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ORIGINAL ARTICLE

Transmission, characterization and occurrence of recombination in Indian strain of *squash leaf curl China virus* associated with yellow mosaic and leaf curl disease of Summer squash

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Abstract

Summer squash is one of the important vegetable crops and its production is hampered by various abiotic and biotic stresses. Of the different biotic stresses, viral infections are responsible for causing great losses to this crop. Diseases caused by begomoviruses are becoming a major constraint in the cultivation of summer squash. Samples from summer squash plants exhibiting severe yellow mosaic and leaf curl symptoms were collected from the Varanasi district of Uttar Pradesh (India) and begomovirus associated with these plants was transmitted through whiteflies (*Bemisia tabaci*) to healthy squash plants. The relationship between causal virus and whitefly vector was determined. The minimum acquisition access period (AAP) and inoculation feeding period (IFP) required by *B. tabaci* to transmit the virus was determined to be 10 min and female insects have greater efficiency in transmitting virus than male insects. The partial genome of the virus was amplified by PCR (1.2 kb), cloned and sequenced from the ten infected plant samples collected from field. Partial genome sequence analysis (1.2 kb) obtained from the ten samples revealed that they are associated with begomovirus species closely related to the Indian strain of *Squash leaf curl China virus* (SLCCNV). Therefore, one representative sample (Sq-1) was selected and complete genome of the virus was amplified by rolling circle amplification (RCA) method. Sequence analysis by Sequence Demarcation Tool (SDT) showed that the current isolate has maximum nucleotide (nt) identity of 93.7–98.4% and 89–98.1% with respect to DNA A DNA B, respectively with Indian strains of SLCCNV infecting cucurbits in India. Recombination analysis of genomes (DNA A and DNA B components) showed that a major part of genomes likely to be originated from already known begomoviruses (ToLCNDV, SLCCNV-CN and SLCCNV-IN) are infecting cucurbitaceous crops. Serological assays such as triple antibody sandwich-enzyme-linked immune-sorbent (TAS-ELISA) assay, dot blot immunobinding assay (DIBA), immuno-capture polymerase chain reaction (IC-PCR) were developed for the detection of SLCCNV.

Keywords *Squash leaf curl China virus* · *Bemisia tabaci* · Squash · PCR · Begomovirus · Recombination · Phylogenetic analysis

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Introduction

Summer squash (*Cucurbita pepo* L.), belongs to the family *Cucurbitaceae* (Whitaker 1975), which was known to be originated from North America. It is best suited for temperate and subtropical regions because it is a short-duration crop and can be grown easily. Immature fruits of summer squash have great economic value due to their use in culinary purposes (Paris 1986). The major drawback in the cultivation and production of summer squash is diseases caused by different plant viruses, which result in a significant decrease in yield. Squash lacks resistance source to many viruses belong to different families and highly susceptible to several viruses (Paris 1986). Important RNA viruses affecting squash crop are well documented in many parts of the world (Chan et al. 2019). Apart from these, the *Squash leaf curl China virus* (SLCCNV) transmitted by whitefly, *Bemisia tabaci* (Cohen et al. 1983) is becoming a major problem in squash and many other cucurbits in India and its neighboring countries. The SLCCNV has a narrow host range and is reported to infect only cucurbits for more than a decade and posing a major limitation for the cultivation of cucurbits in South East Asia (Varma and Malathi 2003). In recent years, the incidence of SLCCNV was increasing at a very fast rate in the cucurbits throughout India (Muniyappa et al. 2003; Singh et al. 2008, 2009; Riyaz et al. 2013, 2015), Pakistan (Tahir et al. 2010), China (Hong et al. 1995; Hui-jie et al. 2020), Philippines (Liao et al. 2007; Kon et al. 2003), Thailand (Ito et al. 2007) and East Timor (Maina et al. 2017). However, little is known about the virus–vector relationship and nature of the begomovirus affecting summer squash in India. Based on the coat protein gene sequence, the begomovirus infecting squash was identified as SLCCNV (Saritha et al. 2011). However, the exact strains of begomovirus infecting squash was not confirmed due to the unavailability of full-length sequence of the begomovirus, which is essential for the nomenclature of any begomovirus. Keeping this in view, samples from squash plants exhibiting different kinds of symptoms like yellow mosaic, downward curl and stunted growth along with whiteflies (*B. tabaci)* were collected from experimental plots at ICAR-Indian Institute of Vegetable Research (IIVR), Varanasi, India. Variation in symptoms was observed and was difficult to identify viruses associated with squash based on the symptoms. With this backdrop, the present study was carried out aiming to characterize of begomovirus causing the yellow mosaic and leaf curl disease on squash.

Materials and methods

Virus isolates

During summer 2017, samples from squash plants exhibiting symptoms typical to begomovirus infection were collected from experimental plots at ICAR- IIVR Research Farm, Varanasi (82.52°E longitude; 25.10°N latitude), Uttar Pradesh State, India. Two samples from non-symptomatic plants were also collected. Samples were stored at − 80 °C for further studies and some samples were used for whitefly transmission. Pure culture of the virus isolate (Sq-1 isolate) was established by whitefly inoculation and maintained continuously in the glasshouse.

Collection of whiteflies

During the survey, 10 whitefly samples were collected from squash plants in 2 ml microcentrifuge tubes having 70% ethanol for identifying the cryptic species. Each sample comprised ten whiteflies in the tube.

Establishment of whitefly culture for transmission studies

Adult whiteflies were collected from squash plants were reared on healthy eggplants maintained under controlled conditions and are allowed to lay the eggs. Laid eggs of whiteflies were collected and tagged to the newly grown eggplant in a separate cage. The eggs were allowed to hatch and a colony of whitefly was established. Genomic DNA was isolated from these whiteflies and subjected to PCR using begomovirus-specific primers (Venkataravanappa et al. 2012) to confirm that whiteflies reared under controlled conditions are free from begomovirus. Then virus-free stock culture of whiteflies was reared separately on healthy eggplant in large wooden cages (45 × 45 × 30 cm) covered with a 40-mesh size nylon net and kept in a controlled glasshouse and maintained for transmission studies (Venkataravanappa et al. 2017).

