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Full Length Research Paper

Detection and diagnosis of tomato leaf curl virus infecting tomato in Northern Karnataka

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Tomato (*Lycopersicon esculentum* Mill.) is an important and most widely grown vegetable crop in India. The begomo viruses' affecting tomato in India is the most devastating and is a major limiting factor in the tomato production. The tomato leaf curl virus (ToLCV) was present in almost all fields of Belgaum, Dharwad and Haveri districts surveyed with the disease incidence ranged from 4-100% in rabi and was in severe form ranging from 60-100% during summer. All the five representative symptomatic samples collected from the different regions of North-Karnataka were found positive for polymerase chain reaction (PCR) amplification with specific primers for deoxyribonucleic acid (DNA); a component of tomato leaf curl Bangalore virus (ToLCBV). To examine the diversity of the sequences, phylogenetic trees were generated for the four CP sequences together with representative sequences available in gene Bank. The isolates under study clustered into two groups. The Dharwad isolate and Belgaum- 2 isolate were closely related (99.4% nucleotide similarity) and formed in to one cluster in which Haveri and Belgaum-1 isolates had comparatively less homology (97.30% nucleotide homology) between themselves and clustered into another sub group. The isolates under study had lowest nucleotide sequence homology of 53.50 to 53.90% with ToLCV 19.Patna (AJ 810358) followed by ToLCV 18.Malvastrum.Pa (AJ 810357) (53.50-54.00%), ToLCV 17. Nasik (AJ 810356) (53.90-54.30%), while they had highest homology of 92.40-96.00% with ToLCBV-AVT 1 (AY 428770). The results revealed that these isolates are entirely different from North Indian isolates and there is some variability within the isolates collected from a geographical location, indicating that there will be continuous variability in gemini viruses.

Key words: ToLCV, detection, diagnosis, cloning, nucleotide sequence.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is an important and most widely grown vegetable crop in India. Virus diseases are the major production constraints. Among the virus diseases, tomato leaf curl disease; tomato leaf curl Bangalore virus (ToLCBV), tomato leaf curl New Delhi virus (ToLCNDV), tomato leaf curl Karnataka virus (ToLCKV) and tomato leaf curl Gujarat virus (ToLCGV) are the important begomo viruses which limit the tomato production.

The begomo viruses affecting tomato in India are the most devastating and the early work on the geographical distribution of twolined chestnut borer (TLCB) species within India has shown that the begomo viruses affecting tomato in northern India are bipartite and those affecting tomato in Southern India are monopartite (Muniyappa et al., 2000; Kirthi et al., 2002). These two groups of viruses are quite distinct in their biological activity and genomic organizations.

The incidence of ToLCV has become a major limiting factor and challenge to farmers and scientists. The tropical climate in Southern India allows year-round tomato cropping, which together with the presence of perennial host plants for both TLCBs and *B. tabaci*

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Abbreviations: ToLCV, Tomato leaf curl virus; **PCR,** polymerase chain reaction; **DNA,** deoxyribonucleic acid; **ToLCBV,** tomato leaf curl Bangalore virus; **ToLCNDV,** tomato leaf curl New Delhi virus; **ToLCKV,** tomato leaf curl Karnataka virus; **ToLCGV,** tomato leaf curl Gujarat virus; **TLCB,** twolined chestnut borer; **ORF,** open reading frame.

(Ramappa et al., 1998; Sastry et al., 1978), enables the easy carry-over of ToLCD between growing seasons. Until the late 1990s, the main control method employed against ToLCD was the intensive use of insecticides targeted at viruliferous immigrant adult *B. tabaci* that spread TLCBs into and within tomato crops. The existence of variability among the virus isolates is the main reason for the break down of resistance in the ruling varieties.

In order to manage the deployment of these resistant varieties to improve the efficacy of the ToLCD-resistant material, an improved understanding of the diversity and distribution of TLCBs present in a region is required. Though the disease has been known for quite a long period with a good amount of literature on various aspects of the disease, the information regarding the extent of diversity among the different isolates of the ToLCV in the tomato growing areas of North Karnataka was lacking. Considering that the present work is initiated in identification of ToLCD isolates in North Karnataka and development of reliable detection method suitable for development of various management strategies.

MATERIALS AND METHODS

Survey for disease incidence

A roving survey was conducted during rabi 2005 and summer 2006 in major tomato growing districts of North Karnataka. In each visited field, a plot area of 10 x 10 m area was selected and the total number of plants and infected plants showing leaf curl symptoms was recorded separately and the percent disease incidence was calculated. Three such plot areas were selected randomly in each field and mean of three plots was calculated. The overall disease incidence and severity was recorded based on visual symptoms.

