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ORIGINAL ARTICLE

Emergence of croton yellow vein mosaic virus in turnip (*Brassica rapa* subsp. *rapa*) indicated new host adaptation by a weed-infecting begomovirus

Alok Kumar[1,3] · Manas Kumar Bag[2,4] · Ranbir Singh[2] · Bikash Mandal[1] · Anirban Roy[1]

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Abstract
Turnip (*Brassica rapa* sub sp. *rapa*) germplasm accessions exhibiting severe leaf curl and stunting symptoms under natural field conditions were evaluated for the presence of begomovirus by PCR using a set of primers specific to begomoviruses. Two germplasm accessions, IC732033 and EC732034 were positive. Furthermore, detailed characterization of the begomovirus from the leaf curl-affected turnip plant (IC732033) revealed an association of croton yellow vein mosaic virus (CYVMV) and croton yellow vein mosaic betasatellite (CroYVMB). Agroinoculation of CYVMV construct alone or in combination with CroYVMB and back inoculation of the progeny virus from agroinoculated plants through whiteflies to healthy plants produced typical disease symptoms. Thus this study provides etiological evidence for the cause of leaf curl disease in turnip plants through the establishment of Koch's postulates, similar to our earlier findings of CYVMV infection in rapeseed-mustard and crambe. Therefore, the present and past studies have shown that CYVMV, a weed-infecting begomovirus, outreaches Brassica crops, which were hitherto not known to be affected by a begomovirus.

Keywords *Croton yellow vein mosaic virus* · *Croton yellow vein mosaic betasatellite* · *Brassica rapa* subsp. *rapa* · Turnip · Agroinoculation

Introduction

The genus *Begomovirus* under the family *Geminiviridae* comprises the largest number of plant virus species, which cause diseases in economically important crops globally (Malathi et al. 2017; Varma and Malathi 2003; Rojas et al. 2005; Seal et al. 2006). The genome of these begomoviruses contains circular single-stranded DNA, encapsidated in twinned quasi-isometric particles of 18-22 nm diameters and is exclusively transmitted by whiteflies *Bemisia tabaci* (Seal et al. 2006). The genome of the majority of begomoviruses from the New World (NW) and some from the Old World (OW) have two separately encapsidated genomic components (bipartite), designated as DNA-A and DNA-B, each of which is 2.7-3.0 kb in size. On the other hand, most of the OW begomoviruses have a monopartite genome, which lack the DNA-B component. DNA-A has six partially overlapping open reading frames (ORFs) that encode six proteins. The coat protein (CP, AV1) and pre-coat protein (AV2) have been expressed from the viral sense strand whereas, the replication initiator protein (Rep, AC1), replication enhancer protein (REn, AC3), transcriptional activator protein (TrAP, AC2) and AC4 protein has been expressed from the complementary-sense strand. The DNA-B genome contains two genes, which encode nuclear shuttle protein (NSP, BV1) and movement protein (MP, BC1) through the viral-sense and complementary-sense strand, respectively (Hanley-Bowdoin et al. 1999). The systemic spread and symptom expression due to the infection of bipartite begomoviruses essentially

✉ Alok Kumar
alok.igib@gmail.com

✉ Anirban Roy
anirbanroy75@yahoo.com

[1] Advanced Centre for Plant Virology, Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, India

[2] Germplasm Evaluation Division, ICAR-National Bureau of Plant Genetic Resources, New Delhi, India

[3] Present Address: College of Agriculture and Environmental Sciences, Haramaya University, Dire Dawa, Ethiopia

[4] Present Address: ICAR - National Rice Research Institute, Cuttack, Odisha, India

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require both the components (Stanley 1983) whereas, in the case of some of the monopartite begomoviruses, DNA-A alone can cause systemic infection and symptom expression (Dry et al. 1993; Kheyr-Pour et al. 1991; Navot et al. 1991). However, the majority of monopartite begomoviruses require the association of a satellite DNA called betasatellite for the induction of severe disease symptoms (Briddon et al. 2001; Jose and Usha 2003; Saunders et al. 2004; Cui et al. 2004; Briddon and Stanley 2006; Kon et al. 2009). Betasatellites are small, circular, single-stranded DNA of about 1.3 kb in size and share negligible sequence similarity with DNA-A and DNA-B (Saunders et al. 2000; Briddon et al. 2003; Zhou et al. 2003) except, a highly conserved nonanucleotide sequence (TAATATTAC), which forms a loop in a stem-loop secondary structure in the intergenic region of the viral genome. This conserved sequence plays a vital role in the initiation of replication in all begomoviruses. The replication, encapsidation, and transmission of satellite molecules depend on the associated begomovirus. It also contains a single ORF, βC1, that determines the host range of associated begomoviruses (Saunders et al. 2000; Jose and Usha 2003; Saunders et al. 2002) and also acts as a suppressor of gene silencing (Cui et al. 2005).

Turnip (*Brassica rapa* subsp. *rapa*) is an herbaceous annual or biennial plant from the family *Brassicaceae,* grown for its edible roots and leaves. Turnip greens are an excellent source of vitamins and minerals and provide special nutrient support for the body's detox system, antioxidant system, and inflammatory/anti-inflammatory system which are closely connected with cancer development. Among all types of cancer, prevention of bladder cancer, breast cancer, colon cancer, lung cancer, prostate cancer, and ovarian cancer are most closely associated with the intake of turnip greens (Ambrosone and Tang 2009, Clarke et al. 2008, Higdon et al. 2007, Hu et al. 2007, Kelemen et al. 2006, Konsue and Ioannides 2010, Larsson et al. 2008, Lin et al. 2009, Moore et al. 2007, Silberstein and Parsons 2010, Tang et al., 2008, Thompson et al. 2010, Zhang 2010).Because of its rich content of crude protein, digestible dry matter, and fibers, the entire turnip plant is also used as high-quality forage.

The productivity of the turnip is mainly affected by the diseases caused by the fungi. Although the crop has also been reported to be infected by other pathogens such as bacteria, viruses, and insects, so far, only three RNA viruses, turnip mosaic virus, turnip crinkle virus, and turnip yellows virus have been reported to infect turnip, however, no begomovirus has been reported to infect this crop worldwide. During a routine evaluation for the presence of diseases in brassica germplasm, a leaf curl disease with severe stunting was observed in some plants of two germplasm accessions of turnip grown in the experimental farm of the National Bureau of Plant Genetic Resources (NBPGR) located at Issapur village (28°34'32"N and 76°51'52"E), Delhi, India. The presence of a significant amount of whitefly populations in the turnip germplasm suggested the association of a begomovirus with such hitherto not known disease of turnip germplasm. For the last few years a similar leaf curl disease caused by begomovirus-betasatellite complex, croton yellow vein mosaic virus (CYVMV) and croton yellow vein mosaic betasatellite (CroYVMB), has been observed in the germplasm of rapeseed-mustard and crambe crops, grown in the nearby experimental fields (Roy et al. 2013; Kumar et al. 2018).