Whitefly transmission of virus

The transmission of virus by whitefly was carried as described by Venkataravanappa et al. (2017). After inoculation through whiteflies, the plants were sprayed with imidacloprid @ 0.05%, (Bayer Crop Science, Ltd., Mumbai, India) and maintained under the insect-proof net house for symptoms expression. The plants showing prominent

yellow mosaic and leaf curl symptoms were utilized for further studies.

Seed transmission of virus

The matured seeds from yellow mosaic and leaf curl disease infected and non-symptomatic squash plants were harvested. The harvested seeds were washed with 2% (v/v) sodium hypochlorite solution for 2 min and rinsed with sterile distilled water several times. Three sets of 25 seeds each from healthy and diseased plants were sown in separate earthen pots. Germination percentage was recorded and the seedling pots were kept in the glasshouse for one month for symptoms development. The seedlings were sprayed with imidacloprid (0.05%) at 10 days interval to avoid chances of insect transmission. The presence of virus in the seedlings was analyzed by PCR.

Amplification and analysis of mitochondrial cytochrome oxidase 1 (*mtCOI*) gene for *B. tabaci* cryptic species identification

To identify *B. tabaci* cryptic species transmitting the yellow mosaic disease of squash, total genomic DNA was isolated from whiteflies maintained controlled glasshouse, as well as ten whitefly samples collected from the field. The extracted DNA was subjected to the PCR amplification using *mtCOI* gene-specific primers (C1-J-2195-TTGATT TTTTGGTC ATCCAGAAGT) and (L2-N- 3014-TCCAAT GCACTAATCTG CCATATTA) (Simon et al. 1994), which are used as a marker for identification of *B. tabaci* cryptic species (Frohlich et al. 1999; Dinsdale et al. 2010). A negative control without DNA was included in each reaction. The PCR amplified products were cloned and sequenced (Venkataravanappa et al. 2017). The sequence similarity of *mtCOI* gene was checked using the BLASTn at the NCBI (National Center for Biotechnology Information, San Diego, CA, USA). Further, the *mtCOI* gene sequence analysis was carried out as described in virus genome sequence analysis given below.

Virus genome amplification and sequencing

The total genomic DNA was isolated from 100 mg often symptomatic and asymptomatic squash samples using cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). The pellet obtained was suspended in TE buffer. The quality of the genomic DNA was checked on 1% agarose gel and stored at – 20^0C till further use. Initially, the status of DNA virus in squash samples was confirmed through PCR using specific primers of DNA A component of the begomovirus (Rajos et al. 1993; Venkataravanappa et al. 2012). Sequencing of 1.2 kb partial genome from the ten virus-infected squash samples revealed that the sequences had more identity with previously identified *Squash leaf curl China virus* (SLCCNV) (data not shown). Therefore, one sample (Sq-1) was selected for complete genome (DNA A and DNA B components) amplification through RCA (Haible et al. 2006) using a TempliPhi illustra amplification kit (GE Healthcare, Piscataway, NJ). The resulting RCA product was digested with *Bam*H1 (DNA A) and *Xba*I (DNA B) to obtain linear fragments of 2.8 kb in size each, which were cloned into pUC19 plasmid with respective sites as described by Venkataravanappa et al. (2016). The ligated products were transformed and positive clones were confirmed by PCR followed by restriction analysis. The confirmed clones were sequenced in both orientations by primer walking method from Eurofins Genomic India Pvt. Ltd DNA Sequencing facility, Bangalore, Karnataka, India.

Sequence analysis

Sequence similarity searches for complete genome sequence of squash infecting begomovirus were carried out at NCBI database using BLASTn (Altschul et al. 1990). Sequences shown maximum blast score with sequences [DNA A (Table S1a) and DNA B (Table S1b)] of the present isolate were retrieved from the database. Alignment of sequences was done using Muscle method implemented in Sequence Demarcation Tool (SDT) version 1.2 (Muhire et al. 2014). The nucleotide (nt) percent pairwise identities of current isolate with representative sequences from the database were calculated. A phylogenetic tree was generated by MEGA X (Kumar et al. 2018) using the Maximum likelihood method with 1000 bootstrapped replications. The recombination breakpoint analysis was done using recombination detection program (RDP) (Martin et al. 2015) with the default setting.

Serological detection of virus

Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA)

The samples from the symptomatic (from Sq-1 isolate inoculated) and asymptomatic squash plants maintained in the glasshouse were ground separately with carbonate buffer (pH 9.6) in 1:9 (w/v) with the help of sterilized pestle and mortar. The extract of each sample was squeezed through double layer of cotton and transferred to a separate test tube. The filtrate thus obtained was treated as 1:10 dilution and serial dilutions of 1:100, 1:1000 and 1:10,000 were made with carbonate buffer and the presence of virus was detected serologically by TAS-ELISA protocol as described by Seepiban et al. (2017) using *Squash leaf curl virus* (SLCV) antibodies (DSMZ, Germany).

Dot immunobinding assay (DIBA)

Crude extract was prepared by grinding the samples collected from healthy and infected squash plants showing yellow mosaic and leaf curl disease (Sq-1 isolate) maintained in the glasshouse in a Tris buffer saline (pH 7.5) in 1:9 (w/v) ratio with the help of a sterilized pestle and mortar. Crude sap (10 µl) of extracted from both infected and healthy leaf tissues were directly spotted on the polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech, USA) without any dilution. The presence of the virus was detected using polyclonal antibody of SLCV (DSMZ, Germany) as described by Powell (1987). Further, to know the sensitivity of the test, the crude extracts of infected and healthy squash plants and partially purified virus (Palmer et al. 1998) were diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions and the presence of virus was detected in both crude extracts and partially purified virus of different dilutions of the samples as described above.