Collection of ToLCV isolates and DNA extraction

A survey was undertaken to collect the samples of ToLCV and the leaves and twigs exhibiting typical symptoms were collected from all the fields visited in Belgaum, Dharwad, Gadag and Haveri districts of North Karnataka. The samples showing some variations in symptoms within a cultivar were chosen for further analysis. The total deoxyribonucleic acid (DNA) was extracted from the representative field samples, following standard DNA extraction protocols of Cetyl trimethyl ammonium bromide method (Srivatasva et al., 1993). The extracted DNA was quantified for the concentration and was diluted appropriately in sterile distilled deionised water before being subjected to polymerase chain reaction (PCR) amplification using tomato leaf curl specific primers.

PCR amplification and cloning

The total DNA extracted was used for PCR amplification of ToLCV DNA. Further, the PCRs were carried out in 25 µl of reaction mixture containing DNA template (50-60 ng) -1.0 µl, deionised nuclease free water-16.0 µl, 10X PCR buffer (supplied with enzyme)-2.5 µl, 2 mM dNTPs -2.0 µl, 25 mM $MgCl_2$ - 2.0 µl and primers- forward primer (20 pmol/µl)- 0.5 µl, reverse primer 20 pmol/µl) -0.5 µl and Taq DNA polymerase (1.5 U/µl) - 0.5 µl. All the reaction components were procured from MBI Fermentas, Germany.

The PCR amplification was carried out in a thermal cycler (Biometra) with initial-denaturation at 94°C for 2 min followed by 35 cycles each consisting of denaturation at 94°C for 45 s, annealing at 55°C for 1 min followed by extension at 72°C for 1.30 min with final extension at 72°C for 20 min. Amplified DNA fragments were electrophoresised in 0.8% agarose gel according to the procedure outlined by Sambrook and Russel (2001).

Sequence of tomato leaf curl specific primers used

Forward primer (ToLCBV33F): 5' GGT CCC CTC CAC TAA ATCAT 3' (20 nt)
Reverse primer (ToLCBV1070R): 5'CAG TTG GTT ACA GAA TCG TAG AAG 3' (24 nt)

Cloning of PCR product

After gel elution of amplified DNA fragment using gel extraction kit (Qiagen, Germany), the PCR amplified DNA fragment was cloned into the plasmid vector pTZ57R/T using T/A cloning kit (MBI, Fermentas) following the manufacturer's instructions. The vector also contains the ampicillin resistant gene for antibiotic selection and the lacZ gene that allows blue/white selection of recombinant colonies by a α- complementation. Ligation was carried out at 22°C for 16 h. The ligation mixture was used for transformation. The competent cells *of Escherichia coli* strain DH5 α were prepared by calcium chloride method as described by Sambrook and Russel (2001).

Screening of clones

Colony PCR was carried out by using the specific primers to confirm the clones carrying the insert. The confirmed colonies were then grown in 3-5 ml LB containing ampicillin (100 mg/ml) at 37°C overnight in a rotary shaker at 100 rpm. Plasmid mini-preparation was done from the culture by alkaline lysis method as described by Sambrook and Russel (2001).

After confirmation of the presence of inserted DNA, the clone was named as pTZ57R+CP and the isolates were named based on the geographical location from which it was collected.

Sequencing of the cloned insert

After successful confirmation of the presence of expected insert in the clone, the plasmid DNA was isolated in large scale using plasmid extraction kit (QIAGEN GmbH, Hilden, Germany) and sequenced using the automated sequencing facility at Microsynth, Switzerland. Sequencing was done in both directions using M 13 forward and reverse primers in an ABI Prism 377 DNA sequencer (MWG Biotech Limited).

Sequence analysis

The complete coat protein sequences of ToLCV isolates from various geographical locations were obtained from NCBI (Table 1). For sequence comparison, published nucleotide and amino acid sequences from related viruses of gemini group were used. Genebank (Benson et al., 1996) searches were done using the BLAST program (Altschul et al., 1990). The nucleotide sequences were translated to amino acid sequences using the bioedit program and the open reading frame (ORF) was also determined using bioedit program. Dendrogram and phylogenetic analysis was

Table 1. Reference geminivirus sequences and their GenBank accession numbers.

