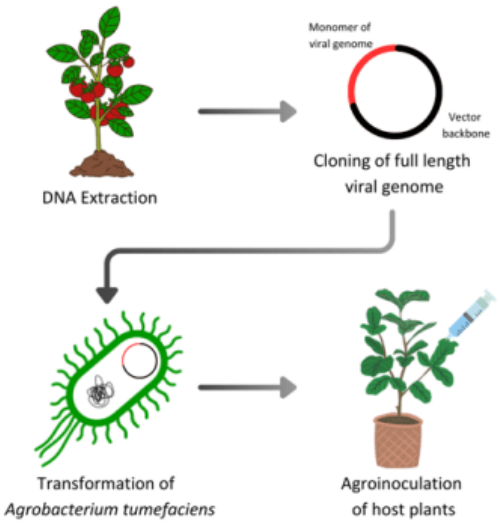
Original Research Article

Study of pathogenicity of infectious clones of Tomato leaf curl Karnataka virus (ToLCKV) by agroinoculation of tomato(*Solanumlycopersicum*L.) plants

Abstract

Tomato leaf curl disease (ToLCD) caused by Tomato leaf curl Karnataka virus (ToLCKV) is a serious threat to tomato production. It is a *Begomovirus* (*Geminiviridae*) with monopartite single stranded DNA and transmitted by whitefly. In this study, we have amplified the whole genome of ToLCKV by rolling circle amplification (RCA) using the bacteriophage Phi 29 DNA polymerase. The infectious clones (ICs) were generated by monomerising the RCA products by partial digestion using a single restriction enzyme followed by cloning into a single digested binary vector. The resultant recombinant vector was sequenced, which revealed the genome to be 2663 bp in length, with the consensus nonanucleotide found in all geminiviruses. The sequence information was used for recombination analysis using Recombination Detection Program (RDP). The recombination analysis using RDP method and gene conversion method showed the virus to be recombinant. The genome was moved to *Agrobacterium* for agroinoculation of tomato plants. Susceptible variety and a resistant hybrid of tomato were agroinoculated to study the pathogenicity of the ICs. The susceptible variety showed leaf curling and yellowing symptoms 15 days post inoculation (dpi), with resistant hybrid not showing much symptoms except on the lower leaves, confirming that monopartite component is sufficient for successful infection.



Keywords: Rolling circle amplification (RCA), infectious clones (ICs), Tomato leaf curl Karnataka virus (ToLCKV), Agroinoculation, Recombination detection program (RDP), *Solanumlycopersicum*

1. Introduction

Tomato is one of the major vegetable crops cultivated throughout the world. Tomato cultivation is affected by many of the diseases with viral diseases causing huge crop losses in tropical and sub-tropical region. Tomato leaf curl disease (ToLCD) caused by Tomato leaf curl virus (ToLCV) belongs to the genus *Begomovirus* of the family *Geminiviridae*, is the largest genera among the 9 genera of *Geminiviridae* as well as the largest genera in the virosphere. The genera has more than 400 species of reported viruses so far [1]. ToLCD is characterized by upward curling of leaves, stunted growth of the plant, reduction in the leaf size, vein clearing, excessive branching, chlorosis and low yield [2]. It was first reported in north India by Vasudeva & Sam Raj (1948) [3].

Begomoviruses are single stranded circular DNA viruses transmitted by white fly (*Bemisia tabaci*) [4]. The genome of *Begomoviruses* can be monopartite or bipartite depending on the presence of one (DNA-A) or two (DNA-A and DNA-B) genomes, respectively, which infect only the dicotyledonous plants. Tomato leaf curl Karnataka virus (ToLCKV) consist of only one genome (DNA-A). The DNA-A component consists of six open reading frames (ORFs), with V1 encoding coat protein, V2 encoding pre-coat protein on virion sense strand. Complementary sense strand has C1 encoding replication associated protein, AC2 encoding transcriptional activator protein, C3 encoding replication enhancer protein and AC4 [4-6].