Immunocapture-PCR

PCR tubes were pre-coated with 50 µl SLCV polyclonal antibody (1:500 dilution) and kept at 4 °C for overnight and washed with distilled water before adding the plant sap. The squash leaf samples ground in 0.05 M phosphate buffer (pH 7.5) in a 1:10 w/v ratio. The leaf extract was centrifuged briefly and then 50 µl of the supernatant was transferred to the SLCV polyclonal antibody pre- and kept at 4 °C for overnight. Sap in the tubes was discarded and washed with sterile distilled water. To destabilize the antibodies bound to the virus particles, 15.5 µl of distilled water was added and subjected to freezing at − 80 °C for 10 min and thawing at 70 °C for 5 min. Further, the virus was detected by PCR using begomovirus-specific primers as described above.

Results

Disease transmission

The yellow mosaic disease symptoms were exhibited on whitefly inoculated squash plants were similar to symptoms recorded in the field such as yellow mosaic, downward curling, and stunted growth (Fig. 1a, b, c). The transmission was successful and the infection rate was 100% on eight days old squash seedlings (100/100) used for transmission studies. Initially, yellow mosaic symptoms were observed in all inoculated squash seedlings with an incubation period of 8–10 days and as the disease advances, the infected leaves showed downwards curling (Fig. 1d). Samples from these plants were used for all other experiments.

Virus–vector relationship

A minimum of two whiteflies (Asia-II-5 cryptic species) per plant was required to transmit SLCCNV (Table 1). However, when the number of whiteflies per plant was increased from 2 to 20 in the multiples of two, the transmission efficiency was increased and attained maximum transmission, when eight or more whiteflies per plant were used. Inoculated squash plants upon success, took a minimum incubation period of 8–10 days to express symptoms typical to begomovirus infection under controlled conditions. The control plants did not show any symptoms.

Viral acquisition assay

The minimum acquisition access feeding period (AAP) required for whiteflies (Asia-II-5) to acquire SLCCNV and effectively transmit the virus onto assay plants was 10 min. Prolonging the AAP from 10 min to 24 h, transmission efficiency was increased from 10 to 100%. However, 24 h of AAP was required for achieving the maximum transmission (100%) of SLCCNV on eight days old squash plants. All successful virus-inoculated plants produced disease symptoms. The plants where non-viruliferous whiteflies were used for inoculation did not show any symptoms (Table 2).

Viral transmission assay

Following 24 h AAP, 10 min of inoculation access feeding period (IAP) is sufficient to attain 10% transmission of SLCCNV by eight adult-viruliferous whiteflies to squash seedlings. Prolonged IAP from 10 min to 24 h, increased transmission efficiency was increased from 10 to 100%. Plants upon successful transmission expressed disease symptoms. The control plants did not show any symptoms (Table 2).

The efficiency of whitefly gender on viral transmission

Both males and females of *B. tabaci* were given acquisition on SLCCNV infected plants and transferred to healthy squash plants for virus transmission. This revealed that females have more transmission ability (87%) compared to males (65%). This may be attributed to the more quantity of viral inoculum that can be harbored by females due to their bigger body size (Table 3).

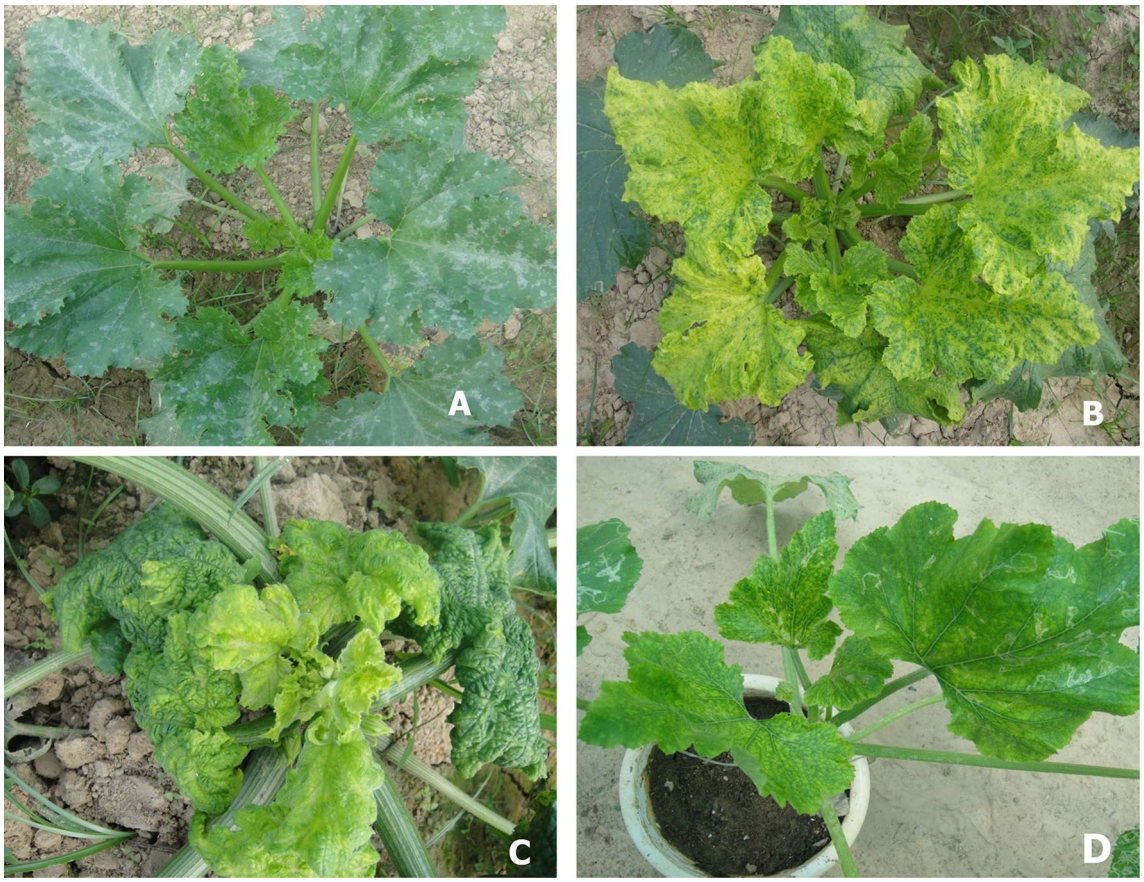

Fig. 1 Symptoms induced *Squash leaf curl China virus* on summer squash **a** Healthy, **b** yellow mosaic and **c** downward curling under natural conditions. **d** Test plant of summer squash inoculated with *Squash leaf curl China virus* isolate using whitefly (*Bemisia tabaci* Asia II-5) showing yellow mosaic symptoms

