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BIOTECHNOLOGY

Molecular Diagnosis and Characterization of a Geminivirus Causing Leaf Curl Disease of Tomato in Mid Hills of Northern India

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Abstract

Geminiviruses are small isometric particles which contain either one or two circular single stranded DNAs. The family *Geminiviridae* comprises of four genera: Mastrevirus, Curtovirus, Topocuvirus and Begomovirus. Among begomoviruses, one of the most important virus is TYLCV which infects an economically important food crop i.e. tomato [*Lycopersicon esculentum*), all over the world. TYLCV is also prevalent in different regions of India. Five samples from different tomato growing areas of Himachal Pradesh were collected for serological detection of TYLCV using DAS-ELISA. All samples reacted positively with the antisera. In further confirmation studies, CP (coat protein) gene based specific primer pairs for TYLCV were designed and tested. CP gene was amplified from two isolates and a 771 bp long CP sequence was amplified, sequenced and submitted to NCBI database (Acc no-KC253231). TYLCV, the test virus was confirmed to be a begomovirus on the basis of phylogenetic analysis while comparing it with other geminiviruses. On comparison with other Indian tomato leaf curl viral isolates, it was found to be closely related to Dharwad isolate (GI 1160428) and not to native isolate (GI 1244840), which indicates it's origin in other than North Indian region and evolution of a new viral strain.

Highlights

- A new recombinant strain of TYLCV was isolated
- CP gene of virus amplified using designed primers
- Phylogenetic analysis were performed to confirm the results

Keywords: Geminivirus, TYLCV, DAS-ELISA, coat protein

Plant viruses belonging to family *Geminiviridae* are characterized by small, twinned, isometric particles containing either one or two circular, single-stranded DNA species. The family comprises four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*, distinguished by insect vector, host range and genomic characteristics (Stanley *et al.,* 2005).

During the last two decades begomoviruses have emerged as devastating pathogens, particularly in the tropics and subtropics, causing huge economic losses and threatening crop production. Epidemics caused by re-emerging and newly emerging gemini viruses are becoming frequent even in regions which were earlier free from these viruses (Varma and Malathi, 2003). Majority of the gemini viruses fall in the genus *Begomovirus*. Members of this genus are transmitted by a whitefly *Bemisia tabaci*, infect dicotyledonous plants and have either monopartite or bipartite genomes.

Tomato leaf curl virus is a monopartite begomovirus (Pandey *et al.,* 2010) containing a covalently closed, single-stranded DNA genome. Many whitefly-transmitted viruses occurring on economically important crop plants are suspected to be gemini

viruses (Singh *et al.*, 2013). The plant disease caused by gemini viruses are yellow mosaic viruses of dolichos, frenchbean, horsegram, limabean, mungbean, pigeonpea and soybean; yellow vein mosaic of bhendi, *Croton sparsiflorus* and *Malvastrum cormandelianum*; leaf curl of tobacco, tomato, chilli and papaya; and mosaic of cassava and introduction of high yielding tomato varieties in India has been accompanied by Tomato leaf curl virus infection (Ramappa *et al.,* 1998 ; Saikia and Muniyappa, 1989).

Table 1: Primer sequences used for detection of Tomato Yellow Leaf Curl Virus (TYLCV) coat protein (CP) gene using PCR

Gene	Primer sequence
TYLCV CP Primer pair 1	Forward CAAAAGCAAAGGCCTGGACC
	Reverse ATCCTGTAGGACGACGGTCA
Primer pair 2	Forward CATATTTCAACGCCCGCAT
	Reverse GGTCCAGGCCTTTGCTTTTG
Primer pair 3	Forward GAAAGTACGCCGACGTCTCA
	Reverse TCATACTTGCCGGCCTCTTG

It was recorded that 100% of the fall- grown tomato plants are usually infected with TYLCV (Tomato Yellow Leaf Curl Virus) and production losses may reach 80% (Moustafa, 1991). As is evident from the name itself (tomato leaf curl), severely stunted plants with deformed leaves showing yellow leaf margins are usually associated with the disease. Leaves may be cupped, reduced in size and the axillary shoots are erect. Plants infected early lose vigor and stop producing marketable fruit. They are called gemini (twin) viruses, because they are usually found in pairs. Each particle has a diameter of just 15 – 20 nm. Gemini viruses belong to the smallest virus particles able to multiply without a helper virus.

They have a circular DNA with a molecular weight of 0.7 – 0.8 × 10^6 daltons (about 2,500 base pairs). In case of some gemini viruses, it has been proven, whereas in case of others, it is assumed, that the genome consists of two molecules of DNA of almost equal size, but different sequence. The nucleotide sequences of only some species are known. Insects (greenhouse whitefly, grasshoppers, and others) help usually in spreading Gemini-viruses in nature. The majority of the begomoviruses are bipartite and the genomic components are referred as DNA-A and DNA-B (Fiallo-Olivé *et al.,* 2015; Fauquet *et al.,* 2008 and Naqvi *et al.,* 2013). Several isolates of Tomato leaf curl virus from India have been cloned and sequenced. It is evident from the work of Pratap *et al.,* 2011 and Naqvi *et al.,* 2013 that Tomato leaf curl isolates from Northern India possess a bipartite genome (DNA-A and DNA-B) while those from Southern India have a monopartite genome (DNA-A like). Discovery of leaf curl virus in the Indian subcontinent together with its spread has aggravated the situation. The existence of different Tomato leaf curl isolates having high genome diversity in India poses a threat to tomato production. The virus is also responsible for damage to potato crop; as potato leaf curl has been found to be caused by a strain of Tomato Leaf Curl New Delhi Virus. Results by Asmaa *et al.,* 2011 showed that TYLCV isolate infected large number of species from family *Solanaceae*. In addition, TYLCV infected a few species of family *Cucurbitaceae, Fabaceae and Chenopodiaceae.*