An infectious clone, generated by cloning the whole genome of a virus into a suitable vector, is important for researchers to carry out studies on viruses at molecular and biological levels. Among the available techniques, rolling circle amplification (RCA) which makes use of bacteriophage Phi 29 DNA polymerase and random hexamer primers, is found to be simple and efficient in generating the infectious clones [7]. Phi 29 DNA polymerase possesses strand displacement activity, generation of long polymerase chain reaction (PCR) products and proof reading activity, making it suitable for the amplification of circular DNA molecules. Since RCA makes use of random primers, many novel viruses have been discovered by cloning and sequencing the genome [8].

Mutation, recombination, reassortment and de novo gene acquisition are the important evolutionary mechanisms that lead to the diversity in viruses [9]. Among them,

recombination provides an advantage to viruses within species, genera and family levels during the process of natural selection [10,11]. Recombination occurs when at least two viral genomes co-infect the same host cell and exchange genetic segments [12].

This study was carried out to develop an infectious clone of ToLCKV. ToLCKV infected tomato leaf samples collected from the field were confirmed by cloning and sequencing the coat protein (CP) gene of ToLCV. The whole genome was amplified using the RCA method, restriction digested and ligated to a suitable vector. The cloned product was sequenced and recombination detection was carried out. The *Agrobacterium* harboring the recombinant vector is used for the Agro-inoculation of the susceptible variety and a resistant hybrid of tomato. The presence of viral DNA in the inoculated plants was confirmed by symptomatology followed by PCR analysis.

2. Materials and methods

2.1 Sample collection and genomic DNA isolation

Tomato plants showing the symptoms of ToLCD such as reduced size, upward curling and chlorosis at the margins are collected from the field. Genomic DNA was isolated from infected leaf samples using a modified CTAB method [13].

2.2 Amplification of ToLCV-CP gene

Preliminary identification of the virus in diseased sample was carried out by PCR using gene-specific primers ToLCV-CP-F: 5'- ATGTCGAAGCGACCAGGCG-3' and TYLCV-CP-R: 5'-TTAATTTGATATTGAATCATAG-3'. Genomic DNA isolated from infected leaf samples was used as a template to amplify the CP gene. PCR was carried out using the ToLCV-CP specific primers in a 10 µL reaction mixture containing 0.4 µL each of 10 pmol forward and reverse primer, 1 µL of 10X reaction buffer, 1.2 µL of 2 mM dNTPs, 5 µL of DNA template (50 ng/µL), 1 µL of nuclease-free water and 1U of *Taq* DNA polymerase (3B Biotech). PCR profile was as follows: initial denaturation for 5 mins at 94 ℃, 40 cycles of denaturation at 94 ℃ for 1 min 20 secs, annealing at 48 ℃ for 1 min and extension at 72 ℃ for 1 min followed by final extension for 10 mins at 72 ℃. The amplified product was electrophoresed on 1 % agarose gel.

2.3 Cloning, sequencing and phylogeny of ToLCV-CP gene

The amplified CP gene was gel extracted using QIAquick Gel Extraction Kit (Qiagen India Pvt. Ltd.) and ligated into a T/A cloning vector, pTZ57R/T (Thermo Fisher Scientific). The ligated product was transferred to *Escherichiacoli*DH5α cells by heat shock method and plated on solid LB media containing 50 mg/l ampicillin.The colonies grown on the selective

media were confirmed by restriction digestion using *Bam* HI – HF (20000 U/ml) and *Sac* I – HF (20000 U/ml) (New England Biolabs). The cloned product was sequenced and the obtained sequence was subjected to BLAST analysis in NCBI database for the confirmation of the ToLCV-CP gene. The CP sequences of other closely related viruses, with the accession numbers, KJ452558, NC003898, NC003897, NC003896, NC003891, NC004153, NC010313, NC055126, NC004558, KY511140, MF429945, NC075075, NC000869, NC005032, NC005842, NC001346 were collected from NCBI database for the construction of the phylogenetic tree. Multiple sequence alignment of the sequences was carried out using Clustal omega [14]. The phylogenetic tree was constructed using the maximum likelihood statistical method in MEGA X program. The reliability of the tree was tested using the bootstrap method with 1000 replicates.

