. This result was well supported by phylogenetic analysis with OY34 isolate closely clustering with BYVMV group (Fig. 2). Based on the current taxonomic criteria for begomoviruses, the threshold cut-off of nt identity for species demarcation at 91% (Brown et al. 2015), the virus isolates displaying more than this should be considered as strains rather than different virus species (Padidam et al. 1995). The current result indicates that OY34 is a variant of BYVMV infecting okra in India.

Identification of genetic species of *B. tabaci*

Silver leaf assay

Differentiation of the genetic species of *B. tabaci* by visual observation is very difficult, due to their morphological similarities. To overcome these constraints, a simple and rapid biological silver leaf technique was carried out using squash (*Cucurbita pepo* cv. Ambassador) as indicator plant. The results showed that the MEAM-1 of *B. tabaci* induced clear silvering symptoms 3 weeks after inoculation, while Asia-I failed to induce silvering symptoms on the squash plants (Fig. 3a).

Identification of genetic species of B. tabaci by SCAR marker and mtCOI gene sequences

The total DNA isolated from both genetic species (Asia-I and MEAM-1) was amplified by PCR using sequence

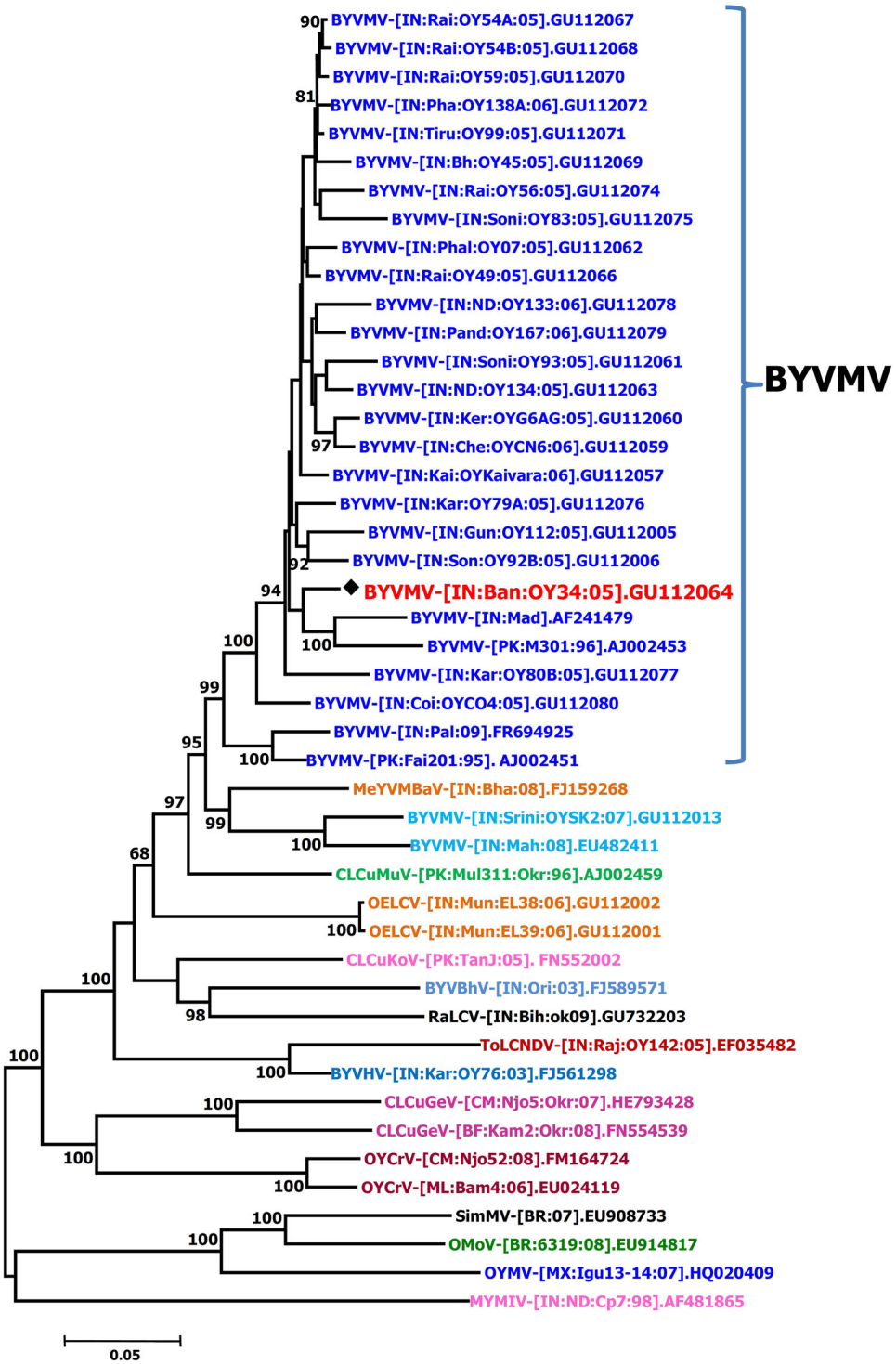

Fig. 2 Phylogenetic tree based on nucleotide sequences of the DNA-A component of the new BYVMV isolate OY34 with other begomoviruses using the Neighbor-Joining method in MEGA6.01 software. Horizontal distances are proportional to sequence distances. Vertical distances are arbitrary. The tree is unrooted. Bootstrap analysis was performed with 1000 replicates and only