A new variant of Croton yellow vein mosaic virus naturally infecting wild sunflower in India

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Abstract During 2015–2016, wild sunflower (*Verbesina encelioides*) was observed with bright yellow vein mosaic symptoms in Noida, Uttar Pradesh, India. The initial analysis by PCR with a pair of coat protein based primers revealed the association of a begomovirus. Further, the virus was identified by rolling circle amplification and cloning of the complete genome. The DNA-A (2671 nucleotides, MH359168) with the genome organisation typical of the Old World begomovirus shared 98% sequence identity with that of Croton yellow vein mosaic virus (CYVMV) reported from India. The betasatellite (MH359169) associated with the disease shared 92% sequence identity with papaya leaf curl betasatellite (KY825245). The results showed that the wild sunflower is a new alternate host of CYVMV. The study also revealed a natural association of heterologous betasatellite with CYVMV in wild sunflower exhibiting yellow vein mosaic symptoms.

Keywords Wild sunflower · Begomovirus · Weeds · Croton yellow vein mosaic virus

Introduction

Wild sunflower scientifically known as *Verbesina encelioides* belongs to Asteraceae family and is commonly found as weed growing along the fields, sides of the roads and isolated open areas. This plant can grow very well in dry weather conditions which helps plant in spreading profusely. It causes losses in cereals because its growing season is same as that of summer wheat, and thus competing for nutrients and space leading to poor growth of crop plant, yield losses and vigor of plant. It is also reported to be used for broadening the genetic base of cultivated sunflower in India and to introduce the desirable traits from wild sunflowers to cultivated sunflower [5]. Also this plant is poisonous for animals if consumed [5].

This plant is mostly used to treat stomach problems. The roots consumed for retention of water in body, bladder inflammation and used as blood purifier. The main pharma compounds from various parts of the plant are terpenoids, flavonoids, aromatic compounds and phenolic complexes etc. Plant leaf is cast-off to cure pain in legs so as to cure rheumatism, and juice of the plant has its own importance as purgative compound. [5].

In literature not many viruses are reported on this plant worldwide. Few unpublished record of sequence data of tobacco curly shoot virus (Accession HQ407395) and Ageratum yellow leaf curl betasatellite (Accession HQ407397) molecules have been reported on wild sunflower. Though there are viruses reported on sunflower viz tomato leaf curl Karnataka virus and potato apical leaf curl betasatellite which are associated with leaf curl disease of sunflower (*Helianthus annuus*) from India [16]. There is partial sequence data on Ageratum enation virus (AEV) (*Verbesina encelioides* yellow vein Lakshmangarh virus) isolate (JN998449) and Ageratum leaf curl betasatellite (*Verbesina* yellow vein betasatellite)

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isolate (JQ693145) infecting wild sunflower (*Verbesina encelioides*) in GenBank database.

In the year 2016 natural occurrence of Ageratum enation virus (AEV) and Ageratum leaf curl betasatellite (ALCuB) infecting wild sunflower plants was reported by Kumar et al. [7] from India.

Begomovirus (family *Geminiviridae*) is characterized by twin geminate icosahedral particle morphology. Geminiviruses have been classified to nine genera based on the characteristic features like host range, vector, organization of the genome viz. *Becurtovirus*, *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Mastrevirus*, *Topocuvirus*, *Turncurtovirus*, *Capulavirus* and *Grablovirus* [2, 18]. Begomoviruses are plant viruses possessing broad host range infecting dicotyledonous plants. Begomoviruses are sub-divided into two different classes based on geographical origin as old world and new world viruses which are either of bipartite or monopartite and bipartite respectively [3, 17].

Begomoviruses which are mostly native to the New World have genomes comprising of two components of ssDNA which are of ~ 2800 nt and are known as DNA A and DNA B and from various studies it is evident that both the components are required for the systemic infection of plants. The DNA A encodes all viral proteins which are required for the replication of viral DNA, for gene expression and transmission to other plants while the DNA B component encodes only two proteins which are essential for inter- and intracellular movement of virus in the host.