2.4 Rolling circle amplification

The total DNA from the diseased leaf sample was subjected to rolling circle amplification (RCA) using phi 29 DNA polymerase (Thermo Fisher Scientific) and Exo-Resistant random hexamer primer (Thermo Fisher Scientific). RCA was carried out in two steps: in the first step, 2 µl of 10X phi 29 buffer, 3 µl of 10 mM dNTPs, 5 µl of 20X random hexamer primers, 5 µl of genomic DNA and 4.75 µl of nuclease-free water were added. The reaction mixture was incubated at 95 ℃ for 3 mins followed by cooling at 4 ℃ for 3 mins. In the second step, 0.25 µl of Phi 29 DNA polymerase (10 U/µl) was added and incubated at 30 ℃ for 18 hrs followed by inactivation at 65 ℃ for 10 mins.

2.5 Restriction digestion of RCA product and the vector

The RCA products were subjected to partial digestion using *Sac*I (Thermo Fisher Scientific) to obtain a monomer copy of the viral genome. The partial digestion was carried out in a 20 µl reaction mixture containing the RCA product, 10X tango buffer, 0.25 µl of *Sac*I (10 U/µl) (Thermo Fisher Scientific) and nuclease-free water. The mixture was incubated at 37 ℃ for 3 mins 15 seconds followed by enzyme inactivation at 65 ℃ for 20 mins.

The infectious clones of ToLCVwere generated using the single-digested pCAMBIA1300 as the backbone. pCAMBIA1300 was subjected to single digestion using *Sac* I (10 U/µl). The digested product was subjected to alkaline phosphatase (20 U/µl) treatment to prevent the circularization of the vector.

2.6 Ligation and transformation

The partial digested RCA product and *Sac* I digested pCAMBIA1300 were gel purified using QIAquick Gel Extraction Kit (Qiagen India Pvt. Ltd.). The ligation reaction was carried out in a 1:1 insert vector ratio. The mixture was incubated at four different temperatures: 12 ℃ for 5 mins, 14 ℃ for 3 mins, 18 ℃ for 3 mins and 21 ℃ for 10 mins. The ligation mixture was used for the transformation of DH5α competent cells using heat shock method. The transformed colonies were plated on solid LB media containing 50 mg/l kanamycin. The colonies grown on the selective media were confirmed by colony PCR and restriction digestion.

2.7 Phylogenetic analysis and recombination detection

The cloned genome was sequenced using Sanger's method and subjected to BLASTn analysis. The whole genome sequences with the accession numbers, NC003897, NC003896, NC003891, NC055126, NC004558, MH255790, MN020537, MZ578458, MN095551 and EU152257 were collected from NCBI database. The collected sequences along with the genome used in this study were subjected to multiple sequence alignment using Clustal omega [14]. Phylogenetic tree was constructed using the maximum likelihood statistical method in MEGA X program. The reliability of the tree was tested using the bootstrap method with 1000 replicates.

The whole genome sequences used for phylogenetic analysis were analysed for recombination. Recombination Detection Program (RDP 4.101) was used for the detection of recombination in the genome identified in the study [15]. The sequences used for phylogenetic analysis were also used for recombination analysis. The sequences were converted to FASTA format and multiple sequence alignment was carried out using Muscle [16]. The aligned sequences were used for the detection of recombination. Within the RDP program, RDP method [17] and the GENECONV method [9] were used for the identification of recombination event and analysis of major parent, minor parent and the recombinant