values >50% are shown at the branch points. Each branch has the GenBank Accession Number of the virus isolate followed by the abbreviated virus names, the country in which it was reported, the location from which it was collected, the isolate number, and the year of collection, in all parentheses. The Begomoviruses acronyms given are *bhendi yellow vein mosaic virus* (BYVMV), *bhendi yellow vein Bhubaneswar virus* (BhVBhV), *cotton leaf curl Multan virus* (CLCuMV), *cotton leaf curl Kokhran virus* (CLCuKoV), *okra enation leaf curl virus* (OELCuV), *tomato leaf curl New Delhi virus* (ToLCNDV), *radish leaf curl virus* (RaLCV), *cotton leaf curl Gezira virus* (CLCuGeV), *okra yellow crinkle virus* (OkYCrV), *okra yellow mosaic Mexico virus* (OkYMMV), *okra mottle virus* (OKMoV), *sida micrantha mosaic virus*(SimMV) and out group is *mungbean yellow mosaic India virus* (MYMIV)

characterized amplified region (SCAR) marker and mtCOI gene primers separately. The expected sequence characterized amplified region (SCAR) marker amplicon size 0.5 kb was present in PCR products obtained from MEAM-1 and absent in Asia-I (Fig. 3b). The *mtCOI* gene amplicon with expected size of 860 bp was obtained in both genetic species. There was no amplification in water sample that served as negative control. The PCR amplified products of *mtCOI* gene was cloned and three positive clones from each *B. tabaci* genetic species were sequenced. The sequences from three clones obtained from each *B. tabaci* genetic species were identical. The sequences are available in NCBI database (under accession number KX904937 (MEAM-1), KX904938–KX904941 (Asia-I), respectively).