Designation	Region/country	GeneBank source
TLCGV-Kelloo	Gujarath, India	AF449999
ToLCGV-Nepal	Gujarath, India	AY234383
TLCGV-Varnasi	Varnasi, India	AY190290
ToLCGV-Vadodara	Vadodara, India	AF413671
ToLCV19.Patna	Patna, India	AJ810358
ToLCV17.Nasik	Nasik, India	AJ810356
ToLCCV-G18		AJ558119
ToLCV18.Malvastrum.Pa		AJ810357
CrYVMV		AJ507777
SiYVV-Madurai	Madurai, India	AM259382
ToLCNDV-Severe	New Delhi, India	U15015
SYLCV		AJ620187
ToLCNDV-PkT1/8	New Delhi, India	AF448059
TLCNDV-Lucknow	Lucknow, India	Y16421
ToLCNDV-PkT5/6	New Delhi, India	AF448058
ToLCNDV-Mild	New Delhi, India	U15016
ToLCNDV-Potato	New Delhi, India	AY286316
ToLCNDV-AVT1	New Delhi, India	AY428769
ToLCNDV-S[Jessore]	New Delhi, India	AJ875157
TLCNDV-Luffa	New Delhi, India	AF102276
SqLCCV-[Pumpkin_Coimb	Coimbatore, India	AY184487
ToLCV-32.Tirupati	Tirupati, India	AJ810371
ToLCV2.Assam	Assam, India	AJ810341
ToLCV-9.Tomato.Calcut	Calcutta, India	AJ810348
LoYMV		AF509739
ToLCBV-Bangalore	Bangalore, India	Z48182
ToLCV27.Thrissur.Kera	Thrisur, India	AJ810366
VS228.Haveri	Haveri, India	
ToLCBV-TNAU1	Tamil Nadu, India	DQ358098
ToLCV24.Ranibennur	Ranebennuru, India	AJ810363
ToLCV7.Belgaum	Belgaum,India	AJ810346
VS229.Belgaum1	Belgaum,India	
VS226.Dharwad	Dharwad, India	
VS230.Belgaum2	Belgaum,India	
ToLCBV-TNAU2	Tamil Nadu, India	DQ358099
ToLCBV-Kolar	Kolar, India	AF428255
ToLCBV-AVT1		AY428770
ToLCV-30.Maderahalli.	Maderahalli, India	AJ810369
ToLCV-Ban5	Bangalore, India	AF295401
ToLCBV-Cotton	Bangalore, India	AY456684
ToLCV-Ban4	Bangalore, India	AF165098
ToLCSLV	Srilanka	AF274349
|ToLCKV-Ban2	Bangalore, India	U38239
ToLCKV.Arskeri	Arsikeri, India	AY753203
ToLCV3.Aurangabad	Aurangabad, India	AJ810342
ToLCV-Tagetes		DQ339120
TbLCV-Kar1		AY007615
ChLCV-[Multan]	Pakistan	AF336806
ToLCND-PRM		DQ629103
CLCuBV		AY705380
MaYVV-[Y47]		AJ457824

Table 1. Contd.

SLCMV-TN6		AJ890228
ICMV-Mah	Maharastra, India	AJ314739
ToLCV28.Ageratum.Ke		AJ810367
ToLCBdV.Bd2	Bangalore, India	AF188481
PaLCV-PD		DQ376036
ToLCJoV		AJ875159
PepLCBV	Bangalore, India	AF314531
EuLCV-[G35]		AJ558121
CLCuKV-Dabawali	Kokhran	AY456683
BYVMV-[Madurai]	India	AF241479
CLCuAV-[802a]	Alababad	AJ002455
CLCuMV-[Bhatinda_05]		DQ191160
CLCuRV [India_Abohar]	India	AY795606
ToLCV-Taiwan		U88692
MYMIV-[Cowpea]		AF481865
MSV-A		Mastrevirus

Table 2. District wise distribution of disease (ToLCV) incidence during 2005-06.

S/N	District	Percent (%) disease incidence		
		Minimum	Maximum	Average
Rabi-05				
1	Belgaum	4.00	75.00	25.83
2	Dharwad	5.00	60.00	45.00
3	Gadag	8.00	20.00	16.00
4	Haveri	8.00	100.00	33.43
Summer-06				
1	Belgaum	60	100	80
2	Dharwad	90	100	95
3	Gadag	60	90	75
4	Haveri	75	90	87

constructed by neighbour joining method using the Tree View program.