2.8Inoculation of pCAMBIA1300:ToLCKV monomer to tomato

The recombinant plasmid was transformed to *Agrobacteriumtumefaciens* strain GV2260 by freeze-thaw method [18] and plated on solid YEP media containing 50 mg/l kanamycin and 25 mg/l rifampicin. Seeds of tomato variety, Arka Vikas (Susceptible) and a hybrid, Arka Vishesh (Resistant) were sown in a horticultural soil mix in a pot-tray. After germination, each plant was transplanted into a plastic pot containing horticultural soil mix and grown in a polyhouse. For agroinoculation, single colony of *A. tumefaciens* strain

GV2260 harboring recombinant binary vector pCAMBIA1300:ToLCKV-monomer was inoculated in 10 ml liquid YEP medium (50 mg/l kanamycin and 25 mg/l rifampicin) and incubated in a 28 ℃ shaker for 24 hrs. The grown culture was centrifuged at 13,200 rpm for 2 mins at 20 ℃. The pellet was resuspended in 5 ml infiltration buffer (10mM $MgCl_2$ and 10 mM MES, pH 5.60) and OD_{600} was adjusted to 0.1. 80 µMacetosyringone was added and the culture was incubated in a 28 ℃ shaker for 4 – 5 h. For, Agroinfiltration of leaves, 1 ml needleless syringe containing the suspension of *A. tumefaciens* was orthogonally pressed against the abaxial sides in between the leaf veins of third or fourth leaves of 3-week-old tomato plants (Fig. 1). The infiltrated region of the leaves turned dark green as soon as the suspension entered the intercellular area of the leaves. *Agrobacterium* transformed with empty pCAMBIA1300 and infiltration buffer were also infiltrated into the tomato plants as controls.

2.9 Viral DNA detection and sequence analysis

The leaf samples from the agroinoculated plants were collected and DNA was extracted using modified CTAB method. PCR was carried out using the ToLCV-CP specific primers in a 10 µL reaction mixture. The amplified product was electrophoresed on 1 % agarose gel.

3. Results

3.1 Detection of ToLCV

Naturally infected tomato leaf samples collected from the field showing symptoms, such as yellowing of the leaf margins, upward leaf curling and reduced leaf size were used for the genomic DNA isolation from modified CTAB method [13]. PCR amplification was carried out using ToLCV-CP gene specific primers for the presence of ToLCV. PCR analysis revealed the presence of the amplicon around ~770 bp (Fig. 2). The amplified product was gel purified using QIAquick gel extraction kit and cloned to a cloning vector, pTZ57R/T. Restriction digestion of the putative recombinant vector confirms the presence of ToLCV-CP gene. The cloned product was sequenced using Sanger method. The BLASTn analysis of the CP gene from the infected samples showed 92.72% nucleotide sequence identity with GenBank sequence NC003897 with 99% of the query cover, confirming the presence of ToLCV in the field collected infected tomato samples. The closely related sequences were collected from NCBI, subjected to multiple sequence alignment using Clustal-Omega and

phylogenetic tree was constructed on MEGA X. The phylogenetic tree showed high similarity of our sample to the CP gene of ToLCKV-NC003897 with bootstrap value of 95 (Fig. 3).

3.2 Rolling circle amplification and cloning of viral genome

To clone full length genome of ToLCV, RCA was employed using Phi 29 DNA polymerase and Exo-resistant random primers. Exo-resistant random primers have two 3'-terminal phosphorothioate modifications that are resistant to the 3' – 5' exonuclease activity of Phi 29 DNA polymerase. Isothermal amplification of genomic DNA using Phi 29 DNA polymerase resulted in high molecular weight DNA. The high molecular weight DNA consisting of concatemers was subjected to partial digestion with *Sac*I, putatively digesting the ToLCKV genome only once. DNA bands were observed around 2.8 kb (monomer) and 5.6 kb (dimer) in an agarose gel (Fig. 4). Monomer of the viral genome was gel purified and cloned into single digested (*Sac*I) and alkaline phosphatase treated pCAMBIA1300. The recombinant vector was transformed to *E. coli*. Plasmid isolation and restriction digestion analysis of pCAMBIA1300:ToLCKV monomer showed a band around 2.8 kb (Fig. 5), confirming the presence of viral genome in the vector.