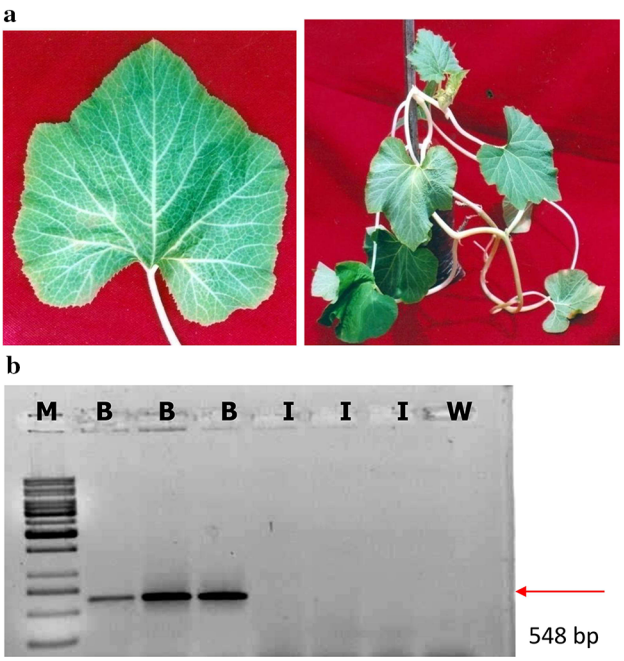

Fig. 3 **a** Silver leaf symptom induced by MEAM-1 genetic species on Squash (cv. Ambassador), **b** PCR-based detection of Asia-I and MEAM-genetic species of *Bemisia tabaci* by using SCAR marker primers, Lanes, M—1kb marker; I—Asia-I stock culture, B—MEAM-1 genetic species stock culture

The phylogentic analysis was carried out by Neighbor-Joining method using *mtCO1* gene sequences of *B. tabaci* genetic species and the sequences of *B. tabaci* genetic species used by Dinsdale et al. (2010). The analysis showed that the *B. tabaci* genetic species used in the transmission of BYVMV under study were closely clustered with MEAM-1 and Asia-I genetic species (Fig. 4).

Virus–vector–host relationships

Number of B. tabaci and transmission efficiency

The viruliferous adults of both genetic species of *B. tabaci* (Asia-I and MEAM-1) were able to transmit the BYVMV to okra with incubation period of 24 h (Table 1). Single Asia-I *B. tabaci* was not able to transmit the disease, whereas single MEAM-1 transmitted BYVMV with 10% efficiency. This indicates the effectiveness and capability of single MEAM-1 genetic species of *B. tabaci* as a potential vector. However, the transmission efficiency was increased to 50% when five Asia-I and four MEAM-1 genetic species were used. It was further increased to 100% with ten or more *B. tabaci* in both the cases. These results showed that the number of *B. tabaci* and transmission efficiency was positively correlated. The results also showed that the MEAM-1 is more efficient transmitter of BYVMV than the Asia-I. Further, dot blot assay was