The CYVMV belongs to the species *Begomovirus crotoflavi* (genus: *Begomovirus*, family:*Geminiviridae*), and is one of the most prevalent begomoviruses in the Indian subcontinent. The occurrence of this virus in India was first reported in 1963 (Varma 1963) from its original host *Croton bonplandianus,* where it causes bright yellow vein mosaic symptoms on the infected leaves (Pramesh et al. 2013). The CYVMV was found to be transmitted by whitefly, *Bemisia tabaci*, and is gradually expanding its host range to other weeds and economically important plant species such as, Acalypha, Crotalaria, Cyamopsis, Jatropha, okra, papaya, radish, rapeseed-mustard, tomato and so on (Khan et al. 2015; Pramesh et al. 2013; Roy et al. 2013; Snehi et al. 2011), whereas under the experimental conditions, the virus was reported to infects of approximately 35 plant species from 11 families (Pramesh et al. 2013). Currently, the complete genome sequence of CYVMV and CroYVMB are available for several isolates in the Genbank database that have been reported from different plant species including the economically important plants from India and Pakistan (Snehi et al. 2011; Zaffalon et al. 2012; Singh et al. 2012; Singh-Pant et al. 2012; Venkataravanappa et al. 2011; Roy et al. 2013), indicating rapid spread and adaptation of this begomovirus complex in different plant species in Indian subcontinent. To understand the etiology of the leaf curl disease of turnip germplasm in this study, for the first time, we are reporting the natural infection of a begomovirus and its cognate betasatellite, CYVMV, and CroYVMB, producing leaf curl and stunting symptoms in germplasm accessions of turnip, and established the causal relationship of the begomovirus-betasatellite complex with the disease through agroinoculation of infectious constructs followed by whitefly transmission of progeny virus and betasatellite to turnip plants.

Materials and methods

Germplasm evaluation for diseases

A total of 15 germplasm accessions of turnip, stored in the National Gene Bank of the National Bureau of Plant Genetic Resources (NBPGR), Delhi, India, were grown

in an augmented block design for the purpose of routine evaluation and seed multiplication during the cropping season with two rows for each accession at the experimental farm of NBPGR located at Issapur village (28°34'32"N and 76°51'52"E), Delhi, India. Upon noticing leaf curling and stunting symptoms in a few plants, a total of 60 symptomatic and asymptomatic plants (4 plants/accession) from all of the 15 accessions grown in the field were collected, and tested for the association of begomovirus with the disease. All methods of sample collection and other experimentation were performed by the relevant guidelines and regulations.

DNA extraction and PCR amplification

Total genomic DNA was extracted from all of the collected symptomatic and asymptomatic leaf samples using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). To ascertain if the CYVMV and CroYVMB, which were reported earlier to be associated with the leaf curl disease of rapeseed-mustard and crambe grown in the nearby fields, were infecting the turnip germplasm, they were tested through PCR with the specific primers, BM90F/BM82R (Pramesh et al. 2013) for CYVMV and BM534F/BM535R (Jailani et al. 2016) for CroYVMB. PCR was carried out in a 25 µl reaction volume containing 100 ng of total plant DNA, 1x PCR buffer, 2 mM dNTPs, 0.1 µg of each primer and 1 unit of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania) in a Thermal Cycler (BIOER Genepro, Shanghai, China) with an initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 52–55°C for 30 s, and 72°C for 60 s and a final extension at 72°C for 10 min. To test the presence of any DNA-B molecule, the samples were tested with universal DNA-B specific primer pairs (Rojas et al. 1993). The amplified fragments were resolved in 0.8% agarose gel by electrophoresis and visualized in a UV gel documentation system (Bio-Rad, Hercules, CA, USA). A plasmid DNA construct containing the genome of CYVMV-mustard isolate and the DNA extracted from glasshouse-grown healthy turnip plants were used as positive and negative controls, respectively, in the PCR reactions.

Amplification of the complete genome of the virus and betasatellite

The complete genome of the CYVMV was amplified using the total DNA extracted from one of the PCR-positive plants of the germplasm accession IC732033 by rolling circle amplification (RCA) using phi-29 DNA polymerase (Fermentas) following the standard protocol (Singh et al. 2011). Briefly, 50 ng of total DNA was mixed with 1x phi-29 enzyme buffer, 10 µM exo-resistant random primer, 2 mM dNTP mix, and denatured for 5 min at 95°C. After cooling on ice for 2 min, 0.02 units of pyrophosphatase, and 5 units of phi-29 DNA polymerase were added and incubated for 18 h at 30°C, followed by enzyme denaturation at 65°C for 10 min. To amplify the betasatellite a universal primer pairs β01 (5' GGTACCACTACGCTACGCAGCAGCC 3') / β02 (5' GGTACCTACCCTCCCAGGGGTACAC 3') were used and PCR was carried out with standard procedure reported earlier (Roy et al. 2013).

Cloning of the viral genome and betasatellite

The obtained RCA product of the viral genome was digested with the restriction enzyme *Bam*HI and the linearized 2.7 kb viral genome was ligated with the *Bam*HI-linearized pUC18 vector. Similarly, a 1.3 kb amplicon of betasatellite was ligated with pJET1.2 positive selection vector (Thermo Scientific). Ligated products were used to transform the competent *Escherichia coli* cells (DH5α) and recombinant clones were initially identified using colony PCR followed by restriction digestion with the restriction enzymes, *Bam*HI and *Sca*I for begomovirus and with *Kpn*I for betasatellite.

Sequencing and sequence analysis

Two clones, each for the begomovirus and the betasatellite were completely sequenced from both directions using the commercial facility of Chromous Biotech, India. As the sequence of two clones from either the virus or the betasatellite was identical within their respective category, only sequences from a single clone each for the virus and betasatellite were submitted to the NCBI database. The sequences were assembled and open reading frames were predicted using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The sequences were analyzed by BLASTn (http://www.ncbi.nlm.nih.gov/blast) and the viral sequences showing high similarity in BLASTn analyses were retrieved from the NCBI database for comparison study. The sequence identity matrix was generated using the BioEdit sequence alignment editor (Hall 1999) and the phylogenetic and molecular evolutionary analyses were conducted by MEGA version 11 (Tamura et al. 2021) using the maximum parsimony method with 1000 bootstrap values.