Table 1 The effect of the number of insects on transmission and incubation period of SLCCNV on Squash after a 24 h acquisition feeding period and inoculation feeding period

Number of whiteflies per plant	No. of plants infected/inoculated	Transmission[a] (%)
0	0/10	0
1	0/10	0
2	2/10	20
4	4/10	50
6	6/10	90
8	8/10	100
10	9/10	100
12	10/10	100
14	10/10	100
20	10/10	100

[a]Positives are confirmed by dot blot

Ten viruliferous flies were released on each plant with 24 h AAP and IAP each

Age of the squash seedling

Seedlings between the age of 6–10 days were found to be more susceptible to the virus. As the age of seedlings advances, the percentage of transmission was decreased indicating that the age of the seedlings also plays an important role in the infectivity of the virus (Table 4).

Seed transmission

Three sets of 25 seeds each from healthy and diseased plants of squash were collected and sown in the controlled condition. Seed germination was very poor even up to 40 days in seeds collected from diseased plants. The results revealed that the virus was not seed-borne. However, the germination percentage of the seeds collected from diseased fruits was lower (60%) compared to seeds from healthy fruits (95%).

Table 2 Determination of minimum acquisition access period and inoculation access period for insect transmission of SLCCNV between squash plants

Determination of minimum AAP[a]				Determination of minimum IAP[#]			
AAP[a]	IAP[b]	Transmission®		AAP[a]	IAP[b]	Transmission®	
		Plants infected/ plants inoculated	Percentage of plants infected			Plants infected/ plants inoculated	Percentage of plants infected
0 min	12 h	0/10	0	12 h	0 min	0/10	0
5 min		0/10	0		5 min	0/10	0
10 min		1/10	10		10 min	1/10	10
15 min		2/10	20		15 min	2/10	20
20 min		3/10	30		20 min	3/10	30
30 min		4/10	40		30 min	4/10	40
1 h		5/10	50		1 h	5/10	50
4 h		6/10	60		4 h	6/10	60
8 h		7/10	70		8 h	7/10	70
12 h		8/10	80		12 h	8/10	80
16 h		9/10	90		16 h	9/10	90
24 h		10/10	100		24 h	10/10	100

Ten viruliferous flies were released on each plant with 24 h AAP and IAP each

[a]Acquisition access period

[b]Inoculation access period

®Positives are confirmed by dot blot

Table 3 Comparative efficiency of sex of an *B. tabaci* on transmission of SLCCNV

Sex of *B. tabaci*	No. of plants infected / inoculated	% Transmission[a]
Female	35/40	85.00
Male	26/40	65.00

Ten viruliferous flies were released on each plant with 24 h AAP and IAP each

[a]Positives are confirmed by dot blot

Table 4 Effect of age of the seedlings on transmission of SLCCNV by *B. tabaci*

Age of the seedlings[a]	No. of plants infected/ inoculated	Transmission® (%)
7 days	10/10	100
10 days	10/10	100
15 days	5/10	50
20 days	4/10	40
25 days	2/10	20
30 days	1/10	10

Ten viruliferous flies were released on each plant with 24 h AAP and IAP each

[a]Days after germination

®Positives are confirmed by dot blot

Identification of cryptic species of *B. tabaci* by *mtCOI* gene sequence

The PCR for amplification of *mtCOI* gene using genomic DNA from whiteflies maintained in the controlled conditions as well as whiteflies collected from the infected squash plants resulted in PCR amplification of 860 bp in size (Data not shown). No amplification was observed in the negative control where water is used as the template. Amplified *mtCOI* gene products were cloned and sequenced. The sequences from three clones obtained from each sample were identical. Therefore, one sequence was deposited at NCBI database under accession number (MT424000). The phylogenetic analysis was carried out using *mtCO1* gene of *B. tabaci* in the present study with sequences of *B. tabaci* cryptic species used by Dinsdale et al. (2010). The analysis clearly showed that the cryptic species of *B. tabaci* used in the transmission of SLCCNV under study was closely related to Asia-II-5 cryptic species (Fig. 2).

Virus genome amplification and sequencing

The total genomic DNA isolated from the infected and healthy squash plants was subjected to the PCR using primers specific to begomovirus. All the ten infected samples showed positive amplification and resulted in the expected amplicon of 1.2 kb and no amplification was observed with a healthy sample (data not shown). Sequence analysis of 1.2