There are several reports of existence of vide variation in gemini viruses in general (Padidam *et al.,* 1995, Krenz *et al.,* 2014 and Shelly *et al.,* 2004) and TYLCV in particular (Shiraji *et al.,* 2014; Xie *et al.,* 2013; Al-Ali *et al.,* 2015, Wan *et al.,* 2015 and Sawangjita *et al.,* 2005). Wide sequence variability amongst Tomato Leaf Curl Virus isolates from India has also been reported Pandey *et al.,* 2010. Variability in geminiviruses has arisen through mutations, recombination and pseudo-recombination. Genomic recombination in gemini viruses, not only between the variants of the same virus but also between species and even between genera, has resulted in rapid diversification. From the disease point of view, most virulent variants have developed through recombination of viral genomes such as those associated with cassava mosaic, cotton leaf curl, and tomato leaf curl diseases (Morya *et al.,* 2015, Belabess *et al.,* 2015 and Varma and Malathi, 2003).

It is clear from the sequence analysis and phylogenetic results by Padidam *et al.,* 1995 and Sawangjita *et al.,* 2005 that the genomes of ToLCV isolates exhibit multiple putative recombination events between themselves and have integrated

Table 2: Representation of Elisa Plate (96 wells) using DAS-ELISA for detection of Tomato yellow leaf curl virus

1	2	3	4	5	6	7	8	9	10	11	12
A											
B	Kotbeja		Tohana		Pathru-1		Manjhed		Pathru-2		
C											
D	Kotbeja		Tohana		Pathru-1		Manjhed		Pathru-2		
E											
F											
G			+ve control		Healthy sample		Extraction buffer		-ve control		
H											

Results

1	2	3	4	5	6	7	8	9	10	11	12
A											
B	Kotbeja		Tohana		Pathru(1)		Manjhed		Pathru(2)		
	1.160		0.965		0.259		0.291		0.247		
C											
D	Kotbeja		Tohana		Pathru-1		Manjhed		Pathru-2		
	1.178		0.884		0.268		0.276		0.231		
E											
F											
G			+ve control		Healthy		Extraction		-ve control		
			1.187		Sample		buffer		0.090		
					0.086		0.075				
H											

Table 3: Acronyms and reference number of other Tomato leaf curl viruses compared with other gemini virus

Sl. No	Reference No.	Virus Isolates
1	195548123	Tomato leaf curl Kerala virus isolate
2	121495484	Tomato Yellow Leaf Curl Virus complete genome, strain TYLCV
3	62204	Tomato yellow leaf curl virus TYLCV virion DNA
4	215400608	Tomato yellow leaf curl virus strain TYLCV-Ir2
5	408004667	Tomato yellow leaf curl virus
6	356892481	Tomato yellow leaf curl virus isolate 20090109-46
7	350529254	Tomato yellow leaf curl virus DNA, complete genome, strain: TYLCV-CC
8	334883211	Tomato yellow leaf curl virus DNA, complete genome, strain: TYLCV-CJ
9	334683422	Tomato yellow leaf curl virus DNA, complete genome, strain: TYLCV-YS
10	334883197	Tomato yellow leaf curl virus DNA, complete genome, strain: TYLCV-GJ
11	62484953	Tomato yellow leaf curl virus-Israel
12	313110006	Tomato yellow leaf curl virus isolate TYLCV-CW
13	284027806	Tomato yellow leaf curl virus isolate TYLCV-Ab1 coat protein (V1) and pre-coat protein (V2) genes, partial cds
14	152148906	Tomato yellow leaf curl virus isolate recombinant
15	149395352|	Tomato yellow leaf curl virus isolate recombinant MmA-C.21
16	149396135	Tomato yellow leaf curl virus isolate recombinant MmB-C.
17	258611299	Tomato leaf curl New Delhi virus isolate ToLCV-186b segment DNA-A

(Cont...)

18	195548123	Tomato leaf curl Kerala virus isolate ToLCV-K5 segment DNA A, complete sequence
19	304557395	Tomato leaf curl New Delhi virus-Severe segment DNA A, complete sequence
20	89077668	Tomato leaf curl virus strain TNAU1 pre-coat protein (AV2) and coat protein (AV1) genes, complete cds
21	124484022	Tomato yellow leaf curl virus rep (partial), V2 (partial) and CP (partial) genes, isolate midhills
22	449085222	Whitefly-transmitted Indian begomovirus strain ToLCV New Delhi AV1 protein gene, partial cds
23	116042153	Tomato leaf curl Dharwad virus
24	411169540	Tomato leaf curl virus isolate pTZ-ToLCV Meerut
25	196049395	Tomato leaf curl Kerala virus, complete genome
26	149396070	Tomato yellow leaf curl virus isolate recombinant Ty-1St-C.19 15dpt type VII REn and TrAP genes, complete cds; and Rep gene, partial cds

other pieces of DNA that have been presumably originated from the other viruses not identified so far.

Materials and Methods

Survey and collection of TYLCV isolates

Extensive surveys were conducted in different tomato (*Lycopersicon esculentum* Mill.) growing regions of Himachal Pradesh during the years 2010 and 2011 in the months of May to July to mark the areas infecting with leaf curl disease and for collection of virus isolates. The cultures of TYLCV isolates were collected from naturally infected tomato plants.

The isolates were collected on the basis of symptoms (reduction in leaf size, curling, interveinal and marginal necrosis, purple discoloration of the abaxial surface of leave, shortening of internodes, development of small branches and reduced fruiting) and were maintained by tongue grafting on healthy plants of 'Him Sona' variety of tomato under insect proof glasshouses of Department of Biotechnology and Department of Mycology and Plant Pathology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.). Insecticidal sprays of malathion (organophosphate 1 ml/l) were applied regularly at an interval of 15 days to control insect population in the glass house.