Croton yellow vein mosaic virus (CYVMV) has been reported from various host plants such as: *Jatropha gossypifolia* [15], *Solanum lycopersicum*, [6], *Crambe abyssinica* [8], Radish, [12], *Solanum lycopersicum* [14]. Number of sequences of CYVMV on other host plants have been submitted in the NCBI; Turnip (ACC no KF888655: India), Chilli (JN663850:India), Acalypha, (FN645898:India), *Cyamopsis tetragonoloba* (FN645915: India), *Brassica rapa* (KF88865:India) Cotton (HG937524: Pakistan), Papaya (KF307208:India), *Alcea rosea* (FN678906:Pakistan), *Calotropis procera*, (JQ407224:India), Croton (KX671964:Pakistan), Croton (AJ507777: India), *Jatropha* (EU727086:India), Cotton (HG937524:Pakistan).

We are reporting CYVMV on wild sunflower for the first time. The natural association of a heterologous betasatellite, papaya leaf curl betasatellite (PLCuB) with CYVMV is also reported in the present study.

Materials and methods

Sampling

Leaves of wild sunflower weed plants displaying leaf vein clearing and yellowing of whole plant (Fig. 1) were

Fig. 1 Symptoms on wild sunflower; vein clearing, yellowing of whole plant

collected from various fields from Noida region India, in the year 2015–2016. Samples were placed in plastic bags and carried to the laboratory for further analysis.

DNA extraction

For DNA extraction leaf samples were taken and homogenized in liquid nitrogen. DNA was extracted by CTAB method [11].

PCR and RCA

The primer pair AV494 and AC1048 [19] was used to amplify the partial coat protein gene AV1 and 550 bp amplification was expected (14). Each reaction contained 1 µl DNA, 1 unit Taq DNA Polymerase (Invitrogen), 0.3 µl dNTP mix, 0.3 µl of each primer (10 mM), 2.5 µl 10× Taq Buffer (Invitrogen) and sterile water to make up total volume of 25 µl. Initial denaturation was done at 94 °C for 2 min and then thermal-cycler (BioRad) for 34 cycles: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 90 s. Final extension was done for 10 min at 72 °C. PCR amplicons were screened by using agarose gel electrophoresis and was stained using EtBr. For betasatellite primers given by Briddon et al. [1] were used. For alphasatellite primers by Singh et al. [13] were used. Amplified products were cloned in TA cloning vector (Invitrogen, USA) and were sequenced.

Rolling circle amplification (RCA) amplifies all circular DNA in the samples in a single reaction without the use of any specific equipment like thermal cycler [4]. DNA was

extracted and RCA was done using a bacteriophage ϕ 29 DNA polymerase (“Ilustra TempliPhi 100 Amplification Kit” GE Healthcare). 5 μ l of sample buffer was incubated at 95 °C for three minutes along with 1 μ l DNA extracted. Five μ l of reaction buffer and 0.02 μ l of enzyme (phi DNA Polymerase) were added subsequently and this was incubated at 30 °C overnight. RCA products were digested using different restriction enzymes such as *Bam*HI, *Xba*I, *Eco*R1, *Sac*I, and 2.7 kb bands were later cloned in pUC19 vector. Restriction digestion pattern of various full length viral clones was analysed and followed by sequencing commercially at Triyat Labs, Bangalore.

Phylogenetic analysis and recombination detection program

Phylogenetic analysis was performed by 1000 bootstrap replications using neighbour joining method in MEGA version 6. To study recombination events, RDP was used in DNA and betasatellite using RDP 4 program [10].

Results

Association of CYVMV with the wild sunflower

In wild sunflower vein clearing in the leaves was recorded along with yellowing of the whole plant (Fig. 1) and these are typical characteristic symptoms of Begomovirus infection. It was interesting to note that the maximum number of plants were showing these symptoms but few plants seems to be healthy without any symptom.