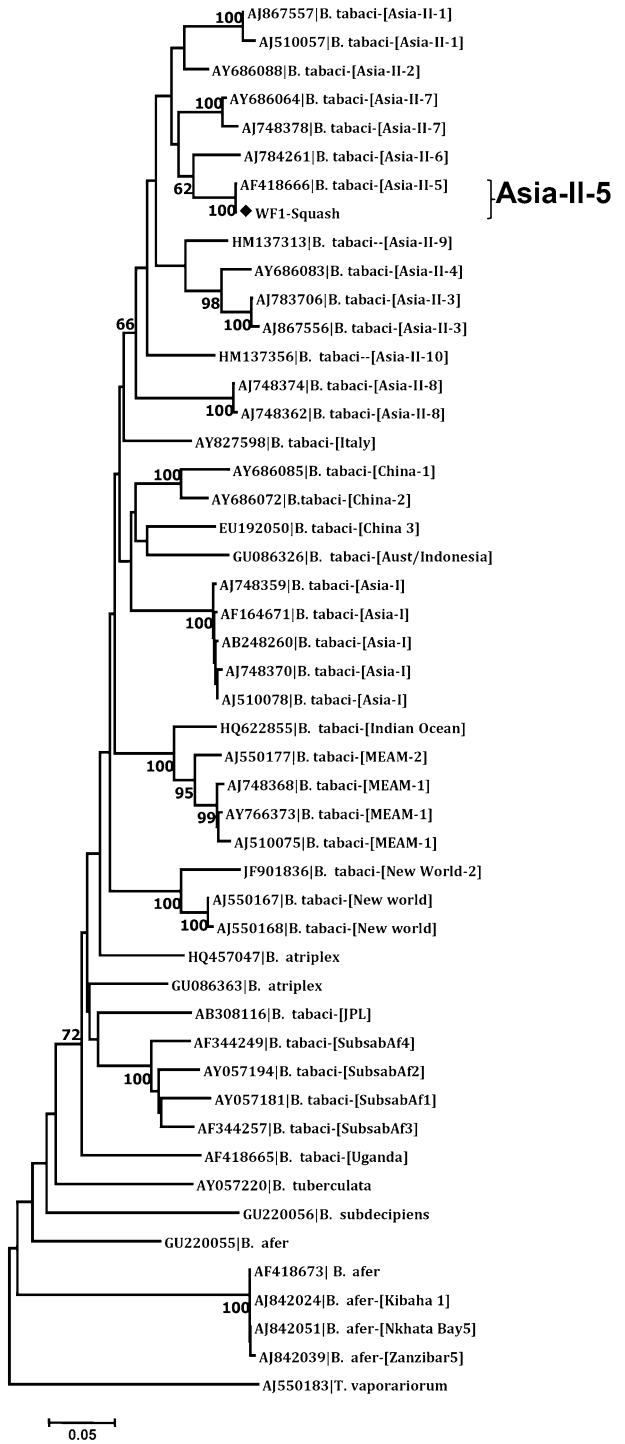

Fig. 2 Phylogenetic tree showing the relationship of cryptic species of *B. tabaci* (Asia-II-5) sequence collected in this study was compared to the sequences of cryptic species of *B. tabaci* as described by Dinsdale et al. (2010) and Kanakala and Ghanim (2019) consensus sequences

kb partial genome showed that all the 10 squash samples are infected SLCCNV (> 98% nt identity among themselves). Therefore, one representative isolate, Sq-1 was selected for the amplification of complete genome of the begomovirus by RCA method.

Genome organization of DNA A and DNA B component of begomovirus

The complete genome (DNA A) of the begomovirus (Sq-1) was determined to be 2738 nt in length (accession number MH836313). The analysis of the DNA A sequence of Sq-1 revealed that it codes for seven open reading frames (ORFs) with an arrangement similar to the DNA A component of the Old World (OW) bipartite begomoviruses. Of these, two ORFs are present in virion-sense strand [encoding the coat protein (281-1051/256) and AV2 (121-459/112, of unknown function)] and five in the complementary-sense strand [encoding the replication-associated protein (Rep, 1500-2585/361), the transcriptional activator protein (TrAP,1193-1597/134), the replication enhancer protein (REn, 1048-1458/136), AC4 (2252-2428/58) and AC5 (331-796/161)]. Further, the complete nt sequence of the DNA B component was determined to be 2716 nt (accession number MH836314) and encode two ORFs; one nuclear shuttle protein (BV1, 480-1286/268) in the virion-sense and the movement protein (BC1, 1340-2185/281) in the complementary-sense.

To compare DNA A component of the begomovirus isolate (Sq-1) with the publically available begomoviruses, the sequence identity searches were performed using the BLASTn algorithm. Sequence comparison and SDT analysis showed that the Sq-1 isolate is closely related to the isolates of SLCCNV reported previously with the sequence identities ranged from 93.7 to 98.4% with Indian strains of SLCCNV (EU573715, AM286794, AM286794, DQ026296, AY184487,KF188433 and JN587811), 90.9–92.1% with China strains of SLCCNV (KC857509, AB330078, EU543562, AF509743, AF509741, KF999983, HM566112, AM260205, KF184993, KF184992, KC171648, AM260206, AB027465), 86.8–87.1% with *Squash leaf curl Philippines virus* (AB085793, EF199774, DQ866135) and 84.7% with *Pumpkin yellow mosaic Malaysia virus* (EF197941) (Fig. 3b). Based on the begomovirus species demarcation threshold (91% nt sequence identity; Adams et al. 2017) and criteria for distinguishing strains of the begomoviruses (Adams et al. 2017), the isolate in the present study is an 'India' strain of SLCCNV. This result was well supported in the phylogenetic analysis by the grouping of Sq-1 isolate with isolates of the 'India' strain of SLCCNV and distinct from isolates of the 'China' strain (Fig. 3a).

Similarly, the DNA B component of Sq-1 was compared with publically available begomoviruses sequences using the SDT tool. The results revealed that SLCCNV shared maximum nt identity (89–98.1%) with Indian strains of SLCCNV (GU967382, FJ859881, AM778959, JN624306,