RESULTS

Detection of tomato leaf curl (begomo) virus in field samples of tomato incidence

The survey data of the rabi season revealed that the ToLCV was present in almost all parts of Belgaum, Dharwad, Gadag and Haveri. Disease incidence ranged from 4-100% and was maximum in Hiremathuru (100.0%) of Haveri taluk, UAS campus (100.0%), Sidenur (70.0%) of Byadgi taluk, Itagi (60.0%) of Ranebennur taluk of Haveri district and Hulikppa (60.0%), Kalaghattagi taluk of Dharwad district (Table 2). While the survey data on summer reveals that ToLCV was present in severe form ranging from 60-100% in almost all the major tomato fields surveyed.

Distribution of TLCBs in North Karnataka (PCR detection)

All the five symptomatic representative samples collected from the different regions of North-Karnataka were found positive for PCR amplification with specific primers for DNA; a component of ToLCBV. These primers amplified a ≈ 1040 bp product. These primers produced expected results on the five samples for which CP sequences had been obtained. Table 3 shows the PCR amplification results of the five representative samples used for screening using ToLCBV primers of DNA-A.

Table 3. PCR detection of ToLCV in the tomato samples collected from different regions of North Karnataka.

S/N	Place	Variety/hybrid	Date of collection	PCR detection
1	UAS campus (Dharwad)	Megha	6-12-05	+
2	Honnihalli (Hukkeri Tq) (Belagao)	Hy 2535	5-12-05	+
3	Hattaragi (Hukkeri Tq) (Belagao)	Utsav	5-12-05	+
4	Hiremuttur (Hirekerur Tq) (Haveri)	Sungro seeds	12-12-05	+
5	Bikadakatti (Gadag Tq) (Gadag)	Sungro seeds	3-12-05	+

Cloning and sequencing of coat protein gene of tomato leaf curl virus isolates

The coat protein gene was amplified by PCR and amplified product was eluted from the gel and ligated to pTZ57R/T of size 2.4 kb with T-overhang. Transformation of *E.coli* strain DH_5 α was done with ligation mixture. The bacterial clones carrying the recombinant DNA molecules were colorless and could easily be distinguished from the non-recombinant blue colonies.

Further, the clones carrying the coat protein gene were analyzed and confirmed by colony PCR, which produced about 1.0 kb product as resolved and analyzed by gel electrophoresis. The clones were sequenced using M13 universal forward and reverse primers.

Sequence analysis and phylogenetics

To examine the diversity of the sequences, phylogenetic trees were generated for the four CP sequences together with representative sequences available in gene Bank. The tree was constructed using Clustal-W multiple alignments programme (Figure 1). The isolates under study clustered into two groups. The Dharwad isolate and Belgaum- 2 isolate were closely related (99.4% nucleotide similarity) and formed into one cluster (Figure 1) and Haveri and Belgaum-1 isolates had comparatively less homology (97.30% nucleotide homology) between themselves and clustered into another sub group (Figure 1). The isolates under study had lowest nucleotide sequence homology of 53.50-53.90% with ToLCV 19.Patna(AJ810358)followedbyToLCV 18.Malvastrum.Pa (AJ 810357) (53.50-54.00%), ToLCV 17. Nasik (AJ 810356) (53.90-54.30%). While they had highest homology of 92.40-96.00% with ToLCBV-AVT 1 (AY 428770).

However, within the isolates under study, Haveri isolate (VS 228) had highest nucleotide sequence homology (97.30%) with Belgaum.1 (VS 229) and had lowest homology (95.1%) with Belgaum-2 (VS-230) isolate. The Belgaum –1 (VS 229) had highest homology (97.30%) with Haveri isolate (VS 228) and lowest (96.30%) homology with the Belgaum-2 (VS230).

The Dharwad isolate had highest homology of 99.40% with Belgaum-2 (VS-230) isolate and had lowest homology of 95.60% with the Haveri isolate (VS 228), while the Belgaum-2 (VS230) isolate had lowest homology of 95.10% with Haveri isolate (VS 228).