Infected leaves exhibiting typical symptoms were taken and brought to the laboratory for serological detection of TYLCV in different isolates of tomato. Biotin specific double antibody sandwich (DAS) form of enzyme linked immunosorbent assay (ELISA) was used for conducting the experiment.

Molecular diagnosis

Double antibody sandwich-enzyme linked immunosorbent assay as devised by Rojas *et al.* 2005a was conducted for molecular diagnosis as per the protocol of suppliers (Sediag SAS, Longvic, France) of ELISA reagents using antisera against TYLCV.

PCR Amplification of DNA Using TYLCV Specific Primers

Total DNA from infected tomato leaf samples showing symptoms, were isolated by CTAB method as experimented by Rojas and co-workers, 2005b with some modifications**.** PCR was carried out in an automated thermocycler (2720 Thermal Cycler, Applied Biosystems, Life Technologies, Singapore).

Total reaction mixture of 25 µl was prepared for each isolate which comprised of 4 µl of DNA product, 1 µl (20 pmol) of upstream (forward) primer, 1 µl (20 pmol) of downstream (reverse) primer, 1.25 µl of 2 mM dNTP mix, 0.5 µl (3U/ µl) of Taq DNA polymerase (Genei, Bangalore), 2.5 µl of 10X PCR Buffer and 14.75 µl of double distilled water. PCR programme used for the amplification of target fragment consisted of initial denaturation step at 94°C for 4 min; 35 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 60 sec and extension at 72°C for 4 min; followed by final primer extension at 72°C for 10 min.

Three primer pairs were designed by applying online primer designing tool "Primer 3" by analysing sequence DQ029202. Out of the three primer pairs designed, primer pair1 amplified 770 bp long cp gene sequence in two DNA samples.

Viral DNA extraction and Sequencing

PCR product of each isolate (10 µl) was mixed with 3 µl of gel loading dye and loaded in wells of agarose gel. Gel was run at 60V for 2 hours in 1X TAE buffer. 10 µl of 1Kb DNA ladder (Genei, Bangalore) was also electrophoresed to serve as molecular weight marker. After run, gel was analyzed under UV trans-illuminator and photographed using gel documentation system (Ingenius, Syngene, UK). After agarose gel electrophoresis, specific DNA bands were eluted using QIAquick gel extraction kit (Qiagen, Germany). DNA sequencing has been performed using the chain termination method given by Sanger and Co-workers, 1977. DNA in specified range (minimum conc. ≥ 30-100 ng/µl) were sent for sequencing to Genei, Bangalore, India along with the primer pair used in cp gene amplification.

In Silico Sequence Analysis

TYLCV CP gene sequence was analyzed using online tools BLASTN and BLASTP to know their homology among themselves and with other sequences of Tomato leaf curl virus, already submitted to NCBI.

CP gene sequence of Tomato leaf curl virus was translated into protein sequence using protein translator tool at ExPASy (Expert Protein Analysis System) server which is a bioinformatics resource portal operated by the Swiss Institute of Bioinformatics. BankIT internet tool was used for the submission of nucleotide sequences to the NCBI, GenBank database. During present investigations, multiple sequence alignment (MSA) of nucleotide sequences were generated between the test virus coat protein gene sequence with other TYLCV isolates of India and world, retrieved from NCBI database. From the resulting MSA, inference of sequence homology and phylogenetic analysis was carried out. MSA was performed using CLUSTAL W program.

Computational Phylogenetics

For the computational phylogenetic studies, MEGA (Molecular Evolutionary Genetics Analysis) version 4 was used. Phylogenetic trees were generated by using Unweighted Pair Group Method of Arithmetic Averages (UPGMA), Neighbor-Joining (NJ), Maximum Parsimony (MP) and Minimum Evolution (ML) methods. MSA for generating phylogenetic trees of the sequences was done using CLUSTAL W program, integrated with MEGA version 4. For creating multiple datasets, a number of replicates for bootstrapped data were adjusted to 1000 with random seed parameter as 33 (any number of the order 4n+1). Finally tree explorer was used to view the constructed trees.

Table 4: Acronyms and reference number of gemini viruses from all over the world compared with test virus

Sl. No.	Reference No.	Virus Isolates
1	291292519	African cassava mosaic virus clone ACMV
2	37591454	Potato yellow mosaic virus replication protein (AC1) and coat protein (AV1) genes, partial cds
3	331486	Bean golden yellow mosaic virus- [Dominican Republic]
4	155184312	Squash leaf curl virus isolate SLCV-JOR coat protein gene, partial cds
5	9626213	Beet curly top virus - California
6	158562141	Beet mild curly top virus strain BMCTV-Mexico, complete genome
7	332651363	Beet severe curly top virus isolate BSCTV_Mex2 C1 protein and V4 protein genes, partial cds
8	10257473	Horseradish curly top virus, complete genome
9	197210074	Pepper yellow dwarf virus - New Mexico
10	395864230	Chloris striate mosaic virus isolate AU-QG29-2011
11	307752551	Digitaria didactyla striate mosaic virus strain DDSMV
12	59357	Maize streak virus - A [Nigeria1] DNA sequence (MSV)
13	166919160	Sugarcane streak Reunion virus isolate SSRV-A[RBas
14	295042143	Wheat dwarf virus complete genome, isolate WDV-HU-Pula
15	20564194	Tomato pseudo-curly top virus, complete genome
16	1419330	Tomoato pseudo-curly top virus complete DNA sequence

Results and Discussion

Serological Detection (DAS-ELISA)

Tomato leaf curl virus was detected in tomato samples brought from different field locations. The mean absorbance value was measured at 405 nm wavelengths. Highest absorbance was recorded in tomato sample brought from Kotbeja (1.178) followed by Tohana (0.965) and least absorbance was found in samples brought from Pathru-2 (0.231). It was clear from the results that all the samples infected with leaf curl virus brought from the field showed positive reaction for TYLCV antisera with OD values ranging from 0.231 to 1.178 (Table 2).