Construction of partial/complete dimeric agro-constructs

A partial tandem repeat (PTR) construct for the begomovirus and a dimeric construct for betasatellite were developed in the binary vector pCambia2300 following the strategies used in previous studies (Jailani et al. 2016; Kumar et al. 2018). The entire schematic representation of the PTR construct development is depicted in Supplementary Fig. 1. Briefly, for the construction of PTR of the begomovirus, based on the sequence information

generated for the isolate, an *Xba*I-*Bam*HI digested fragment (1.0 kb, 0.4 mer) from the full-length begomovirus clone in pUC18 was used to ligate with *Xba*I-*Bam*HI digested pCambia2300 vector to generate a 0.4 mer partial construct (pCTur-A-0.4). The full-length *Bam*HI-*Bam*HI fragment (2.7 kb, 1.0-mer) of the begomovirus obtained from the pUC18 clone was then ligated with *Bam*HI-linearized pCTur-A-0.4 clone, resulting in the formation of a PTR construct pCTur-A-1.4. For creating the dimeric CroYVMB construct, initially, the betasatellite was amplified from the pJET1.2 clone using a mutated forward primer, β-01* (Jailani et al. 2016; Kumar et al. 2018) and a universal reverse primer, β-02 (Briddon et al. 2002) in a PCR based strategy. The amplified full-length betasatellite with a single base pair mutation at the 5 terminal nucleotide of the *Kpn*I restriction site was first cloned into the pGEMT easy vector (Promega) and subsequently transferred in pCambia2300 as *Sac*I and *Kpn*I fragment to generate pCTur-β-1.0. Further, a full-length betasatellite, released as a *Kpn*I fragment from a pJET1.2 clone, was inserted into *Kpn*I-linearized pCTur-β-1.0 to generate the dimeric construct pCTur-β-2.0. The tandem orientation in both the constructs was verified by restriction digestion using the enzyme *Xba*I, which was expected to release a 2.7 kb and 1.3 kb fragment after digesting the pCTur-A-1.4 and pCTur-β-2.0 clones, respectively. Both the constructs (pCTur-A-1.4 and pCTur-β-2.0) and the vector pC2300 were mobilized into the *Agrobacterium* strain EHA105 by transformation.

Infectivity assay of the agro-constructs

The agro-mobilized constructs (pCTur-A-1.4 and pCTur-β-2.0) were grown separately for 48 hours on Luria Bertani Agar and harvested in 500 µl of B5 medium (Gamborg et al. 1968). Both the agromobilized constructs alone and in combination were prick-inoculated to young seedlings (3-4 leaf stage) of turnip plants. Turnip plants inoculated with only vector pC2300 served as a negative control. All the agroinoculated plants were maintained in an insect-free glasshouse for 45 days and observations were recorded periodically. The infectivity of the constructs was confirmed by their ability to induce typical symptoms in agroinoculated turnip plants. Further, total genomic DNA from the leaves of each agroinoculated plant was extracted at 30 days post inoculation (dpi) using the DNA isolation kit (Gene-aid, Taiwan). The presence of viral/satellite DNA in the agroinoculated plants was confirmed by PCR using the CYVMV-specific primers, BM90F/BM82R (Pramesh et al. 2013) and CroYVMB-specific primers, BM534F/BM535R (Jailani et al. 2016). These experiments were repeated twice to confirm their reproducibility.

Whitefly transmission

The adult aviruliferous whiteflies (*B. tabaci*), which were maintained on different solanaceous hosts in a whitefly rearing chamber were allowed to feed on symptomatic agroinoculated turnip plants for an acquisition access period of 24 h. These viruliferous whiteflies (8-10 whiteflies per plant) were then allowed to feed on healthy seedlings of turnip for an inoculation access period of 12 h. Further, all the inoculated plants were sprayed with 0.01% imidacloprid (Confidor) to kill the viruliferous whiteflies used for transmission. After whitefly transmission plants were kept in insect free cages until symptom development. Symptoms were recorded periodically and transmission of virus by whiteflies was confirmed by PCR amplification using CYVMV- and CroYVMB-specific primers.

Results

Plants from two germplasm accessions of turnip exhibit leaf curl symptoms

The routine germplasm health assessment of the 15 germplasm accessions of turnip plants grown in the NBPGR field located at Issapur village, revealed the presence of leaf curl disease in eight plants, 4 each from two germplasm accessions, IC732033 and EC732034. While IC732033 is an indigenous collection from India, the EC732034 is an exotic collection. The typical symptoms include upward curling of the leaf with rolling of the leaf margin, vein thickening, and stunted growth (Fig. 1a). The plants of other germplasm accessions grown in the same field did not exhibit any such symptoms.

Initial detection through PCR indicated presence of CYVMV and CroYVMB with the symptomatic samples

The PCR was performed with CYVMV-specific primers for 60 plant samples belonging to 15 germplasm accessions, collected from the field. Only the eight symptomatic plants, belonging to the germplasm accession nos. IC732033 and EC732034 gave a desired 750 bp amplicon similar to that observed with the positive control (Fig. 1b). However, among those eight plants which were tested positive for the CYVMV, only two plants from accession no., IC732033, and one plant from accession no., EC732034 showed the presence of CroYVMB (Fig. 1c). None of the other asymptomatic plants from other 13 accessions of turnip plants, as well as the glasshouse grown healthy turnip plants, showed any positive amplification either with CYVMV or CroYVMB primers (data not shown). Also, none of the samples

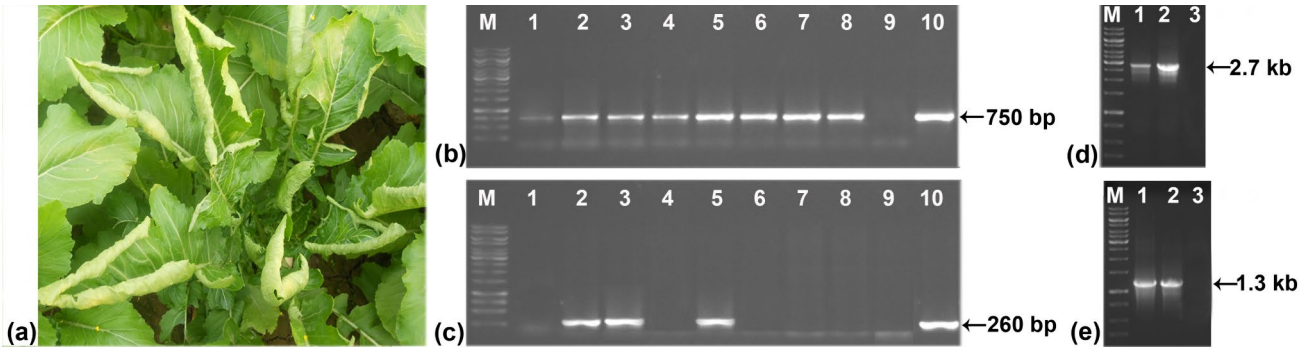

Fig. 1 Field detection and complete genome amplification of begomovirus and associated betasatellite in the turnip germplasm. (a) A plant of *Brassica rapa* sub sp. *rapa* showing leaf curl symptoms in the field. Amplification of begomovirus (b) and betasatellite (c) from the field samples using CYVMV-specific primers, using total DNA extracted from individual symptomatic plant samples from accession nos. IC732033 (Lane 1-4), and EC732034 (Lane 5-8), and glasshouse grown healthy plant served as -ve control (Lane 9). Plasmid DNA of a clone of CYVMV and CroYVMB served as positive control(Lane 10). (d) *Bam*HI restricted RCA product of the DNA isolated from leaf showing leafcurl symptom, Lane 1: sample from the accession no. IC732033, Lane 2: sample from the accession no. EC732034, Lane 3: Healthy, (e) PCR amplification of full length betasatellite from the DNA isolated from the symptomatic field samples, Lane 1: sample from the accession no. IC732033, Lane 2: sample from the accession no. EC732034, Lane 3: Healthy sample

(symptomatic/asymptomatic) gave any amplification with the universal DNA-B primers (Rojas et al. 1993) even after repeated attempts (data not shown), indicating the association of only monopartite begomovirus (CYVMV) with the symptomatic samples. The presence of cognate betasatellite (CroYVMB) with only a few symptomatic samples indicated that association of such betasatellite is not required for symptom development. Further, the presence of full-length CYVMV and CroYVMB in both the accessions of turnip was confirmed by RCA (Fig. 1d) and PCR (Fig. 1e), respectively, of which only the amplification product from accession no. IC732033 were cloned and sequenced.