From Figure 1, it could be concluded that all the isolates under study, collected from North Karnataka were entirely distinct from the North Indian (Gen Bank Accessions) (TLCGV-Varnasi (AY190290)- average of 69.20% homology, ToLCV19.Patna (AJ810358)- average of 53.60% homology and ToLCV18.Malvastrum.Pa (AJ810357) – average of 53.80% homology), North Eastern (ToLCV 2.Assam (AJ810341) - average of 57.00% homology) and North Western Indian (ToLCGV-Vadodara (AF413671) average of 69.0% homology) isolates. However, the isolates under study had less homology with only few South Indian isolates (ToLCV-30.Maderahalli (AJ810369) average of 72.15% homology and ToLCV-24. Ranebennur (AJ810363) average of 71.97% homology). But these had highest homology of 96.00% with the South Indian isolate, ToLCBV-AVT1 (AY428770). The homology with South Indian begomo viruses was ranging from 63.90% with ToLCKV. Arsikeri (AY753203) isolate to 96.00% with ToLCBV-AVT-1 (AY428770) isolate. However, they have got more than 90% homology with majority of the South Indian isolates.

Genetic relationship of ToLCV based on amino acid sequence

To look at the functional diversity of the CPs, their nucleotide sequences were translated into amino acid sequences. Amino acid sequence similarity identity reveals that all the isolates under study had highest amino acid homology 97.60-99.60% with ToLCV Ban-5 (AF295401) and ToLCBV-Cotton (AY456684) followed by ToLCV 27 Thrissur. Kerala (AJ810366). While the lowest homology of 42.90-43.30% was found with SiYVV-Madurai isolate (AF259382) followed by ToLCCV-G18 (AJ558119) with 71.2-71.9% homology.

DISCUSSION

The tests on the samples collected during survey revealed that the ToLCV was present in all the fields visited based on the symptoms in the field and also by

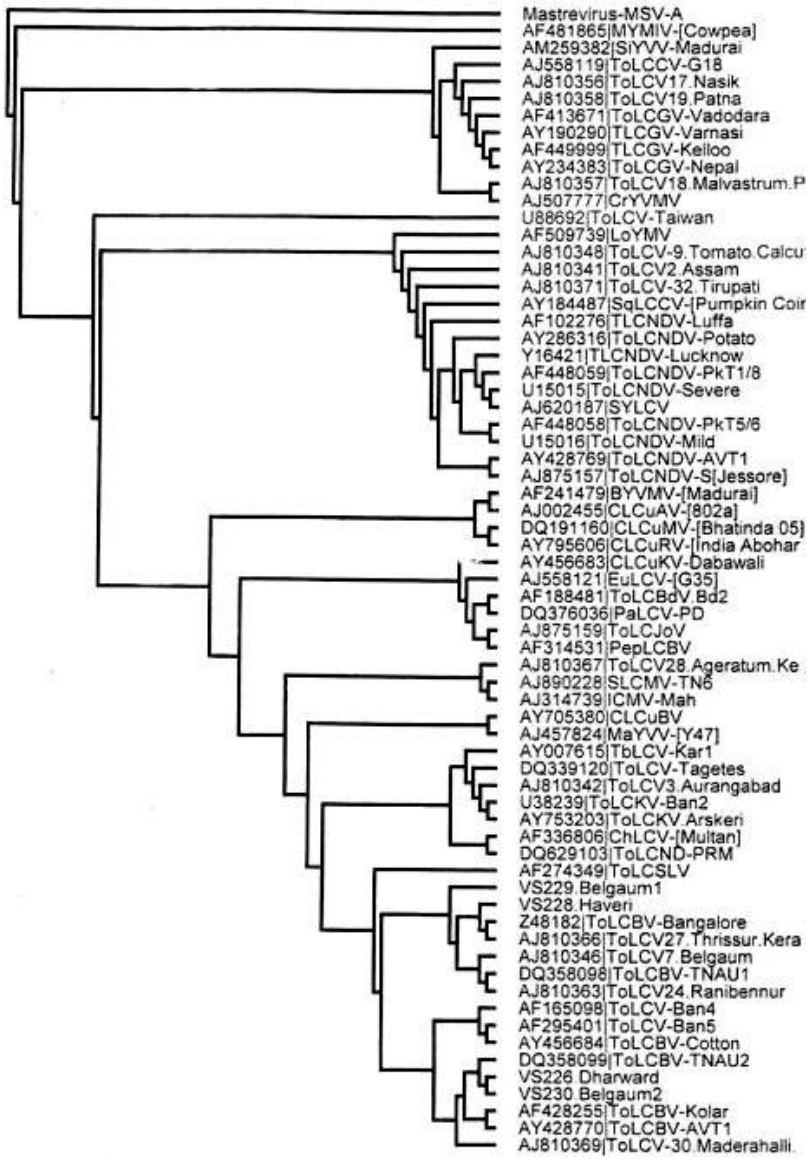

Figure 1. Dendrogram showing clustering pattern of nucleotide sequences of ToLCV coat protein gene from different geographical locations.