PCR Amplification of CP gene using gene specific primer

Young tomato leaves (2-3gm) from plants showing typical leaf curl symptoms were used for DNA isolation. DNA from each sample (Manjhed, Tohana, Kotbeja, Pathru) was analysed using agarose gel electrophoresis and samples from Tohana and Manjhed showed high molecular weight DNA bands, when electrophoresis was performed in 1% agarose gel, using 1kb DNA ladder as molecular weight marker (Fig. 3). Primers were designed using already submitted CP gene sequence from the same laboratory (NCBI Nucleotide Database Accession No DQ029202). For designing primer sequences online NCBI tool Primer3 was used (Fig. 4). DNA isolated was subjected to PCR amplification using "primer pair 1" (Fig. 4).

The PCR product thus obtained from every sample was then made to undergo electrophoresis using 100 bp DNA ladder as molecular weight marker. Lane no 5 and 9 showed presence of amplified fragment band, just below 800bp band of marker in the gel, corresponding to Tohana and Kotbeja samples respectively (Fig. 5). Whereas, other samples from Pathru-1, Pathru-2, and Manjhed did not show any cp gene specific band in the agarose gel. For further investigations Tohana isolate was selected. The PCR amplified products were electrophoresed on 1% agarose gel using 100bp DNA ladder as weight marker. DNA from the gel pieces was extracted after the required fragments were cut out. After agarose gel electrophoresis, DNA elution was performed to check integrity and presence of PCR amplified product. The eluted DNA was found to be intact and of desirable length.

Sequencing

Purity of DNA was investigated by quantification using a spectrophotometer. DNA was in specified range i.e. ≥ 30-100 ng/µl and sent for sequencing to Genei, Bangalore, India along with the primer pair used in cp gene amplification. Coat protein gene sequence obtained from TYLCV isolate was found to be 771 nucleotides long.

In Silico sequence analysis

Different online/offline tools were used to determine the relationship between TYCV test virus and other begomoviruses. These included BLAST searches using cp gene nucleotide sequence at NCBI, multiple sequence alignment using CLUSTAL W and construction of phylogenetic trees. Analysis of coat protein (CP) gene sequence of TYLCV revealed its homology with various other complete and partial coat protein gene sequences of viruses causing leaf curl disease abroad and from India. The cp gene nucleotide information was available in the database of National Centre for Biotechnology Information (NCBI).

Accession numbers of the begomoviruses included in the comparison are given in Table 3. The sequence shared similarity with cp gene of North Indian isolates in range 90-99%, where the maximum similarity (99%) was found to be with accession no DQ029202.1 (Fig. 9). Using online NCBI tool Bank IT, Tomato leaf curl virus CP gene sequence was submitted to the publicly accessible GenBank database and allotted accession No- KC 253231 (Fig. 8).

Phylogenetic studies

Phylograms were prepared using sequences of all viruses taken under investigation using CLUSTAL W offline tool. The trees so constructed were based on cp gene sequences and it was evident from figure 6 that TYLCV test virus constituted a group of viruses distinct from South Indian isolates. In second phylogenetic tree the test virus was compared with other geminiviruses and placed itself among begomoviruses forming a different cluster. Phylogenetic tree (Fig. 6) depicts that TYLCV placed itself in the begomovirus group and showed

(a) leaf in ward curling

(b) Yellow leaf margins

Fig. 1: Symptoms of tomato yellow leaf curl virus in the field conditions

a,b,- Maintenance of healthy plants under fine net ,to prevent mixed infection by other virus vectors

c,d- inoculation of virus from viral isolates through tongue grafting

Fig 2. Healthy plants under glass-house and inoculation of virus through tongue grafting method

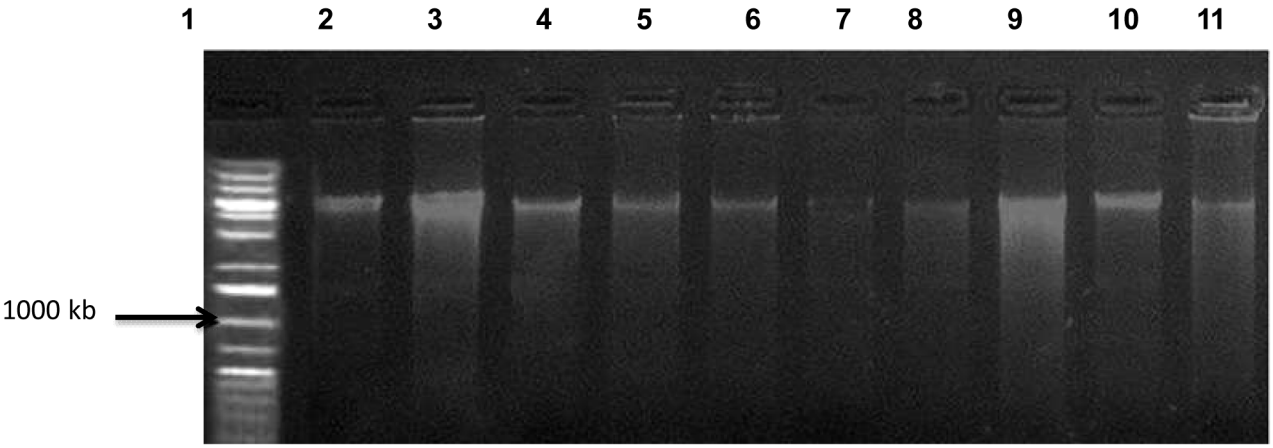

Fig. 3: Gel Electrophoresis of plant genomic DNA in 1% agarose gel

LANE 1 – 1 kb DNA ladder as molecular weight marker

LANE	AREA
2 & 3	Manjhed
4 & 5	Tohana
6 & 7	Pathru 1
8 & 9	Kotbeja
0 & 11	Pathru 2