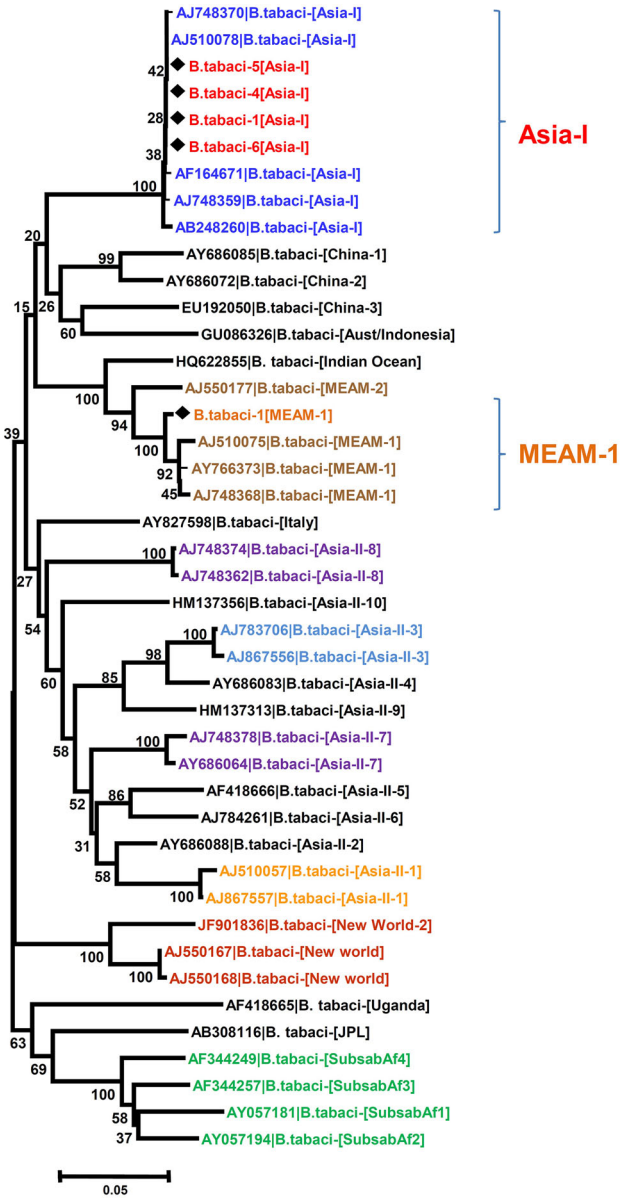

Fig. 4 Phylogenetic tree showing the relationship of the two genetic species (Asia-I and MEAM-1) of *Bemisia tabaci* sequences collected in this study to the Dinsdale et al. (2010) consensus sequences

carried out to detect the virus in two genetic species of *B. tabaci*. The results revealed that the virus was detected in single *B. tabaci* of both Asia-I and MEAM-1 genetic species. As the number of *B. tabaci* increased from 1 to 20/spot, the intensity of the color in each spot was increased (Fig. 5 Panel 1). Based on the intensity of the color on the nylon membrane, the detection level could be quantified and correlated with the titer of the virus in the insect vector.

Table 1 Determination of minimum number of Asia-1 and MEAM-1 genetic species of *B. tabaci* required for BYVMV transmission

Number of *B. tabaci*[a] per plant	Asia-1		MEAM-1	
	No. of plants infected/ inoculated[b]	Transmission (%)	No. of plants infected/ inoculated	Transmission (%)
0	0/10	0	0/10	0
1	0/10	00	1/10	10
2	3/10	30	4/10	40
4	4/10	40	5/10	50
6	6/10	60	6/10	60
8	7/10	70	8/10	80
10	8/10	80	9/10	100
12	10/10	100	10/10	100
14	10/10	100	10/10	100
20	10/10	100	10/10	100