3.3 Phylogenetic analysis and recombination detection

Complete sequencing of the cloned product revealed the length of ~2.8 kb. BLASTn analysis showed the sequenced genome to have 88.80 % identity with GenBank sequence MN020537 with the query cover of 97 %. The whole genome sequences of closely related viruses were retrieved from NCBI, subjected to to multiple sequence alignment using Clustal-

Omega and phylogenetic tree was constructed on MEGA X. The phylogenetic tree showed high similarity of our sample to the of MN020537 with bootstrap value of 100 (Fig. 6).

The sequences used for phylogenetic analysis were also used for recombination analysis using recombination detection program (RDP). In the RDP software, RDP method and gene conversion method were used for the identification of the recombinant and analysis of the parents and the recombinant. RDP analysis revealed that the genome identified in this study (ToLCKV) to be the recombinant of ToLCKV-MN020537 as major parent with 90.1 % similarity and ToLCKV-NC003987 as minor parent with 93.2 % similarity (Fig. 7, 8 and 9). One sequence was detected as recombinant in each of the RDP, Chimera, MaxChi, 3Seq, bootscan, SiScan and gene conversion methods (Fig. 8).

The tree obtained from the RDP software also showed the possibility of recombination with ToLCKV as recombinant and ToLCKV-MN020537 as major parent and ToLCKV-NC003987 as minor parent (Fig. 9). The region 1-399 and 1092-2816 of the recombinant ToLCKV were derived from the major parent ToLCKV-MN020537 and the region 400-1091 of the recombinant was derived from the minor parent ToLCKV-NC003897.

RDP method (Fig. 10) predicted the pairwise identity of ToLCKV-NC003987 (minor parent) – ToLCKV (recombinant) to be found from ~1 bp to ~400 bp and the pairwise identity of ToLCKV-MN020537 (major parent) and ToLCKV (recombinant) predicted in between ~400 bp and ~1100 bp.

The gene conversion also supported that the ToLCKV is the recombinant of ToLCKV-NC003897 and ToLCKV-MN020537. Gene conversion data shows that there is a higher probability of gene conversion between the recombinant ToLCKV and the minor parent ToLCKV-NC003897 in the region ~400 bp to ~1100 bp. On the other hand, the gene conversion between the recombinant ToLCKV and the major parent ToLCKV-MN020537 has occurred at 3 regions(Fig. 11).

3.4 Viral DNA detection in agroinoculated plants

pCAMBIA1300:ToLCKV-monomer was transformed to *A. tumefaciens* strain GV2260 via freeze-thaw method and was used for the agroinoculation of a resistant hybrid (Arka Vishesh) and a susceptible variety (Arka Vikas). *A. tumefaciens* carrying empty pCAMBIA1300 and the infiltration buffer were also infiltrated as controls (Table 1). As expected, susceptible variety showed typical symptoms of ToLCV 15 dpi (leaf yellowing and

curling) (Fig. 12). The resistant hybrid showed symptoms only on the lower leaves with healthier younger leaves (Fig. 13). For the confirmation of presence of viral DNA in agroinoculated plants, DNA was extracted from the agroinoculated plants and the control plants. PCR was carried out with ToLCKV-CP gene specific primers. PCR analysis showed amplification of ToLCKV-CP gene around ~770 bp in three of the six inoculated susceptible plants and the resistant hybrids did not show any amplification. There was no amplification in case of control plants (Fig. 14).