Sequence comparison and phylogenetic relationships

The sequences of two clones, each from the CYVMV and the CroYVMB, shared >99 % similarity within their respective group, and thus only one sequence each from CYVMV and the CroYVMB was further used for analysis. The assembled complete genome sequence of CYVMV and CroYVMB isolated from turnip were determined as 2749 and 1345 nucleotides (nt), respectively. Both the sequences were submitted to the NCBI database under the accession nos., KF888655 and KM229763 with an acronym of CYVMV-Del-Turnip and CroYVMB-Del-Turnip, respectively. Initial BLASTn analysis showed the present isolates of CYVMV and CroYVMB shared high similarity with other isolates of CYVMV and CroYVMB, respectively, reported worldwide. The CYVMV-Del-Turnip sequences showed a typical genome organization of a begomovirus with six ORFs encoding AV2 (146-502 nt, pre-coat protein) and AV1 (306-1076 nt, coat protein) in the viral sense, and AC3 (1079-1483 nt, replication enhancer protein), AC2 (1224-1628 nt, transcriptional activator protein), AC1 (1531-2616 nt, replication-associated protein) and AC4 (2202-2459 nt, C4 protein) in antisense orientation. The intergenic regions (IR) span both sides of the origin of replication and were found to be located at 1-145 nt and 2617-2749 nt region, respectively. Similarly, the CroYVMB-Del-Turnip sequence contains a single ORF encoding βC1 protein (220-576 nt) together with a satellite conserved, and an A-rich region, two common features to all the beta satellites. The conserved nonanucleotide sequence (TAATATTAC), which is necessary for stem-loop formation and serves as the origin of replication during the replication of the begomoviruses, was found in the IR region of both CYVMV-Del-Turnip and CroYVMB-Del-Turnip sequences.

A pairwise sequence comparison of the isolate revealed CYVMV-Del-Turnip shared 88.0-99.7% identity with twenty-four other CYVMV isolates reported from different hosts, whereas it shared 80.0-93.3% identity with other begomoviruses, showing similarity in BLASTn analyses (Table 1). It shared maximum (99.7%) and minimum (88%) percent nucleotide sequence identity with the CYVMV isolates reported earlier from Crambe (CYVMV-Del-Crambe, KJ747958) and *Alcea rosea* (FN678906), respectively (Table 1). The ORF-wise sequence comparison of CYVMV-Del-Turnip with other begomovirus isolates showed maximum identity with different CYVMV isolates both at nucleotide (nt) and amino acid (aa) levels. ORF AV2 shared 94.9-100% nt and 93.2-100% aa sequence identity. Similarly, AV1 shared 83-99.7% nt and 72.5-99.6% aa, AC3 shared 66.3-100% nt and 53.3-100% aa, AC2 shared 70.8-100% nt and 59.2-100% aa, AC1 shared 73.6-99.8% nt and 68.9-99.4% aa, and AC4 shared 66-100% nt and 57.1-100% aa sequence identity with all other CYVMV isolates (Table 1).

Table 1 Percent sequence identity of CYVMV-Del-Turnip isolate (KF888655) with other croton yellow vein mosaic virus (CYVMV) and related begomovirus isolates infecting a number of crops worldwide

KJ747958	CYVMV	Crambe	Delhi, India	**99.7**	100	100	99.4	99.2	100	100	100	100	99.8	99.4	100	100
JX270684	CYVMV	Rapeseed-Mustard	Delhi, India	94.9	100	100	99.7	99.6	97.7	96.2	97	95.5	96	93.9	96.8	92.9
AJ507777	CYVMV	Croton *bonplandianum*	Delhi, India	94.3	99.4	98.3	99.7	99.6	97.7	96.2	96.7	95.5	95.5	93	94.9	90.5
JN817516	CYVMV	*C. bonplandianum*	Delhi, India	98.7	99.4	98.3	99.7	99.6	99.5	99.2	99.2	98.5	99.1	98.6	99.2	98.8
JN817517	CYVMV	*C. bonplandianum*	West Bengal, India	92.7	100	100	84.5	**72.5**	99.5	99.2	96.2	94	96.1	93.9	98.4	96.4
JN831446	CYVMV	*C. bonplandianum*	Bangalore, India	91.9	100	100	99.7	99.6	98.2	97	97.5	96.2	92.5	89.1	**66**	**57.1**
LN886647	CYVMV	*Croton sparsiflorus*	Panjab, Pakistan	97.9	100	100	99.7	99.6	99.2	98.5	98	96.2	98.1	97.2	99.6	98.8
LN871569	CYVMV	*C. sparsiflorus*	Panjab, Pakistan	97.6	100	100	99.7	99.6	99	98.5	98.7	97.7	98.3	97.2	99.2	97.6
FN645915	CYVMV	*Cyamopis tetragonoloba*	Haryana, India	94.1	95.7	94	97.5	97.3	94	89.5	92.7	89.5	86.2	84.4	96.8	92.9
FN645901	CYVMV	Acalypha	Haryana, India	95.7	99.4	99.1	99.7	99.6	99.5	99.2	97.2	95.5	96.4	94.4	97.2	94.1
FN645926	CYVMV	Acalypha	Punjab, India	95.4	100	100	99.7	99.6	99.5	99.2	97.2	95.5	96.4	94.4	96.8	92.9
FN645898	CYVMV	Acalypha	Haryana, India	94.6	100	100	99.7	99.6	99	98.5	98	96.2	75.5	72.2	95.2	89.4
FN645902	CYVMV	Acalypha	Haryana, India	97.8	100	100	99.7	99.6	98.2	97	96.5	94.7	98.5	97.5	99.6	98.8
EU727086	CYVMV	Jatropha	Lucknow, India	91.5	99.4	98.3	97.9	96.8	88.5	85.8	91.5	86.5	95.6	93	98.4	96.4
JN663850	CYVMV	Chilli	Bhubneshwar, India	94.8	100	100	99.3	99.2	96.2	94	96.5	94.7	96.4	94.4	96.8	92.9
FN678906	CYVMV	*Alcea rosea*	Lahore, Pakistan	**88**	**94.9**	**93.2**	89.5	83.5	94.2	89.5	89.8	85.8	96.2	93.9	94.9	89.4
HG937524	CYVMV	Cotton	Jaranwala, Pakistan	94.1	98.3	97.4	98.9	98	98.5	97.7	93.5	90.2	96.3	93.9	96.8	92.9
LN878119	CYVMV	Tomato	Lahore, Pakistan	94.1	96.8	94.9	**83**	81.6	99.5	99.2	98.2	97	95.7	93.3	95.2	89.4
LN878120	CYVMV	Tomato	Lahore, Pakistan	94.1	96.8	94.9	98.6	97.6	99.5	99.2	98.2	97	95.7	93.3	95.2	89.4
LN886525	CYVMV	Tomato	Lahore, Pakistan	93.8	96.8	94.9	98.3	96.8	95.8	93.3	96.7	94.7	95.7	93.3	95.2	89.4
MH359168	CYVMV	Wild Sunflower	India	93.2	98.8	97.4	99.6	99.6	**66.3**	**53.3**	**70.8**	**59.2**	90	87.1	94.9	90.5
MN885537	CYVMV	Brinjal	Bangladesh	94.8	100	100	99.7	99.6	95	91.7	93.2	91	96.6	94.1	96.8	92.9
MH765696	CYVMV	Papapya	Ranchi, India	90.1	96.3	93.2	90.7	83.5	91.5	85.8	88.5	82	**73.6**	**68.9**	89.8	78.8
KY612431	CYVMV	*Sidastrum micranthum*	Ballia, India	91.4	99.4	98.3	97.9	96.8	88.5	85.8	91.5	86.5	95.6	93	98.4	96.4
KF307208	PaLCuV	Papaya	Indore, India	93.3	92.9	91.5	99.6	99.2	95.2	91.7	96	93.2	98.3	96.9	95.2	89.4
FN543112	CYVV	Croton	Punjab, Pakistan	90.9	100	100	89.9	83.9	94	88.8	89.8	85.8	98.7	97.5	98	95.2
DQ629102	ToLCNDV	Tomato	Delhi, India	87.9	93.5	89.8	88.9	82.4	95.5	92.5	97	94.7	93.4	88.6	74.2	58.1
KC713784	ToLCuV	Tomato	Coimbatore, India	83.9	92.9	90.6	98.8	98	95.7	92.5	91.5	88	84.1	75.9	48.7	29.8
FJ589571	BYVBV	Okra	Bhubneshwar, India	83.8	97.5	97.5	95	92.1	94	92.5	84.4	82	84	76.7	56.6	36
KF551579	ToLCKV	Tomato	Coimbatore, India	83.9	94.6	92.3	98.3	97.2	93.7	89.5	87.5	83.5	84.8	76.7	48.7	29.8
HM007097	PeLCBV	Chilli	Ghazipur, India	85	93.2	88.1	88.9	82	83.3	75.3	84.5	77.6	96.7	94.1	97.6	94.1
FM877858	ChLCIV	Chilli	North India	83.2	89.5	84.2	88.9	82	92	86.5	84.1	79.2	91.7	88.3	94.5	89.4
HM007106	ChLCKV	Chilli	Kanpur, India	82.9	92.9	88.9	89.3	82.8	81.8	72.3	84.8	79.1	95.5	92.7	95.6	89.4
JX436472	AgEV	Tomato	Pantnagar, India	80.3	94.6	91.5	89.1	82.8	95	89.5	87.5	83.5	85	76.7	66.2	50.5
EU194914	RadLCV	Tobacco	Pusa, Bihar, India	80	83.8	81.3	89.4	83.2	94.2	88.8	87.8	82.8	84.2	75.9	57.6	39