[a]Acquisition and Inoculation access periods—24 h each

[b]Eight-day-old bhendi (cv. 1685) seedlings were used

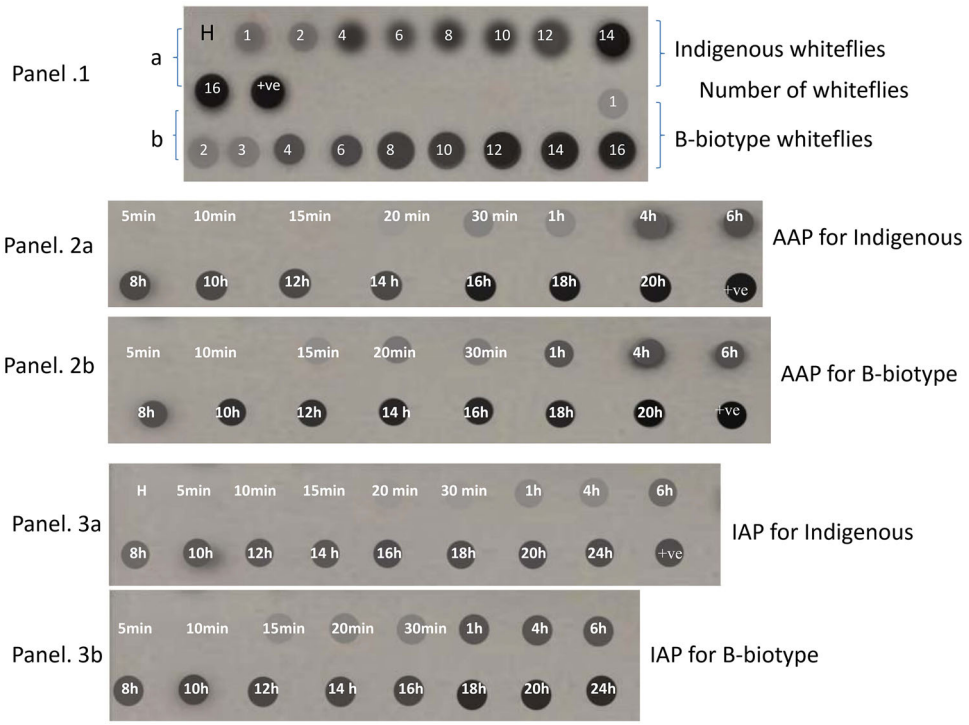

Fig. 5 Detection of BYVMV in Asia-I and MEAM-1 genetic species of *Bemisia tabaci* by dot-blot hybridization using a digoxigenin-labeled Coat Protein gene probe of DNA-A component. **1a** dots 1–16 indicate number of Asia-I genetic species of *B. tabaci*, **1b** dots 1–16 indicate number of MEAM-1 genetic species of *B. tabaci.* **2a** Acquisition access period of Asia-I genetic species of *B. tabaci* at different intervals of time (5 min to 20 h). **2b** Acquisition access period of MEAM-1 genetic species of *B. tabaci* at different intervals of time (5 min to 24 h). **3a** Inoculation access period of Asia-I genetic species of *B. tabaci* at different intervals of time (5 min to 24 h). **3b** Inoculation access period of MEAM-1 genetic species of *B. tabaci* at different time intervals (5 min to 24 h)

Acquisition access period assay

The minimum AAP of 20 and 15 min is required for adult *B. tabaci* of both Asia-I and MEAM-1 genetic species, respectively, to acquire BYVMV,;however, effective vectoring of the virus to the assay plants was with AAP of 24 h. Less than 20 and 15 min, respectively. Thirty percent or more plants became infected with BYVMV from *B. tabaci*, when AAP were increased to 24 h in both genetic species (Table 2). However, using dot blot assay the virus could be detected in both genetic species before the minimum AAP. As the time of AAP increased the intensity of

Table 2 Comparative efficiency of acquisition access period on transmission of BYVMV by Asia-1 and MEAM-1 genetic species of *B. tabaci*

Acquisition access period	Asia-1		MEAM-1	
	No. of plants infected/inoculated[a,b]	Transmission (%)	No. of plants infected/inoculated	Transmission (%)
0 min	0/10	0.0	0/10	0.0
5 min	0/10	0.0	0/10	0.0
10 min	0/10	0.0	0/10	0.0
15 min	0/10	0.0	1/10	10.0
20 min	1/10	10.0	2/10	20.0
30 min	2/10	20.0	3/10	30.0
1 h	3/10	30.0	3/10	30.0
4 h	4/10	40.0	5/10	50.0
8 h	6/10	60.0	7/10	70.0
12 h	7/10	70.0	8/10	80.0
16 h	8/10	80.0	9/10	90.0
24 h	10/10	100.0	10/10	100.0

[a]Inoculation access period of 24 h

[b]Group of 10–15 adult *B. tabaci*

Table 3 Comparative efficiency of inoculation access period on transmission of BYVMV by Asia-1 and MEAM-1 genetic species of *B. tabaci*

Inoculation access period	Asia-1		MEAM-1	
	No. of plants infected/inoculated[a,b]	Transmission (%)	No. of plants infected/inoculated	Transmission (%)
0 min	0/10	0	0/10	0
5 min	0/10	0	0/10	0
10 min	0/10	0	0/10	0
15 min	0/10	0	1/10	10
20 min	1/10	10	2/10	20
30 min	3/10	30	4/10	40
1 h	4/10	40	5/10	50
4 h	5/10	50	7/10	70
8 h	6/10	60	9/10	90
12 h	7/10	70	10/10	100
16 h	9/10	90	10/10	100
24 h	10/10	100	10/10	100

[a]Acquisition access period—24 h

[b]10–15 adult *B. tabaci*

color of in each spot was also increased (Fig. 5 Panel 2a and 2b).

Inoculation access period assay

Transmission of BYVMV was observed in okra with IAP of 20 and 15 min for Asia-I and MEAM-1 genetic species, respectively, and the incidence of viral infection increased with the increase in IAP (Table 3). Transmission reached 100% by 12 h of IAP in both the genetic species. The incubation period of virus was determined by assaying the plant tissue from *B. tabaci* inoculated okra plants by dot blot assay using CP gene probe specific to BYVMV (Fig. 5 Panel 3a and 3b). The virus was detected by PCR in okra plants from 2nd and 3rd day after inoculation in both genetic species (Data not shown). Our data indicated that the virus was unable to be detected in the day 1 because the titer of the virus may be very low in the infected plants and it may need some latent period to replicate in the plant after inoculation. But 2nd and 3rd day onwards the virus was detected in plants without any visual symptoms, even though the virus required a minimum of 8–10 days incubation period to express clear YVMD symptoms on okra plants. None of the healthy control samples gave the positive results.