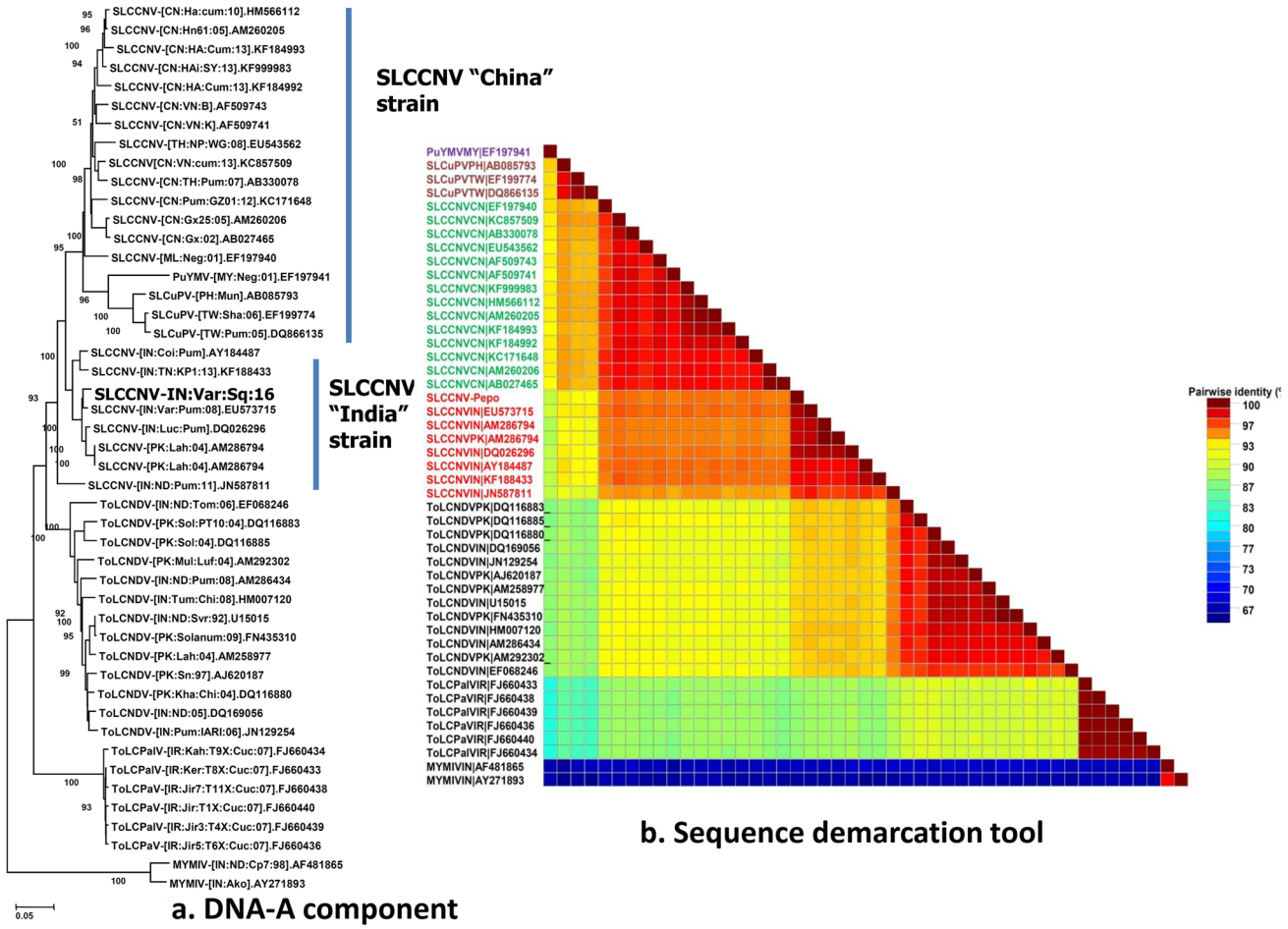

Fig. 3 Dendrograms were constructed using the Maximum Likelihood method. **a** DNA A sequence of begomovirus (Sq-1, Acc. No.MH836313) associated with yellow mosaic and leaf curl disease of Squash in India. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown below the branches. The two-dimensional color-coded matrix of pairwise identity scores of the DNA A of squash isolate **b** under study was obtained using SDT. The accessions and their details used for this study are listed in Supplementary Table 1

AY184488, KJ004521), 85.1–86.1% with China strains of SLCCNV(AF509742, KC857510, HM566113, AM260207, KF999984, AM260208, KC171649) and 83.4–83.8% with *Squash leaf curl Philippines virus* (AB085794, EU479711, JF746196) (Fig. 4b). Phylogenetic tree generated using DNA B component of the current virus isolate (Sq-1) and the selected other begomoviruses sequences from NCBI database showed that Sq-1 isolate grouped with the other SLCCNV isolates closely related to 'India' strain isolates (Fig. 4a).

Recombination analysis

The recombination analysis was carried out using RDP4, based on the alignment of SLCCNV-Sq1 isolate along with the other selected begomoviruses indicated that SLCCNV-Sq1 isolate has both intra- and inter-specific recombination in both DNA A and DNA B components. Recombination fragment of 1935 nt was detected in DNA A component of SLCCNV-Sq1 isolate with major and minor parents resembling *Tomato leaf curl New Delhi virus* (ToLCNDV) (AJ620187) and SLCCNV-CN (AB330078), respectively. The breakpoints were determined at 956 and 2891 nt with an average probability value of 6.101×10^{-22}. Similarly, a recombination fragment of 2303 nt was detected in the DNA B component of Sq-1 with major and minor parents resembling isolates of SLCCNV-IN (AM778959, GU967382). The breakpoints were predicted at 68 and 2371 nt with an average probability value of 5.464×10^{-9}. Another recombination fragment of 317 nt was detected in the DNA B component of Sq-1 with the major and minor parent resembling isolates of SLCCNV-IN (GU967382, AM778959) and the breakpoints were predicted at nt 2372 and 2689 with an average probability value of 4.121×10^{-20}.