A comparison of the associated cognate betasatellite (CroYVMB-Del-Turnip) with twenty-six different CroYVMB and eleven related betasatellite sequences showed 76.2-93% identity with the CroYVMB isolates (Table 2). It has shared maximum percent nucleotide sequence identity with the isolate reported from rapeseed-mustard (JX270685) whereas <62% identity was observed with other non-cognate betasatellites except with papaya leaf curl betasatellite (PaLCuB; JN663874) that showed 92.9% identity with the present isolate. A similar trend was observed in the βC1 gene and IR region of the genome. The βC1 gene shared 88.5-98.5% nt and 66.9-99.1% aa sequence identity with

Table 2 Percent sequence identity of CroYVMB-Del-Turnip isolate (KM229763) with other croton yellow vein mosaic betasatellites and related betasatellites infecting a number of crops worldwide

Accession No.	Virus[a]	Host	Location	DNA-β	IR	βC1	
						nt	aa
KM229762	CroYVMB	Crambe	Delhi, India	92.7	91.2	96.9	94.9
JX270685	CroYVMB	Rapeseed-Mustard	Delhi, India	**93**	**91.5**	97.4	95.7
JN831448	CroYVMB	*Croton bonplandianum*	Delhi, India	84.4	81.7	91.5	87.2
JN831447	CroYVMB	*C. bonplandianum*	Banglore, India	84.5	79.4	91.3	86.4
JQ354987	CroYVMB	*C. bonplandianum*	Banglore, India	83.6	79.2	**88.5**	81.3
EF597245	CroYVMB	*C. bonplandianum*	Barrackpore, India	82.3	77.3	88.7	80.5
AM410551	CroYVMB	*C. bonplandianum*	Punjab, Pakistan	85.9	81.3	91.5	86.4
LT600709	CroYVMB	*C. sparsiflorus*	Faisalabad, Pakistan	86.3	82.4	90.4	86.4
LT600710	CroYVMB	*C. sparsiflorus*	Faisalabad, Pakistan	86.4	82.4	90.4	86.4
EU557375	CroYVMB	Sunn hemp	Barrackpore, India	87.5	85	89.6	79.6
GQ183865	CroYVMB	Sunn hemp	Barrackpore, India	87.4	84.9	89.6	79.6
HM143903	CroYVMB	Papaya	Panipat, India	92.8	90.8	**98.5**	**99.1**
HM143908	CroYVMB	Papaya	Panipat, India	91.1	88.4	**98.5**	**99.1**
JN663878	CroYVMB	Chilli	Coimbatore, India	85.3	83	91	85.5
JN663857	CroYVMB	Chilli	Coimbatore, India	85.4	83	91	85.5
GU111995	CroYVMB	Okra	Bhubaneswar, India	84.4	79.3	91.5	86.4
MH252994	CroYVMB	Okra	Mardan, Pakistan	85.8	81.3	91	85.5
EU604296	CroYVMB	Jatropha	Lucknow, India	83.7	78.7	91	84.7
JX050198	CroYVMB	*Ipomoea purpurea*	Delhi, India	92.3	90.8	96.6	95.7
HQ631430	CroYVMB	*Calotropis procera*	Rajasthan, India	84.1	81.1	89.6	**66.9**
FJ593630	CroYVMB	Radish	Pataudi, India	85.2	82.8	91	85.5
KC545814	CroYVMB	*Cucumis sativus*	Delhi, India	92.7	91.2	96.9	94
KF964649	CroYVMB	*Tomato*	Bhubaneswar, India	84.1	79.2	88.7	81.3
KY612432	CroYVMB	*Sidastrum micranthum*	Ballia, India	82	77	88.7	80.5
KT390489	CroYVMB	*Hibiscus cannabinus*	Mirjapur, India	83.2	80.5	91	85.5
KM588256	CroYVMB	*Benincasa hispida*	Perambalur, India	**72.6**	**65.7**	90.4	85.5
JN663874	PaLCuB	Chilli	Delhi, India	92.9	91	98.3	96.6
KJ605117	ToLCJB	Tomato	Kolkatta, India	58.6	55	55.6	46.8
HQ180394	ToLCPB	Tobacco	Pusa, Bihar, India	60	55.9	62.1	49.1
JN663873	RaLCuB	Chilli	Salem, India	60.2	55.7	60.7	53.3
HM007105	ToLCBB	Chilli	Jodhpur, India	57.9	52.8	60	50.8
KF471033	ToYLCTB	*Amaranthus*	Banswara, India	61.2	56.4	61.9	52.5
AM279663	ChLCuB	Chilli	Sialkot, Pakistan	58	52.7	61.7	52.5
EF614158	CoLCMB	*Hibiscus cannabinus*	Bongaon, India	50.1	50	47.3	29.6
AM260735	TbCSB	Tomato	Yunnan, China	58.2	54.9	60.2	57.6
AM701771	AgLCuB	Turnip	Faisalabad, Pakistan	56.4	49.1	53.4	47.8
FN432358	OkLCuB	*Sonchus arvensis*	Faisalabad, Pakistan	55	49.2	55.1	49.2