Table 1 Infectivity assay of agroinoculated constructs of ToLCKV on tomato plants

Sl. No.	**Construct inoculated**	**Number of Plants inoculated**		**Number of plants showing symptoms**	
		Susceptible	**Resistant**	**Susceptible**	**Resistant**
1.	pCAMBIA1300:ToLCKV-Monomer	6	6	3	0
2.	Empty pCAMBIA1300	5	5	0	0
3.	Infiltration buffer	5	5	0	0
4.	Control plants	5	5	0	0

3. Discussion

The amplification of viral genome using RCA method has been proven to be highly efficient technique, especially for the analysis of *Begomoviruses* [7]. RCA technique is more advantageous than the conventional PCR technique. RCA is simple which does not require the sequence information of the genome of interest for the purpose of cloning and is less expensive than the PCR method [19]. It also has advantages over antibody-based technique as

a diagnostic tool [20]. In addition, RCA technique can also be applied for the amplification of genomes of animal pathogen, circovirus [21] and nanovirus [22]. The results from our study confirm these advantages, leading to identification, cloning of full-length genome and construction of infectious clones of ToLCKV.

The tomato leaf samples showing symptoms of leaf curling, yellowing and reduced leaf size were collected from the field. DNA was extracted and subjected to PCR analysis using CP gene specific primers. Agarose gel electrophoresis of PCR product revealed the amplicon size of ~770 bp, which is same as that of CP gene of ToLCV [23,24]. The sequenced CP gene was used for phylogenetic analysis, which showed the highest similarity with ToLCKV-NC003897, with a bootstrap value of 95.

To obtain the full-length genome of ToLCKV, viral sequences were amplified using the RCA method. The RCA product was partially digested and monomer copy of the genome was cloned to a binary vector. The sequencing results revealed the putative full-length genome of ToLCKV to be ~2700 bp long [25]. Further analysis of the genome revealed to have the nonanucleotide sequence (TAATATTAC), which is conserved among the geminiviruses [26].

Recombination is one of the evolutionary important mechanisms that can lead to diversity in viruses, upon which natural selection acts [15]. It can provide selective advantage in evolution of viruses. Such recombination mechanism was also identified in the virus genome of our study. RDP 4 software [15] was used for the detection of recombination

based on RDP method and gene conversion method. It reveals that ToLCKV used in the present study to be the recombinant of the major parent, ToLCKV-MN020537 and the minor parent, ToLCKV-NC003897. According to the recombination analysis, the recombinant is the major parent ToLCKV-MN020537 with the CP gene derived from the minor parent, ToLCKV-NC003897. Chatchawankanphanich & Maxwell [27], reported that the ToLCKV from Bangalore to be a recombinant virus. Earlier reports also suggested that the recombination in field isolates of *Begomoviruses* is well documented [9, 28]. The results of phylogenetic analysis were in line with the results obtained in recombination analysis. Phylogenetic tree shows ToLCKV to be closely related to MN020537 and also not so far from NC003897, either.

To test the infectivity of the full length ToLCKV infectious clones, the infectious clones constructed using RCA technique were transferred to *A. tumefaciens*. Susceptible variety and a resistant hybrid of tomato were agroinoculated with the infectious clones containing monomer of ToLCKV. 15 days after agroinoculation, the susceptible variety showed leaf curling and yellowing symptoms, similarly to the report ofDokka et al. [2]. The PCR analysis using ToLCKV-CP specific primers showed amplification only in susceptible variety, but did not show any amplification in resistant hybrids, which is in line with the studies conducted by Aguilera et al. [29], Sudha et al. [30].

According to Dry et al. [25], single genomic component of ToLCV was sufficient for the successful infection of plant and to develop typical symptoms, which suggests that ToLCKV reported in this study to be a monopartite virus. Reports of Kirthi et al. [31] and Muniyappa et al. [32] further establishes the proof that ToLCKV to have a monopartite genome.

4. Conclusion

In the present study, RCA was employed to produce infectious clones of ToLCKV. The monomerised genome of ToLCKV was subjected for recombination analysis, which showed the cloned monomer copy of the genome to the recombinant one. Further, the role of ToLCKV in pathogenesis was demonstrated by agroinoculation of susceptible variety and a resistant hybrid of tomato, resulting in production of yellowing and leaf curling symptoms in the susceptible variety with very few leaves showing symptoms in a resistant hybrid.

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