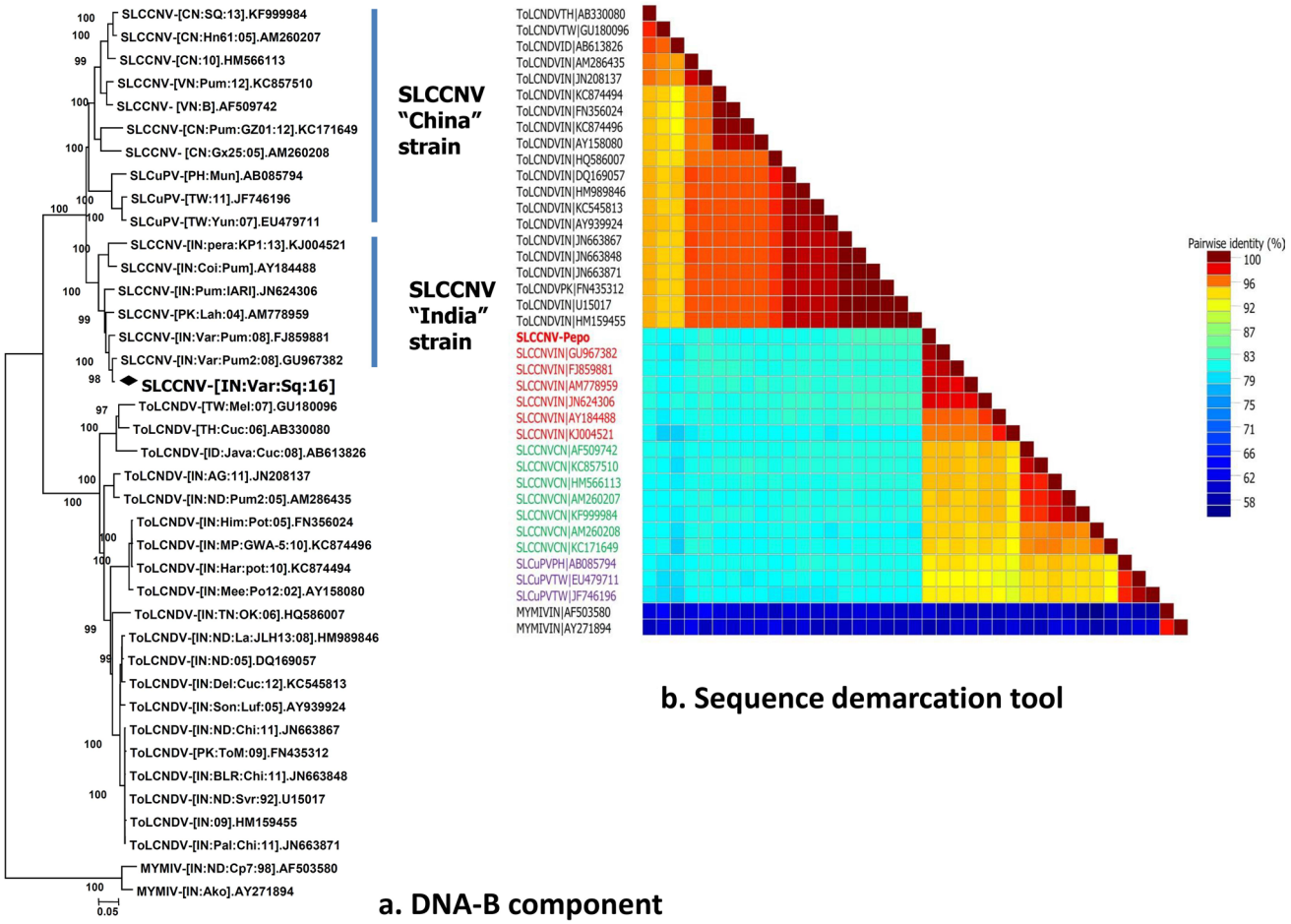

Fig. 4 Dendrograms were constructed using the Maximum Likelihood method. **a** DNA B sequence of begomovirus (Sq-1, Acc. No.MH836314) associated with yellow mosaic and leaf curl disease of Squash in India. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown below the branches. The two-dimensional color-coded matrix of pairwise identity scores of the DNA B of squash isolate **b** under study was obtained using SDT. The accessions and their details used for this study are listed in Supplementary Table 1

Serodiagnostic for detection SLCCNV

TAS-ELISA was used to detect the virus in squash samples using the polyclonal antisera of SLCV (DSMZ, Germany). The virus was detected in the dilutions 1:10, 1:100, 1:1000 and 1:10,000 of the crude samples from symptomatic squash plants at A_{405} nm with OD values of 1.845, 1.42, 1.25 and 1.23, respectively, as compared with the control (0.018) and buffer (0.016).

Dot immunobinding assay (DIBA)

The crude extract and partially purified virus from the squash plants showing yellow mosaic and leaf curl symptoms were strongly reacted with polyclonal antisera of SLCV without any dilution (Fig. 5a). Further, the presence of a virus was detected in the dilution up to 10^{-3} and 10^{-4} in the crude extract and partially purified virus, respectively (Fig. 5b). There was no reaction in healthy control samples.

Immuno-capture PCR (IC-PCR)

The PCR amplification of begomovirus genome segment using SLCV specific polyclonal antibody pre-coated PCR tubes from crude extract of virus-infected squash plant sample resulted in the expected amplicon of 1.0 kb size for the primers used to detect begomoviruses (Venkataravanappa et al. 2012). No such amplification was observed from the extract of healthy plants (Fig. 6).

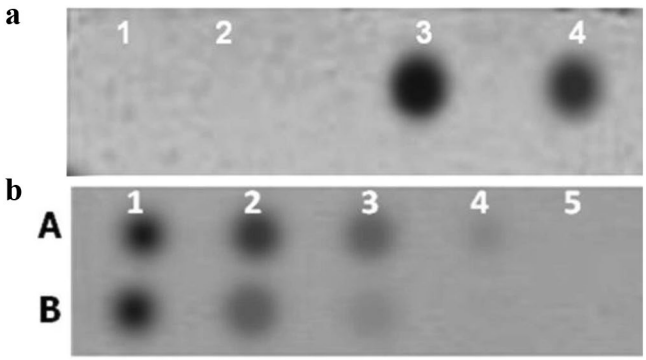

Fig. 5 a Dot Immuno binding assay developed for detection of squash leaf curl china virus using the polyclonal antibody of SLCV, Lane (1) Water sample, (2) Healthy sample, (3) Partially purified virus, (4) Infected sample. **b** Dot immunobinding assay for detection of *Squash leaf curl China virus* with the different dilution of virus and infected crude sap, (A1) Partial purified virus, (A1) 10^{-1}, (A2) 10^{-2}, (A3) 10^{-3}, (A4) 10^{-4}, (A5) Healthy sample; (B1) infected squash, (B2) 10^{-1}, (B3) 10^{-2}, (B4) 10^{-3}, (B5) 10^{-4}, (B5) Healthy sample

Discussion

The viruses belong to the family *Geminiviridae* have more specific relationships with their insect vector than with host plants (Power 2000). More than 300 plant viruses belonging to different groups viz., begomoviruses, crinivi-ruses, carlaviruses, torradoviruses and ipomoviruses trans-mitted by whitefly were reported across the globe (Jones 2003; Polston et al. 2014). The factors responsible for the emergence of whitefly-transmitted viral diseases include mutation and recombination in the virus, changes in the vector biotype complex and long-distance traffic of plant material or vector insects due to global trade. The role of human beings and climate change may also one of the major factors for the emergence of novel viral diseases in many crops (Navas-Castillo et al. 2011).