PCR amplification in the laboratory.

CP genes represent the most conserved gene in the family Geminiviridae and CP sequences can be used as preliminary virus identification or to infer geographic and vector relationships (Brown et al., 2001). The phylogenetic analysis of the four CP sequences obtained from North Karnataka samples showed that they are all closely related to the isolates from South India (Ban-04, Ban-05, ToLCBV-cotton and ToLCBV-TNAU 2). However, there is only slight variation among the isolates under study and there is greater variation when compared with the isolates/accessions (GenBank) from North India, North Eastern India and also of North Western India. Percentage identity figures for members within each cluster were in agreement with the criteria of <90% CP nucleotide identity representative in general of different geminivirus species (Faquet and Stanley, 2003). However, recently recombination between begomovirus DNA-A molecules has been reported to be a frequent occurrence (Fondong et al., 2000; Kirthi et al., 2002; Zhou et al., 1997). Although the sequencing of the full length DNA-A component is desirable, a partial sequence approach based on CPs was adopted to enable a wider scale study to be conducted on Indian TLCBs diversityand phylogeography.

The isolates under study had very close (95.10-99.6%) homology among themselves with respect to nucleotide sequence, they belonged to one group and they show very wide variation of up to 46.50% divergence with respect to nucleotide sequence with some of the North

Indian ToLCV 17. Nasik (AJ 810356), ToLCV 19.Patna (AJ 810358) isolates and only with few south Indian isolates like ToLCV-30.Maderahalli (AJ810369), ToLCKV. Arsikeri (AY 753203) and ToLCV3.Aurangabad (AJ 810342). And high amino acid homology of 96.8-99.6% among themselves and up to 57.10% with SiYVV-Madurai (AM259382) amino acid sequence diversity with the other North Indian isolates of begomo viruses and up to 29.00% diversity with tomato isolate ToLCCV-G18 (AJ 558119). Similar results were reported by few workers (Padidam et al., 1995), where he sequenced the genomes of two isolates of tomato leaf curl geminivirus from India (ToLCNDV-Mild and ToLCNDV-severe). The ToLCV-Indian isolate contains A and B components and the two isolates have 94% sequence identity. Srivastava et al. (1993) compared the amino acid sequence of the putative coat protein product of ToLCNDV- Lucknow with some other mono and bipartite gemini viruses and revealed a maximum of 86% homology with Indian cassava mosaic virus. Similarly, few authors (Shimizu and Ikegami, 1999) reported that in total nucleotide sequence comparisons with other gemini viruses, tomato leaf curl virus was most closely related to tomato leaf curl virus from Taiwan (TwToLCV) (76% identity), tomato leaf curl virus from Bangalore (ToLCV-Ban) (74%) and ageratum yellow vein virus (AYVV) (74%), all possessing a monopartite genome.

The full length 2759 nucleotide long DNA-A like viral genome was sequenced and sequence comparisons indicated that ToLCV-Ban4 is similar to the other three isolates from Bangalore previously sequenced and is closely related to ToLCV-Ban2 (approximately 91% nucleotide sequence identity). Phylogenetic analysis showed that the ToLCV isolates from Bangalore constitute a group of viruses separated from those of Northern India (Muniyappa et al., 2000).

The diversity and distribution of begomo viruses in tomato in Northern Karnataka determined in this study are therefore are likely to only represent general trends and understands true situation. A CP sequence represents the most conserved region of the begomo virus genome and hence there is least diversity among the isolates studied. However, when we compared these isolates with the other ToLCV isolates from Kolar district (AJ810369|ToLCV-30.Maderahalli), which is a highly potential area for commercial cultivation of tomato and also the area of first report of B-biotype in India, it has got only around 72.00% nucleotide homology. This indicates that there is diversity among the gemini viruses infecting tomato with in the state.

Breeders working towards the incorporation of begomovirus resistance genes into tomato must take into account the high degree of genetic diversity among tomato leaf curl viruses in Karnataka. A given resistance gene might be extremely effective against a particular begomovirus species and totally ineffective against distinct unrelated species. Also, the rate of evolution of begomoviruses seems to be quite fast (Padidam et al., 1999), leading to the quick emergence of new strains or species that might overcome resistance genes. The durability of resistance based on a single gene is therefore questionable. The genetic diversity of begomoviruses present in a region must be taken into consideration, in order to orient breeding programs towards resistance to the species prevalent in each region.

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