Table 4 Effect of age of the seedlings on transmission of BYVMV by Asia-1 and MEAM-1 genetic species of *B. tabaci*

Age of the seedlings	Asia-1		MEAM-1	
	No. of plants infected/inoculated[a,b]	Transmission (%)	No. of plants infected/inoculated[a,b]	Transmission (%)
7 days	10/10	100	10/10	100
10 days	10/10	100	10/10	100
15 days	9/10	90	10/10	100
20 days	7/10	70	8/10	80
25 days	5/10	50	6/10	60
30 days	4/10	40	5/10	50

[a]Acquisition and inoculation access period—24 h

[b]Groups of 10–15 *B. tabaci* adults

Table 5 Comparative efficiency of sex of Asia-1 and MEAM-1 genetic species of *B. tabaci* on transmission of BYVMV

Sex of *B. tabaci*	Asia-1		MEAM-1	
	No. of plants infected/inoculated[a]	Transmission (%)	No. of plants infected/inoculated[a]	Transmission (%)
Female	25/40	63.00	34/40	85.00
Male	18/40	45.00	25/40	63.00

AAP and IAP of 24 h each

[a]10–15 *B. tabaci* per bhendi seedling

Effect of age of host plant and susceptibility to BYVMV

The susceptibility of the okra seedling to BYVMV was directly proportional to their age. One-week-old okra seedlings are highly susceptible to BYVMV infection and expressed 100% symptoms as early as 10 days after inoculation (Table 4). This indicated that as the age of the seedlings increased, their susceptibility to virus infection decreased. In the Asia-I, inoculation to 7–10 day-old seedlings were highly susceptible with the transmission efficiency of 100% and subsequently decreased as the age of plants used for inoculation increased. However, when MEAM-1 genetic species was used, the transmission efficiency was 100% even on 2-week-old okra seedling.

Efficiency of B. tabaci gender on viral transmission

Investigation into possible efficiency disparity between males and females of genetic species of *B. tabaci* revealed that the female *B. tabaci* of both Asia-I and MEAM-1 genetic species were more efficient in transmitting the disease with 63 and 85% compared to males with 45 and 63%, respectively (Table 5).

Discussion

The *B. tabaci* is the most efficient vector for members of the genus begomovirus (Idris and Brown 1998) and begomoviruses are currently emerging as a major threat for cultivation of many agricultural and horticultural crops in tropical and subtropical regions of world (Varma and Malathi 2003). Yellow vein mosaic disease of okra is caused by more than one begomovirus, is a major threat for production of okra by infecting at all growth stages of the plant resulting in losses through reduced growth and yield, distortion, and mottling of fruit which are unmarketable (Capoor and Varma 1950; Singh 1980). PCR detection, genome sequencing and phylogenetic relationship indicate that YVMD of okra is associated with variant of BYVMV infecting okra in India according to the guidelines of ICTV (Brown et al. 2015).

The different species of whitefly can be distinguished by morphological characteristics, but within *B. tabaci* there are numerous genetic species that cannot be distinguished by morphological traits. These genetic species can be differentiated using biological characteristics with respect to invasiveness, insecticide resistance profiles, pathogen vectoring, and host range (Bedford et al. 1994). In the present study, the genetic species (Asia-I and MEAM-1) of *B. tabaci* were differentiated by biological and molecular markers which are specific to the MEAM-1. Similarly, biological and molecular approaches were used for identification of genetic species of *B. tabaci* (Botstein et al. 1980; Brown et al. 1995; De Barro and Driver 1997; Cervera et al. 2000; Shankarappa et al. 2007; Dinsdale et al. 2010; Chowda-Reddy et al. 2012).