[a] CroYVMB= Croton yellow vein mosaic betasatellite, PaLCuB= Papaya leaf curl betasatellite, ToLCJB= Tomato leaf curl Joydebpur betasatellite, ToLCPB= Tomato leaf curl Patna betasatellite, RaLCuB= Radish leaf curl betasatellite, ToLCBB= Tomato leaf curl Bangladesh betasatellites, ToYLCTB= Tomato yellow leaf curl Thailand betasatellite, ChLCuB=Chilli leaf curl betasatellite, CoLCMB= Cotton leaf curl Multan betasatellite, TbCSB= Tobacco curly shoot betasatellite, AgLCuB= Ageratum leaf curl betasatellite, OkLCuB= Okra leaf curl betasatellite

the isolates of CroYVMB with maximum identity with papaya isolates (HM143903 and HM14390) from Panipat but shared <63% nt and <54% aa sequence identity with other non-cognate betasatellites (Table 2). The IR region shared 65.7-91.5% identity with the CroYVMB isolates and <57% identity with other betasatellites with the exception of PaLCuB, which shared 91% sequence identity with the present isolate (Table 2).

Phylogenetic analyses of CYVMV and related begomoviruses showed that the six CYVMV isolates (KJ747958, JN817516, LN871569, LN886647, FN645902, and KF888655) including the present isolate formed a cluster in which the present isolate showed close similarity with CYVMV-Del-Crambe (KJ747958), but it showed an evolutionary distant relationship with the CYVMV-Del-Brassica isolate (JX270684) (Fig. 2a). It was also observed that the one isolate of CYVMV (FN678906) together with the isolate of CYVV (FN 543112) formed a separate cluster away from all other CYVMV isolates. In the case of beta satellites, the present isolate formed a cluster together with the isolate, CroYVMB-Del-Ilc (JX050198) whereas it showed a distant relationship with the isolates, JX270685 and KM229762 reported from brassica and crambe, respectively (Fig. 2b). It was also observed that the isolate of PaLCuB (JN663874) clustered together with the CYVMV isolates in the tree constructed (Fig. 2b).

CYVMV can alone induce typical disease symptoms in turnip accessions and betasatellite aggravates the disease

The agro-infectivity was only studied with germplasm accession IC732033 due to the limited seed availability for conducting such experiments. Unfortunately, agroinoculation could not be carried out on other germplasm accessions because of the scarcity of seeds for the exotic germplasm accessions.. The agro-construct of CYVMV (pCTur-A-1.4) alone resulted in curling and upward leaf rolling symptoms on turnip plants (Accession no.IC732033) at 25-30 dpi, at a temperature ranging from 20-25°C (Table 3, Fig. 3b). On the other hand, co-inoculation of CroYVMB (pCTur-β-2.0) with CYVMV (pCTur-A-1.4) increased the disease severity and produced similar leaf curl symptoms with severe stunting in 2-5 days less incubation period (Table 3, Fig. 3c). However, no symptoms were observed in the plants inoculated with either the empty vector pC2300 (mock) (Fig. 3a) or CroYVMB (pCTur-β-2.0) alone even at 60 dpi (Table 3). A 750 bp amplicon specific to CYVMV was obtained from all the CYVMV and CYVMV+CroYVMB inoculated plants, while a 260 bp amplicon specific to CroYVMB, was obtained from plants where both CYVMV and CroYVMB were inoculated (Table 3, Fig. 3d). This indicated that CYVMV alone can multiply in the turnip plant of the specified germplasm accession and produces typical disease symptoms whereas CroYVMB can multiply only in the presence of CYVMV. As expected, no amplification in mock-inoculated plants, and plants inoculated with only pCTur-β-2.0 were observed.

Whitefly inoculation of progeny virus and betasatellite from agro-inoculated plants reproduced the disease in healthy turnip plants

Back inoculation of CYVMV and CroYVMV from agroinoculated symptomatic plants of accession IC732033 to the healthy turnip plants (IC732033) through whitefly transmission resulted in expression of typical symptoms. In the case of CYVMV incoculated plants, symptoms appeared at 30-35 dpi, while it took 25-32 dpi when whitefly transmission was carried from the plants which were inoculated with both CYVMV and CroYVMB (Table 3). The presence of viral DNA and associated betasatellite in the whitefly inoculated plants were confirmed by PCR.

Discussion

Turnip plants at the experimental farm of NBPGR, Delhi, displaying symptoms of leaf curl and severe stunting, were confirmed to be infected with CYVMV and CroYVMB by PCR using pair of specific primers to CYVMV (Pramesh et al. 2013) and CroYVMB (Jailani et al. 2016) followed by sequencing of clones of CYVMV, and CroYVMB. The virus was initially reported to affect the weed croton (Mandal and Muniyappa 1991) and is currently known to affect 14 other crop plants based on the sequences available in the NCBI database (Table 1). Here, we report turnip (*Brassica rapa* subsp. *rapa*) as a new natural and experimental host for CYVMV and its cognate betasatellite. However, irrespective of the presence of a considerable amount of whiteflies in the field, the distribution of the disease was not uniform across all the grown turnip accessions, which indicates a differential response of turnip germplasm accessions to the disease.

The genome of CYVMV exhibited a maximum sequence identity and demonstrated close phyologenetic relationships with the isolate reported from crambe. However, it showed an evolutionary distant relationship with the isolate reported from rapseed-mustard. The associated betasatellite shared maximum sequence identity with the isolate reported from rapeseed-mustard, suggesting that the associated betasatellites are more diverse than those of CYVMV.