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAAAAGCAAAGGCCTGGACC	Plus	20	116	135	59.97	55.00	6.00	3.00
Reverse primer	ATCCTGTAGGACGACGGTCA	Minus	20	442	423	60.03	55.00	6.00	3.00
Product length	327								

Primer pair 2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CATCATTTCAACGCCCGCAT	Plus	20	24	43	59.90	50.00	3.00	2.00
Reverse primer	GGTCCAGGCCTTTGCTTTTG	Minus	20	135	116	59.97	55.00	6.00	0.00
Product length	112								

Primer pair 3

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAAAGTACGCCGACGTCTCA	Plus	20	45	64	60.11	55.00	6.00	2.00
Reverse primer	TCATACTTGCCGGCCTCTTG	Minus	20	659	640	60.11	55.00	6.00	2.00
Product length	615								

Fig. 4: Primers designed using online tool Primer3 based on TYLC V sequence DQ029202 from NCBI database (star represents the primer sequence which successfully amplified TYLCV coat protein

similarity with PYMV, SLCV and BSCTV. However, within the group it showed close resemblance with SLCV and indicates its evolution from a common origin. Further, among geminiviruses, TYCV (test virus) is found more closely related to WDV, SSRV and CSV belonging to Mastre virus group, whereas, least similarity was found with Curto viruses.

Amino acid sequence of CP gene of Tomato Yellow Leaf Curl Virus was analyzed with 26 other CP gene sequences from different parts of the world available in the database of NCBI. Details of virus strains used for such comparisons are given in the Table 4. It is clear from the figure 7 that the test virus was more closely related to Dharwad isolate than as compared to other north Indian isolates like

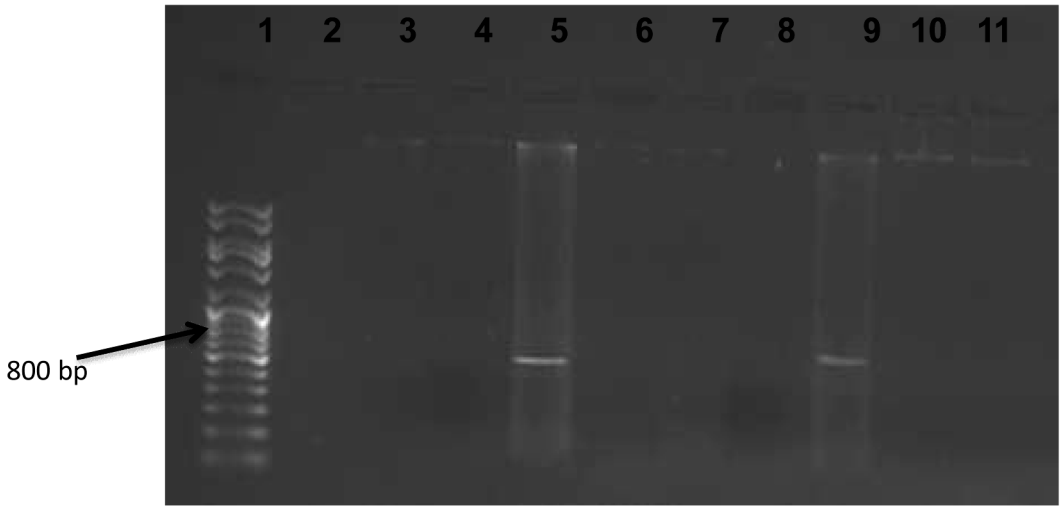

Fig. 5: Gel Electrophoresis of PCR amplified products (~800 bp) using coat protein gene specific markers for TYLCV. Lane1 represents 100 bp DNA ladder, 5 and 9 denotes isolates of TYLCV from Tohana and Kotbeja areas respectively