Amplification of 550 bp was obtained in PCR which confirmed the presence of a begomovirus infection in the sample. After restriction digestion of 2.7 kb was obtained in *Bam*HI digestion. After cloning in pUC and sequencing (primer walking) 2671 nt was obtained. Full length sequence was analysed by BLAST in NCBI showing maximum homology of 98% with CYVMV (AJ507777), reported from Croton (India). When comparison was done at nucleotide level with other CYVMV isolates reported from India and world minimum homology percentage of 78% was found to be with *Tobacco curly shoot virus* (TCSV) reported from India in wild sunflower. A Blast search of the GenBank nucleotide sequence database with 19 other CYVMV sequences was studied both at nucleotide and amino acid level for all the ORF's of DNA-A. Overall these 20 sequences (including the one identified in the present study) shows homology between 98 and 78% at nucleotide level (Table 1).

The highest amino acid sequence homology of the proteins encoded by the ORFs AV1 (99.1%), AV2 (99.2%), AC2 (58.9%), AC1 (93.6%), and AC3 (54.8%),

AC4(100%), AC5(96.2%) with that of CYVMV Acc. No. AJ507777 reported on Croton from India. The highest nucleotide sequence homology of ORF's AV1 (97.9%), AV2 (98.8%), AC2 (86.9%), AC1 (94%), and AC3 (51.3%), AC4(98.8%), AC5(63.2%) with that of CYVMV AJ507777 except that for AC3 where it showed maximum homology to CYVMV reported from croton from India Acc. No. JN817516 (Table 1).

Phylogenetic relationships

In phylogenetic analysis of CYVMV-DNA A sequences four clades were distinctly visible. CYVMV isolate of the present study clustered with CYVMV reported on croton from India forming clade II. Other viruses in the same cluster were CYVMV infecting *Jatropha gossypifolia* and *Sidastrum micranthum*. In clade I, CYVMV reported on chilli, radish, cotton, cymopsis, tomato and *Acalypha* sp. from India and Pakistan. In clade III CYVMV on croton reported from Pakistan and India and on *Crambe* and *Brassica rapa* from India are falling. Whereas in clade IV, CYVMV infecting *Calotropis* and *Alcea rosea* reported from India and Pakistan are coming (Fig. 2). Color-coded pairwise identity matrix was also generated using CYVMV infecting other host plants (Fig. 3). TCSV and AEV were included in the phylogenetic study as other than CYVMV these viruses also infect wild sunflower and therefore these viruses are falling separately in the phylogenetic tree.

RDP

In RDP analysis CYVMV reported from wild sunflower comes out as recombinant of major parent CYVMV reported from *Sidastrum micranthum* in India and minor parent AEV reported from wild sunflower from India (Supplementary Table 1).

Betasatellite

By using betasatellite primers 1.3 kb amplification was obtained, which further indicated towards a betasatellite in the infected sample. No amplification was observed in PCR where alphasatellite primers were used indicating absence of alphasatellite. After cloning and sequencing BLASTn analysis was done and it showed similarity to Papaya leaf curl betasatellite (PLCuB) on Chilli from Pakistan (Acc no. KY825245).

BLAST/CLUSTALW analysis was done for the Papaya leaf curl betasatellite which was found along with DNA-A. Papaya leaf curl betasatellite showed maximum nucleotide homology (92.2%) to PLCuB on Chilli from Pakistan (Acc no. KY825245) and minimum homology percentage of

Table 1 Percentage nucleotide and amino acid sequence similarities between DNA-A of Croton yellow vein mosaic virus reported in the study(MH359168) with 19 other Croton yellow vein mosaic virus infecting other host plants