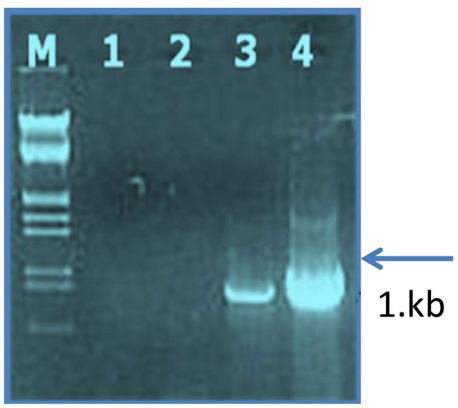

Fig. 6 Immunocapture PCR for detection of *Squash leaf curl China virus* infected with Squash, Lane (1) Water sample, (2) Healthy sample, (3, 4) infected samples

The begomoviruses are transmitted by whitefly in a circulative and non-propagative manner under natural conditions (Gray and Banerjee 1999). The whitefly transmission characteristic of SLCCNV was similar to that of other begomoviruses reported so far. The present study revealed that the minimum AAP required for whiteflies for transmission of SLCCNV was 10 min and transmission efficiency increased with increased AAP. A similar kind of trend was well documented in the previous reports (Patil et al. 2017; Venkataravanappa et al. 2017; Fiallo-Olive et al. 2020). Transmission of 100 percent was obtained following an initial AAP of 24 h, indicating the latent period has been satisfied in 24 h or less, which was also a characteristic of begomoviruses vector transmission (Brown and Nelson 1987; Retuerma et al. 1971). Generally, female whiteflies have more transmission efficiency of the begomoviruses than males (Muniyappa et al. 2000; Czosnek et al. 2001). The reason for the differing ability transmission of begomoviruses by male and female insects remains unclear. However, the difference in the size of males and females might be the reason, which probably determines the amount of viral inoculum taken them. In the present investigation, the virus was not transmitted by seed. Similar results were noticed in other begomoviruses (Ghanem 2003; Singh et al. 1994; Polston et al. 1993).

The squash samples showing symptoms of mosaic and leaf curl were confirmed with the presence of begomovirus infection, which was closely related to the Indian strain of SLCCNV. This species occurs as two geographically distinct strains which include 'China' and 'India' strains. The 'India' strain so far reported only in the Indian subcontinent, while the 'China' strain was recorded from both China and Vietnam (Revill et al. 2003). This indicates the strains are evolved independently and might be due to geographic isolation and environmental conditions. The Himalayan mountain range with the extreme climate will affect the free movement of the virus and its insect

vector, between India and China. The greater diversity of SLCCNV has occurred in southern China. This shows the virus might have originated there and was subsequently introduced to India/Pakistan by trade in agricultural products across the mountain range (Tahir et al. 2010).

Recombination and mutation allow a quick evolution of viruses, resulting in promoting changes in their virulence and host range (Garcia-Arenal et al. 2001). The role of recombination in the emergence of novel viruses has been well documented worldwide (Lima et al. 2013; Rocha et al. 2013; Silva et al. 2014). A major molecular variation in begomoviruses is recombination events occurring between DNA A components of different viruses leading to new strains/viruses with more virulence. The effect of recombination might be positive in some viruses' example, recombination between different *Maize streak virus* isolates resulted in increased pathogenicity in maize, indicating greater adaptation through recombinant (Van der Walt et al. 2009). The recombination may also have a negative effect on some viruses, for example, the recombination between *Tomato yellow leaf curl virus* and *Tomato yellow leaf curl Sardinia virus* lowered the replicative capacity of the viruses (Davino et al. 2009). The present study revealed the presence of both intra- and inter-specific of recombination in DNA A and DNA B of SLCCNV with ToLCNDV, SLCCNV-CN, and SLCCNV-IN, which might be the reason for the evolution of the new recombinant virus severely infecting squash and other cucurbits in India.

For many years, ELISA is one of the important methods for detecting plant viruses due to its reliability, sensitivity and less cost requirement. The diseased squash sample showing yellow mosaic and leaf curl samples were showed a positive reaction to the antisera raised against SLCV begomovirus antibody. The advantage of this technique (ELISA) is to quantify virus titer in the test sample and can be extended to the field application through the development of immunostrips. Further, the detection technique, DIBA developed is a simple, efficient, low-cost method that can be successfully employed for SLCCNV detection in infected squash plants. The advantage of DIBA is that the membranes can be stored for a longer time and have field-level applicability as well as in epidemiological studies (Powell 1987).

Data presented here clearly showed that SLCCNV infection was more prevalent, wherever the summer squash is grown in India. Insect transmission studies, genome analysis and serological tests clearly showed that the squash plants showing typical begomovirus yellowing and leaf curl were infected with the Indian strain of SLCCNV. Further recombination analysis of the complete genome of the virus showed that the virus under study is recombinant and has a potential threat to the cultivation of many cucurbits in this region.

Conclusion

Our results revealed that SLCCNV infection is caused by a begomovirus and is prevalent in the summer squash crops in India. The begomovirus strain is transmitted by whitefly. Serological assays have also successfully developed for the detection of virus in field samples. Further, recombination breakpoint analysis of the complete genome of the virus revealed that a major part of its genomes is likely to be originated from already known begomoviruses (ToLCNDV, SLCCNV-CN and SLCCNV-IN) which are reported to infect cucurbitaceous crops in India. This recombinant SCCCNV-Indian strain might be emerged as a potential threat to the cultivation of many cucurbits in India and needs immediate attention.

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Author contributions We declare that all authors participated in the research and article preparation. VV design of the experiments and drafted the article. CNLR designed the study and revision of the manuscript, KSS participated in analysis of the data, HDV and MN participated in the collection of the data. MKR guided and designed the study and revised the article. All authors approved the submitted version of the article.

Declarations

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

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