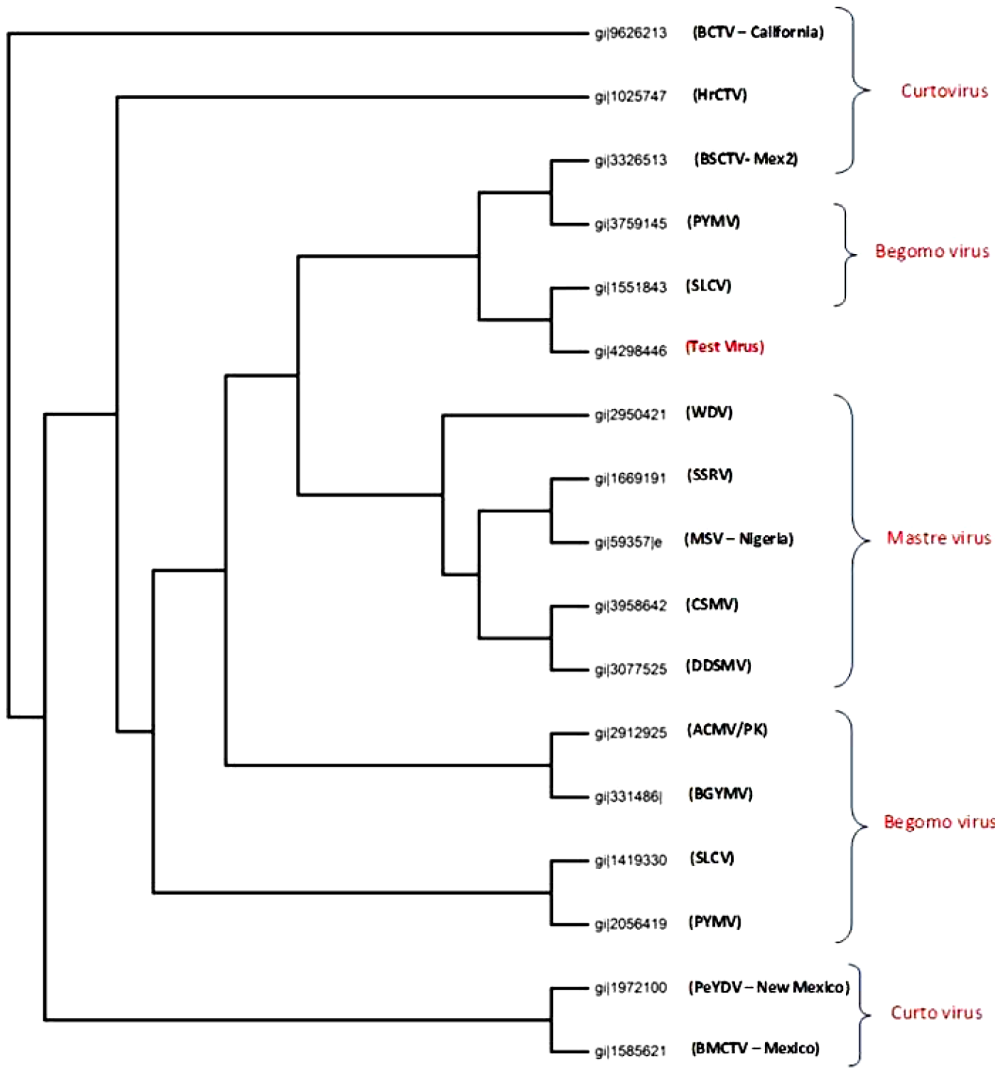

Fig. 6: Phylogenetic tree depicting the relationship of Test virus with other Gemini viruses

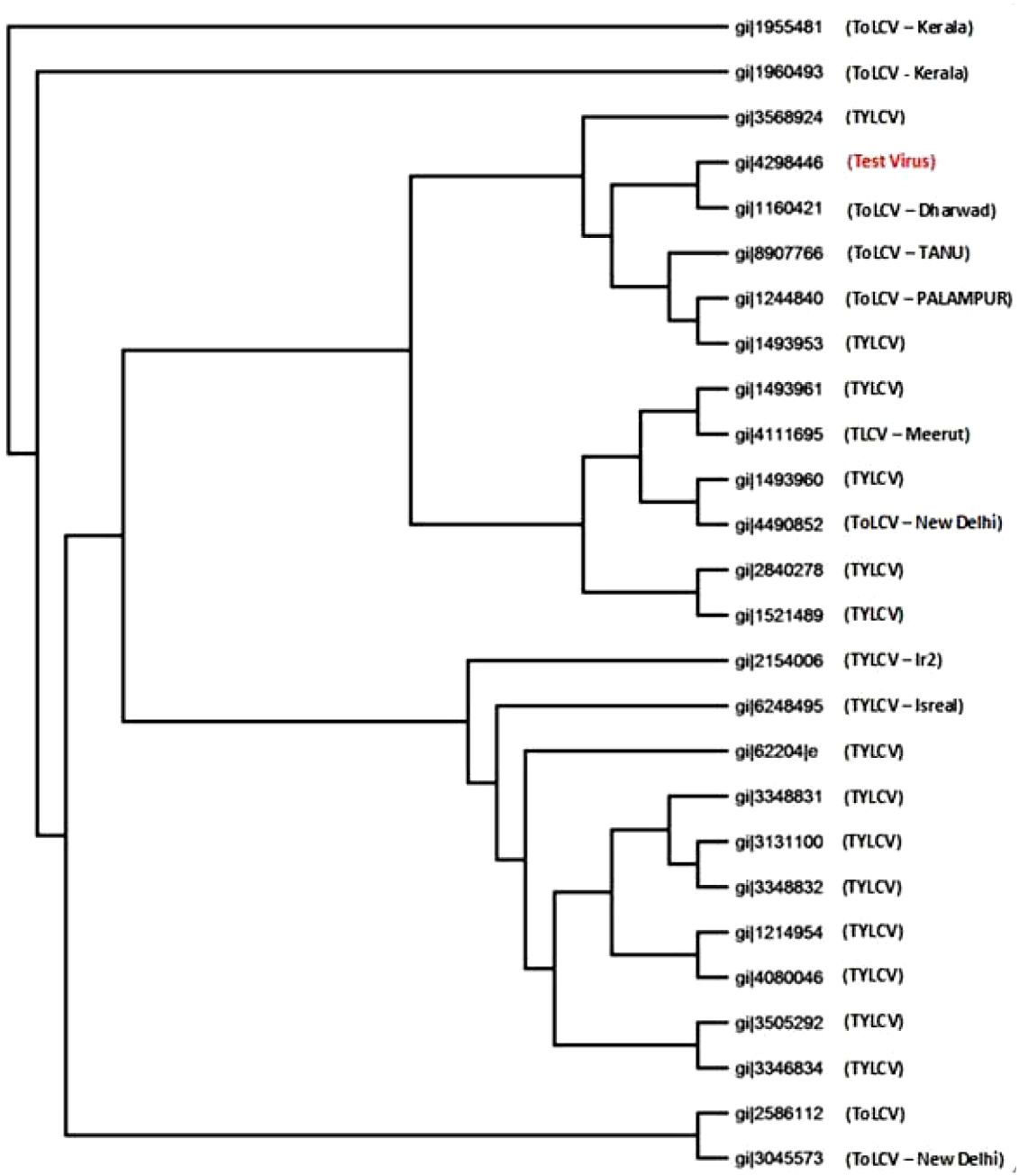

Fig. 7: Phylogenetic analysis of Test virus with tomato leaf curl isolates from world over

Palampur and Ludhiana, which gives a clue about its origin, way back, from central India. Further, it gives an indication that the possible spread through nursery plantations or fruits may have occurred, while the test virus showed some similarity with isolates from New Delhi, Meerut and other Indian isolates. It further showed least similarity with isolates from Kerala, Israel and isolates from other parts of the world.