The present isolate was tested for infectivity using cloned DNA agroinoculation in turnip plants and subsequent transmission of the progeny virus to healthy turnip plants via whiteflies. This confirmed the association of CYVMV and its betasatellite with leaf curl disease in turnip and established Koch's postulates. The virus and betasatellite,

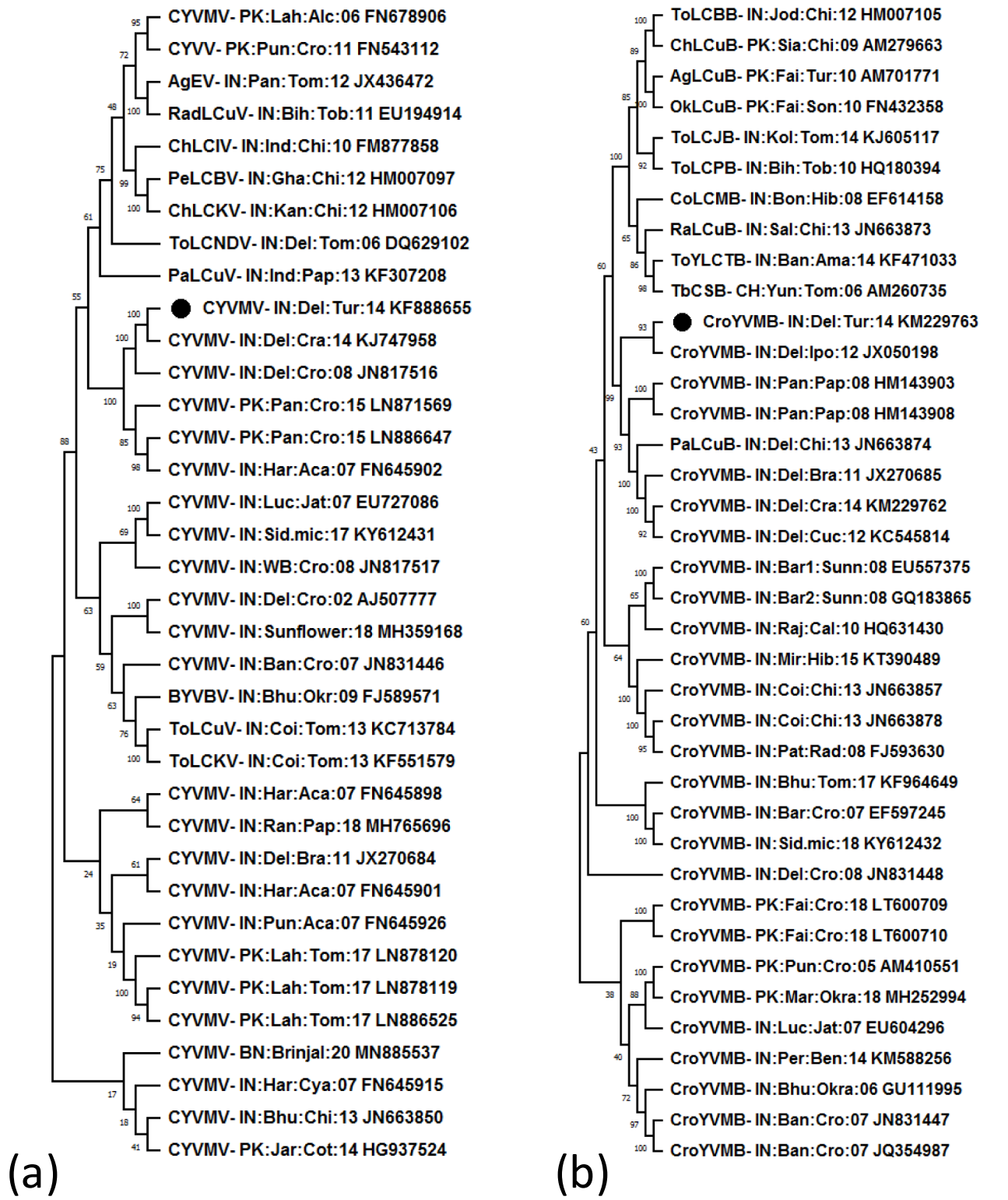

Fig. 2 Maximum parsimony tree showing phylogenetic relationships of the present isolate of croton yellow vein mosaic virus (CYVMV-Del-Turnip) and croton yellow vein mosaic betasattelite (CroYVMB-Del-Turnip) with other related isolates from NCBI database. (a) Tree based on DNA-A sequences and (b) Tree based on the betasatellite sequences. The evolutionary analysis was conducted by MEGA version 11 using 1000 bootstrape values. The isolate detail for each accession of CYVMV is listed in Table 1 and for CroYVMB in Table 2

Table 3 Agroinoculation of infectious croton yellow vein mosaic virus and croton yellow vein mosaic betasatellite constructs, and whitefly transmission of progeny virus, to the turnip plants

Agroinoculation					
Test plant (Accession no.)	DNA construct (Virus/betasatellite/vector)	No. of plants produced symptoms/ No. of plants inoculated[x]	dpi	PCR response	Types of symptoms
Turnip (IC732033)	pC-A1.4 (CYVMV)	6/8	25-30	+	curling and upward leaf rolling
	pC-β2.0 (CroYVMB)	0/5	-	-	No symptoms
	pC-A1.4 + pC-β2.0 (CYVMV + CroYVMB)	7/8	25-28	+	curling and upward leaf rolling with severe stunting
	pCAMBIA 2300 (Mock)	0/5	-	-	No symptoms
Whitefly transmission					
Test plant (Accession no.)	Source plant[y]	No. of plants produced symptoms/ No. of plants inoculated	dpi	PCR response	Types of symptoms
Turnip (IC732033)	Turnip-A	3/5	30-35	+	curling and upward leaf rolling
	Turnip-A+β	4/5	28-32	+	curling and upward leaf rolling with severe stunting

[x] data from the two replication combined

[y] Turnip plants showing symptoms after agroinoculation of the DNA components

dpi= days post inoculation

replicated in agroinoculated plants, were successfully transmitted to healthy turnip plants, confirming that turnip ia a new host for this virus.

Previous studies have shown that many begomoviruses are found in various weed species that typically infect crop plants. This indicates that weeds, when the main crops are not present, can act as alternative hosts for the survival and spread of many begomoviruses (Bedford et al. 1998). Weeds such as Ageratum, Asystasia, Clerodendrum, Emilia, and Malvastrum have been identified as reservoirs of begomoviruses that infect numerous crop species (Leke et al. 2015). Some of these weeds are naturally infected with begomoviruses that are harmful to important crops (Barbosa et al. 2009; Bedford et al. 1998; Kashina et al.2003; McGovern et al. 1994; Salati et al. 2002). However, their significance as virus reservoirs for crops still needs to be demonstrated. In recent years, several begomoviruses originally reported to infect weed hosts have adapted to infect crop plants. CYVMV is an example of a weed-infecting virus that was initially discovered in a weed called Croton bonplandianus (Pramesh et al. 2013), but has since extended its host range to include crops such as tomato, okra, radish, papaya, crambe, and rapeseed-mustard (Khan et al. 2015; Venkataravanappa et al. 2011; Singh et al. 2012; Singh-Pant et al. 2012; Kumar et al. 2018; Roy et al. 2013). Similarly, two other begomoviruses that infect weeds, Ageratum enation virus (AEV) and Ageratum yellow vein virus (AYVV), have expanded their host range to include crop plants. AEV has been reported to naturally infect crop plants such as tobacco, tomato (Tahir et al. 2015), cat's whiskers (Raj et al. 2010), carrot (Kumar et al. 2013), and zinnia (Kumar et al. 2010), whereas AYVV has been found to infect tomato plants in Vietnam (Choi et al. 2019) and papaya plants in Indonesia (Helina et al. 2024). These findings clearly indicate that weeds not only act as alternate hosts for crop-infecting begomoviruses but also serve as reservoirs for begomoviruses that have the potential to cause disease in crop plants.