The YVMD was successfully transmitted by both Asia-I and single MEAM-1 genetic species of *B. tabaci*. The efficiency of begomoviruses transmission by genetic

species of *B. tabaci* was reported in many crops like tomato (Chowda-Reddy et al. 2012), horsegram (Muniyappa and Reddy 1976), mungbean (Chenulu et al. 1979), croton (Mandal and Muniyappa 1991), cassava (Mathew and Muniyappa 1991), cotton (Nateshan et al. 1996), and pumpkin (Maruthi et al. 2007). The titre of the virus in *B. tabaci* was also detected in squash yellow mosaic caused by *Squash leaf curl virus* (SLCV) and showed that the viral DNA in single *B. tabaci* increases with the length of the access period until saturation (Polston et al. 1990). Similarly, the virus titre was estimated in different number of insect genetic species using non-radioactive digoxigenic-labeled CP gene probe; the probe could detect the BYVMV DNA in single *B. tabaci* of both the genetic species and detection limit was increased with number of insects in each case. Similar techniques were applied to detect viral titre in plants as well as individual insects in tomato and squash crops (Czosnek et al. 1988; Polston et al. 1990).

The maximum AAP and IAP was 24 h for 100% transmission of BYVMV by both the genetic species. The MEAM-1 required less time to acquire and transmit the virus as compared to Asia-I. Similarly, MEAM-1 transmits *pumpkin yellow vein mosaic virus* and *tomato leaf curl virus* quickly and more efficiently than the Asia-I in India (Maruthi et al. 2007: Chowda-Reddy et al. 2012). This may be due to broader host range, higher fecundity, dispersal capacity, virus-transmission efficiency, and resistance to insecticides (Brown et al. 1995). Further, these transmissions were confirmed by using digoxigenic-labeled DNA probe; the probe could detect viral DNA in minimum of 20 and 15 min AAP and IAP in both Asia-I and MEAM-1 genetic species, respectively. The detection of viral DNA in both genetic species increases with the length of the AAP and IAP until saturation. The required AAP and IAP for begomoviruses transmission were explained in mechanism of many *B. tabaci* transmitted geminiviruses (Atzmon et al. 1998). Similar results were obtained with study of acquisition of the SLCV by its *B. tabaci* vector using DNA probes and antibodies (Polston et al. 1990). Although radioactive methods have been widely used for several purposes including plant viral detection (Rodriguez et al. 2003), the introduction of non-radioactive probes has been necessary due to the environmental and technological disadvantages of the radioactive probes. Several authors have reported the use of the non-radioactive probe using markers such as digoxigenine, biotin, and fotobiotin, which are able to detect viral concentrations as low as compared to the radioactive probes (Singh et al. 1994; Li et al. 1995; Romero-Durban et al. 1995; Nakahara et al. 1998).

The sexes of genetic species also play a major role in the transmission of many begomoviruses to the crops plants (Maruthi et al. 2007; Chowda-Reddy et al. 2012; Venkataravanappaa et al. 2014). In the present study we have found that the females of both the genetic species are more efficient in transmitting YVMD in okra than the males. The reason for the differing ability of male and female insects to transmit begomoviruses remains unclear. The early stage of okra seedlings are highly sensitive to YVMD infection and thereafter, the susceptibility turn to reverse showing less susceptible to the BYVMV infection (Pun and Doraiswamy 1999) demonstrated that infection decreases with increase in age of the plants.

Geminiviruses are group of plant-infecting viruses, which have more specific relationships with their insect vector than host plant (Power 2000). The relationship of virus–vector interaction in one begomovirus-one insect species might be not understood in post evolution due to the capsid structure and insect receptors which are likely to have one-to-one relationship. Evolution of begomoviruses might have been towards a better adaptation of the capsid to putative receptors of the local *B. tabaci* to ensure optimization of virus transmission (Czosnek et al. 2002). Hence a begomovirus infecting a given host tends to possess a CP with epitopes more closely related to those of other begomoviruses in the same geographical region than to a virus infecting the same host in other regions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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