S/N	Acronym	Acc. Number	DNA-A	AV1 ^P	AV1 ^N	AV2 ^P	AV2 ^N	AC1 ^P	AC1 ^N	AC2 ^P	AC2 ^N	AC3 ^P	AC3 ^N	AC4 ^P	AC4 ^N	AC5 ^P	AC5 ^N
1	CYVMV[IN: Croton:2002]	AJ507777	98.4	99.1	97.9	99.2	98.8	93.6	94.0	58.9	86.9	54.8	50.3	100	98.8	96.2	63.2
2	CYVMV[PK: Tomato:2015]	LN886525	93.0	92.3	94.9	96.4	94.1	88.1	87.5	56.7	85.5	48.1	47.7	89.4	93.4	NA	NA
3	CYVMV[PK: Croton:2016]	KX671964	91.5	97.4	97.2	99.2	97.7	85.5	85.6	52.2	77.8	49.6	51.3	91.7	93.0	NA	NA
4	CYVMV[IN: DEL: Crambe abyssinica:2012]	KJ747958	93.2	97.4	97.2	98.8	97.7	86.8	86.8	55.9	85.0	53.3	50.5	90.5	92.6	NA	NA
5	CYVMV[PK: Jaranwala: Cotton:2014]	HG937524	93.6	94.9	96.6	97.6	96.0	87.8	86.8	52.9	82.0	53.3	51.3	90.5	93.7	NA	NA
6	CYVMV[IN: DEL: Brassica rapa:2013]	KF888655	93.2	97.4	97.4	99.6	97.7	87.1	86.9	55.9	85.0	53.3	51.3	90.5	92.6	NA	NA
7	CYVMV[IN: Papaya:2013]	KF307208	91.0	89.8	96.7	98.8	90.7	87.0	86.9	57.4	85.0	53.3	49.9	88.2	NA	NA	NA
8	CYVMV[IN: Bhubaneswar: Chilli:2011]	JN663850	94.4	97.4	97.0	98.8	97.4	88.4	88.3	57.0	85.1	50.3	22.9	90.5	93.7	NA	NA
9	CYVMV[IN: Rajasthan: Calotropis procera:2010]	JQ407224	83.1	NA	45.9	NA	43.4	NA	56.8	NA	53.2	NA	47.4	NA	NA	NA	NA
10	CYVMV[PK: Alcea rosea :2006]	FN678906	87.6	92.3	79.7	83.9	93.7	88.1	87.4	58.2	82.5	48.1	51.2	89.4	93.4	NA	NA
11	CYVMV[IN: Croton bonplandianus:2008]	JN817516	93.3	99.1	97.9	99.2	98.8	86.8	86.9	57.4	84.7	52.5	50.0	NA	NA	96.2	23.4
12	CYVMV[IN: Radish:2008]	FJ593629	94.4	97.4	97.1	99.2	97.4	88.6	88.4	57.4	85.0	50.3	51.3	90.5	93.7	92.5	23.2
13	CYVMV[IN: Acalypha sp:2007]	FN645898	94.0	97.4	97.5	99.2	97.4	68.9	71.0	58.2	83.5	53.3	48.7	89.4	93.4	93.4	23.4
14	CYVMV[IN: Cyamopsis tetragonoloba :2007]	FN645915	93.4	91.5	94.1	96.9	94.9	84.6	84.5	58.2	83.5	49.6	46.3	90.5	93.7	86.9	61.9
15	CYVMV[IN: Jatropha gossypifolia: 2007]	EU727086	93.8	99.1	96.7	96.4	96.9	90.0	87.8	58.2	85.7	45.1	52.2	92.9	94.9	95.3	61.9
16	CYVMV[IN: Croton bonplandianum:2002]	NC_004300	98.4	99.1	97.9	99.2	98.8	93.6	94.0	58.9	86.9	54.8	46.3	100	98.8	96.2	60.4
17	CYVMV[IN: Sidastrum micranthum: 2016]	KY612431	93.7	99.1	96.7	96.4	96.9	90.0	87.8	58.2	85.7	45.1	51.2	92.9	94.9	95.3	62.9
18	CYVMV[PK: Croton:2014]	LN871569	93.3	97.4	97.2	76.5	97.7	86.5	87.1	58.2	85.7	53.3	47.2	92.9	93.4	94.3	63.2
19	TCSV [IN: Wild Sunflower: 2015]	HQ407395	78.0	88.1	79.5	82.8	92.9	63.4	70.8	55.9	81.0	47.4	46.9	31.9	56.8	NA	NA
20	AEV [IN: Wild sunflower:2015]	KY089033	78.7	87.2	78.8	82.0	92.9	72.6	73.6	52.9	78.8	44.8	50.3	58.8	74.8	NA	NA