Previous studies of CYVMV and its associated cognate betasatellite (CroYVMB) have reported that CYVMV is a monopartite begomovirus. It can cause disease symptoms in tomato plants (Shilpi et al. 2015) and *Nicotiana* species (Jailani et al. 2016). However, in other hosts such as Croton (Pramesh et al. 2013) and Crambe (Kumar et al. 2018), betasatellites are required for symptom development. The current study supports these findings and adds a new host plant to the list of CYVMV. This study also revealed that CYVMV alone can cause disease symptoms in turnip plants. Additionally, the presence of betasatellite worsens the severity of the disease and its symptoms, even though the present isolate closely resembles and is phylogenetically related to the CYVMV isolate from Crambe, which alone is unable to cause disease symptoms in Crambe.

This study provided the etiological evidence of the cause of leaf curl disease in turnip germplasm accession through the establishment of Koch's postulates. This confirmed the emergence of CYVMV and CroYVMB on turnip plants, a new economically important winter vegetable crop. Previously, CYVMVwas found in rapeseed-mustard

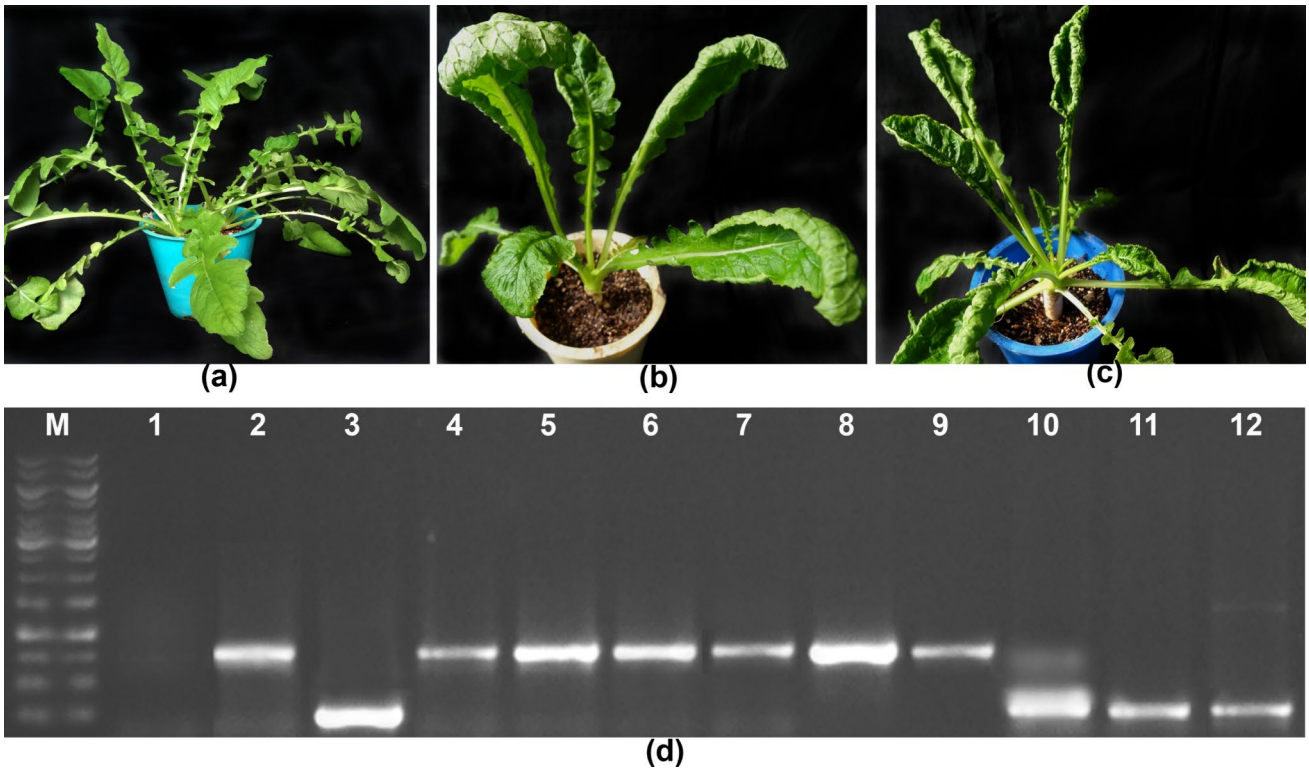

Fig. 3 Infectivity of cloned DNA of croton yellow vein mosaic virus and associated betasatellite on turnip plant (Accession no. IC732033). Symptoms on Mock inoculated turnip plant (a), CYVMV inoculated turnip plant (b) and CYVMV + CroYVMB inoculated turnip plant (c). (d) Amplification of CYVMV and CroYVMB from the DNA isolated from agroinoculated plants. M: Marker, Lane 1: Healthy, Lane 2: cloned plasmid DNA of CYVMV-mustard as positive control, Lane 3: cloned plasmid DNA of CroYVMB-mustard as positive control, Lane 4-6: Amplification from the symptomatic leaf of CYVMV inoculated plants using CYVMV specific primers, Lane 7-9: Amplification from the symptomatic leaf of plants inoculated with CYVMV and CroYVMB using CYVMV specific primers, Lane 10-12: Amplification from the symptomatic leaf of plants inoculated with CYVMV and CroYVMB using CroYVMB specific primers

and crambe. Before these studies, winter-season crops such as rapeseed-mustard, crambe, and turnip were not known to be infected by any begomovirus. The present and past studies showed that CYVMV, a weed-infecting begomovirus, infecting Brassica crops, which were previously not known to be infected by begomoviruses.Effective management practices must be developed to prevent the spread of this disease and its associated losses.

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Author's contribution MKB, RS conducted the field trial of the crop; MKB, RS and AR performed the disease surveillance in the field and collected the samples from infected plants; AK, BM and RY designed the experiment; AK performed the experiments, interprets the data, and prepared the first draft of the manuscript; MKB, RS BM and AR corrected the manuscript. The final draft was read and approved by all the authors.

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Data availability The datasets generated during and/or analyzed during the current study are available in the NCBI database (http://www.ncbi.nlm.nih.gov). The complete genome sequence of CYVMV and associated betasatellite generated during this study are deposited in NCBI database and are publically available under the accession no. KF888655 (https://www.ncbi.nlm.nih.gov/nuccore/KF888655) and KM229763 (https://www.ncbi.nlm.nih.gov/nuccore/KM229763), respectively.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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