Tomato (*Lycopersicon esculentum* Mill.) is one of the major economic vegetable crops of *Solanaceae* family after potato in the world. Microbial pathogens cause heavy losses to the vegetable and fruit crops every year, with tomato being no exception to it. Out of these pathogens, viruses are most important with no remedy available against the infection. Many of these viruses belong to begomoviruses and produce similar symptoms upon infection and tomato leaf curl virus is the most common virus infecting tomato crop all over the Indian subcontinent. Therefore

```
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            partial cds.
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VERSION     KC253231.1  GI:429844638
KEYWORDS    .
SOURCE      Tomato leaf curl virus
  ORGANISM  Tomato leaf curl virus
            Viruses; ssDNA viruses; Geminiviridae; Begomovirus.
REFERENCE   1  (bases 1 to 771)
  AUTHORS   Verma,Y.S., Bhardwaj,S.V. and Sharma,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-NOV-2012) Biotechnology, Dr YS Parmar University of
            Horticulture and Forestry, Solan, Himachal Pradesh 173230, India
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                     YENHTENALKLYMACTHASNPVYATLKIRIYFSDSVTNE"
ORIGIN
        1 atggtgaagc gaccagcaga tatcatcatt tcaacgcccg catcgaaagt acgccgacgt
       61 ctcaatttcg acagccccta tggagctcgt gcagttgtcc ccattgcccg cgtcacaaaa
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      541 acgtggcatg cgactgtgac gggaggaaca tatgcatcta gggagcaagc attagttagg
      601 aagtttgtta gggttaataa ttatgttgtt tataatcaac aagaggccgg caagtatgag
      661 aatcatactg aaaacgcatt aaagttgtat atggcctgta ctcatgcatc aaatcctgta
      721 tatgctactt tgaaaatccg gatctacttt tctgattcgg taacaaatga a
//
```

Fig. 8: Submission of TYLCV cp gene nucleotide sequence to NCBI database

molecular techniques for detection and analysis of the disease are very important for effective disease management. Further virus specific drug-development is a costly and time consuming affair but is need of the hour for reliable management of the diseases and the first step towards drug development is the *in silico* identification of putative inhibitors (Sivakumar *et al.*, 2012; Prajapat *et al.*, 2011; Shikhi *et al.*, 2013). Based on the symptoms like inward curling of leaves, stunted growth, puckering, samples were collected from Kotbeja, Tohana, Manjhed, Pathru-1 and Pathru-2; five localities within Solan district of Himachal Pradesh. The field samples reacted positively with the antisera to TYLCV. Sambyal, 2005 also showed the presence of TYLCV from different areas of Himachal Pradesh while characterizing the leaf curl causing virus in tomato. ELISA is a broad test for detection of viruses

Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Tomato leaf curl virus isolate YSPDBT-1206 coat protein gene, partial cds	1424	1424	100%	0.0	100%	KC253231.1
Tomato leaf curl virus isolate Himachal coat protein gene, complete cds	1391	1391	99%	0.0	99%	DQ029202.1
Tomato leaf curl New Delhi virus from bottle gourd coat protein gene, complete cds	1286	1286	99%	0.0	97%	DQ272540.2
Tomato leaf curl New Delhi virus-Severe segment A, complete sequence	1286	1286	99%	0.0	97%	U15015.2
Tomato leaf curl virus AV1 gene for coat protein, isolate 26	1277	1277	98%	0.0	97%	AJ810365.1
Tomato leaf curl New Delhi virus-India [India/Tumkur/Chilli/2008] clone pChTumB2 segment DNA-A, complete sequence	1275	1275	99%	0.0	97%	HM007120.1
Tomato leaf curl New Delhi virus isolate ToLCNDV-[Pum:IARI:06] segment DNA A, complete sequence	1275	1275	99%	0.0	97%	JN129254.1
Tomato leaf curl New Delhi virus [Pakistan:Solanum:2009] complete segment DNA-A, clone MS4	1275	1275	99%	0.0	97%	FN435310.1
Tomato leaf curl New Delhi virus-[Pumpkin:New Delhi] segment DNA-A, complete sequence, isolate 2	1275	1275	99%	0.0	97%	AM286434.1
Luffa begomovirus coat protein (AV1), AC3 (AC3), and AC2 (AC2) genes, complete cds	1275	1275	99%	0.0	97%	AY309957.1
Tomato leaf curl New Delhi virus isolate ToLCNDV-Severe segment A, complete sequence	1269	1269	99%	0.0	96%	HM345979.1
Tomato leaf curl New Delhi virus-Severe segment DNA A, complete sequence	1269	1269	99%	0.0	96%	HM159454.1
Tomato leaf curl New Delhi virus, complete genome, clone ToA2	1269	1269	99%	0.0	96%	AM258977.1
Tomato leaf curl New Delhi virus-[Multan:Luffa] V2 gene, AV3 gene, CP gene, AC1 gene, REn gene, TrAP gene, AC4 gene and AC5 gene	1269	1269	99%	0.0	96%	AM292302.1
Tomato leaf curl virus coat protein (AV1) gene, complete cds	1269	1269	99%	0.0	97%	AY838895.1
Tomato leaf curl New Delhi virus - [Pakistan:Solanum:1997] segment A, complete sequence	1269	1269	99%	0.0	96%	AJ620187.1
Tomato leaf curl New Delhi virus-JID27 segment DNA-A, complete sequence	1264	1264	99%	0.0	96%	HQ141673.1
Tomato leaf curl New Delhi virus-[Pumpkin:New Delhi] segment DNA-A, complete sequence, isolate 1	1264	1264	99%	0.0	96%	AM286433.1
Tomato leaf curl New Delhi virus - [Pakistan:Solanum] isolate Rahim Yar Khan 2 clone PT6 segment A, complete sequence	1264	1264	99%	0.0	96%	DQ116885.1
Tomato leaf curl New Delhi virus - chili pepper clone PC2No10 segment A, complete sequence	1264	1264	99%	0.0	96%	DQ116880.1
Tomato leaf curl virus coat protein (AV1) gene, complete cds	1264	1264	99%	0.0	96%	AY691902.1
Tomato leaf curl virus coat protein (AV1) gene, complete cds	1264	1264	99%	0.0	96%	AY691900.1
Tomato leaf curl New Delhi virus isolate X 8 segment DNA-A, complete sequence	1258	1258	99%	0.0	96%	FJ468356.1
Tomato leaf curl virus coat protein (AV1) gene, complete cds	1258	1258	99%	0.0	96%	AY691899.1
Tomato leaf curl virus isolate Raebareli segment DNA-A, complete sequence	1253	1253	99%	0.0	96%	JX232220.1
Tomato leaf curl New Delhi virus segment DNA A, complete sequence	1253	1253	99%	0.0	96%	DQ169056.1
Tomato leaf curl New Delhi virus - [PkT1/8] segment DNA-A, complete sequence	1253	1253	99%	0.0	96%	AF448059.2
Tomato leaf curl New Delhi virus segment A, complete sequence	1253	1253	99%	0.0	96%	AY939926.1
Tomato leaf curl New Delhi virus-Mild coat protein AV1, AV2, AV3, replication-associated protein AC1, AC2, AC3, AC4 and AC5 genes, compl	1253	1253	99%	0.0	96%	U15016.1