^NNucleotide sequence identity^PAmino acid sequence identity

NA sequence not available

Highlighted are maximum and minimum values

Fig. 2 Phylogenetic tree was constructed based on nucleotide sequence of DNA-A Croton yellow vein mosaic virus infecting different host plants. Bootstrap values at the node indicate the strength of branching is supported (2000 replicates). Identified virus is falling in clade II

Fig. 3 Color-coded pairwise identity matrix generated from 20 complete DNA-A genome segments of CYVMV. The matrix was generated using the program Sequence Demarcation Tool (SDT v.1.2; <http://web.cbio.uct.ac.za/>), and each colored cell represents a

percentage identity score for two sequences. Colored keys indicating the correspondence between pairwise identities and the colors displayed in the matrix are presented

55.5% to Tomato leaf curl Joydebpur betasatellite from India. The present isolate also shared maximum amino acid sequence identity (100%) of bC1 with PLCuB [PK:Chilli:2017] (Table 2).

In phylogenetic analysis also, PLCuB formed a branch with PLCuB[PK:Chilli:2017] and clustered with some of the betasatellites associated with begomovirus-induced diseases of chilli occurring in the Indian sub-continent (Fig. 4). RDP analysis was done for betasatellite also but no significant recombination event was recorded.

Discussion

Wild sunflower (*Verbesina*) is common weed plant found in India and is reported as host to viruses such as *Tobacco curly shoot virus* from Southern part of India, and *Ageratum enation virus* from Jaipur, India [7].

CYVMV was first reported in 1969 from India and was later reported on croton from India by Mandal et al. [9]. After that it has been reported from many crops as well as weed plants. During sample collection very typical symptoms of total vein clearing and yellowing of leaves were noticed (Fig. 1). After PCR and sequence analysis presence of a new variant of *Croton yellow vein mosaic virus*

Table 2 Percentage nucleotide similarities between full length Papaya leaf curl betasatellite and Bc1 gene identified in the study(MH359169) with other reported betasatellites

S. no	Acronym	Acc. number	Betasatellite	Bc1
1	PLCuB[PK:Chilli:2017]	KY825245	92.2	100
2	CYVMB [IN: Papaya:2008]	HM143903	87.1	100
3	CYVMB[IN:Brassica rapa:2012]	JX270685	86.7	93.2
4	CYVMB [IN:Crambe abyssinica:2014]	KM229762	86.4	92.3
5	CYVMB [PK:Croton spp:2011]	AM410551	83.2	47.4
6	PLCuB [IN:Cluster bean:2016]	KT253636	79.8	83.8
7	PLCuB [PK:Chilli:2017]	KY825246	89.2	99.1
8	PLCuB[IRAN: Papaya:2015]	KJ397535	56.7	53.3
9	CYVMB [India: Bhubaneswar:Okra: 2006]	GU111995	81.4	88.1
10	TLCuJoB [India:Bihar-SBO:Chili:2016]	KY271069	55.5	46.8

Fig. 4 Phylogenetic tree was constructed based on nucleotide sequence of Papaya leaf curl betasatellite reported from different host plants. Bootstrap values at the node indicate the strength of branching is supported (2000 replicates). Highlighted box shows the clade where PCLuB identified in the study falls

infecting wild sunflower from Noida, India is being reported. Along with DNA-A, Papaya leaf curl betasatellite was also found to be associated with CYVMV. This suggests that virus is monopartite having DNA-A and associated with betsatellite. In phylogenetic analysis the identified CYVMV grouped with other CYVMV reported from croton in India. This virus was first reported on croton from India in 2002 and in 2007 it was found infecting *Jatropha* and from *Sidastrum* in 2016 and this indicates towards varied host range of the virus. Papaya leaf curl betasatellite which was found associated with CYVMV infecting wild sunflower, when subjected to phylogenetic analysis grouped with Papaya leaf curl betasatellite on chilli from Pakistan.

RDP shows CYVMV reported on wild sunflower as recombinant of CYVMV reported on *Sidastrum*

micranthum and AEV reported from wild sunflower from India. Results clearly indicate that the Begomoviruses are expanding their host range and weeds are serving as reservoir host of the viruses.

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