Fig. 9: NCBI Blast results for TYLCV test virus sequence

based on the antisera produced for a specific virus, however, may detect different strains of the same virus which may have evolved from the present strain in due course of time. Therefore for further characterization, polymerase chain reaction (PCR) based on specific primers is the procedure of choice (Kumar *et al.,* 2013 ; Ponsubrya *et al.,* 2014) which was also performed during these investigations.

Among the five isolates collected for investigation only the DNA samples from Tohana and Kotbeja localities showed positive amplification of cp gene using specific primers. Non amplification of other isolate viz; Pathu-1, Pathru-2 and Manjhed is indicative of strainal differences in the causal virus due to changes at nucleotide level. Such evolution of different viruses within a group is also supported by the work of Rojas and co-workers, 2005 indicating development of Tomato Severe Leaf Curl Virus (ToSLCV) from Tomato chino La Paz Virus (ToChLPV) through evolution. Similar symptoms are also reported to have been produced by different strains of a particular virus of TYLCV in this case or some other viruses as well. However under these investigations presence of viruses other than TYLCV is ruled out because of positive reaction with ELISA. For sequence comparison, classification and phylogenetic studies, sequencing is necessary (Padidam *et al.*,1995 and Krenz *et al.,* 2014). In the present studies, we report a PCR method based on coat protein specific primers, which were designed by using genomic sequence of TYLCV (accession no DQ029202). A similar approach was followed by research workers (Li *et al.,* 2012) for detection of TYLCV. However, the amplified band obtained

in the present study for TYLCV is 771 bp which is specific for CP gene where as in similar study, 543 bp TYLCV coat protein specific band was amplified which signifies different strains of TYLCV in India and China (Li *et al.,* 2012).

The amplified products of polymerase chain reaction for TYLCV test virus coat protein coded for 257 amino acids. Present procedure is in similitude to that followed by many other researchers who worked on different begomoviruses including TYLCV (Shiraji *et al.,* 2014; Hererra *et al.,* 1999; Lim *et al.,* 2012; Pratap *et al.,* 2011 and Rojas *et al.,* 2005). They, however, got products of different lengths depending upon the viruses under test. The CP gene sequence of the test virus was compared with the Indian isolates and isolates from the world and with other geminiviruses using offline tools CLUSTAL W and PHYLIP. When compared with geminiviruses world over, the test virus formed a cluster with SLCV, PYMV and BSCTV, which placed it in the begomovirus group; though BSCTV is a curtovirus. Its close resemblance with SLCV indicates their common origin and evolutionary similarity.

In similar investigations based on phylogenetic studies, the involvement of ToChLPV (Tomato Chino La Paz Virus) in evolution of ToSLCV (Tomato Severe Leaf Curl Virus), was pointed out by Rojas and co-workers (2005). The test virus in present studies did not show resemblance to other begomoviruses like ACMV, BGYMV which formed a separate cluster within the phylogram, which gives an indication about the evolution of new virus strain of TYLCV. This fact was further strengthened when the test virus was compared with other isolates from Northern India. Although, it showed similarity from 70 to 99 percent with many of the north Indian isolates of TYLCV, in phylogram it formed a group with Tomato Leaf Curl Virus isolate from Dharwad (GI 1160421) and not with ToLCV isolate from Palampur (GI 1244840).

Scientists from world over, (Kumar *et al.,* 2012, Li *et al.,* 2012; Mohana Rao J.K. 1985; Pandey *et al.,* 2010; Sawangjita *et al.,* 2005; Zhang *et al.,* 2009) also have used computational phylogenetics to study the course of evolution and to record genetic variability of various begomoviruses like TYLCV, TYLCTHV (Tomato Yellow Leaf Curl Thailand Virus), TbCSV (Tobacco Curly Shoot Virus) and ACMV (African Cassava Mosaic Virus). It is a must that the diversity of geminiviruses infecting tomato should be considered and studied, so as to develop new and effective strategies like gene silencing, which depend on precise sequence information about the target.

Conclusion

The research paper highlights the evolution of new strain of TYLCV through in silico analysis and also emphasises on a need for proper diagnosis and new methods for development of antiviral strategies utilizing the genomic content of infecting virus.

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