टमाटर में पाए जाने वाले *Ty*-जीनों के प्रतिरोध व्यवहार का मूल्यांकन एवं उन्हें संक्रमित करने वाले बेगोमोविषाणु का अनुक्रमण

Evaluation of resistance behaviour of *Ty*-Gene(s) containing donor lines of tomato and indexing of naturally occurring begomoviruses infecting them

Md. Firoz Mondal

Roll: 21328

DIVISION OF PLANT PATHOLOGY

ICAR-INDIAN AGRICULTURAL RESEARCH INSTITUTE

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Evaluation of resistance behaviour of *Ty*-Gene(s) containing donor lines of tomato and indexing of naturally occurring begomoviruses infecting them

A Thesis

By

Md Firoz Mondal
Roll: 21328

Submitted to the

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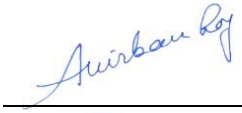
Chairman	:	Dr. Anirban Roy	
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Member	:	Dr. Basavaraj	
	:	Dr. Zakir Hussain	

Division of Plant Pathology
ICAR-Indian Agricultural Research Institute
New Delhi –110012, India

Dr. Anirban Roy
Principal Scientist
anirbanroy75@yahoo.com

CERTIFICATE

This is to certify that the thesis entitled, **"Evaluation of resistance behaviour of *Ty*-gene(s) containing donor lines of tomato and indexing of naturally occurring begomoviruses infecting them"** submitted to the **Faculty of the Post-Graduate School, ICAR-Indian Agricultural Research Institute, New Delhi,** in partial fulfillment of the requirements for the award of **MASTER OF SCIENCE IN PLANT PATHOLOGY,** embodies the results of *bona fide* research work carried out by **Md. Firoz Mondal, Roll No: 21328,** under my guidance and supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by him.

Place: New Delhi
Date: 27.08.2021

Dr. Anirban Roy
Chairman
(Advisory Committee)

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Place: ICAR-IARI, New Delhi

Md. Firoz Mondal

Md Firoz Mondal

DEDICATED TO:

My dear Parents and Nafisa (Niece)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND SYMBOLS

µg	: Microgram
µl	: Microliter
µM	: Micromolar
%	: Percent
~	: Approximate
°C	: Degree Celsius
(PCR)	: Polymerase chain reaction
A_{260}	: Absorbance at 260nm
A_{280}	: Absorbance at 280nm
aa	: Amino acid
BLAST	: Basic local alignment search tool
bp	: Base pair
$CaCl_2$	: Calcium chloride
CP	: Coat Protein
Dpi	: Days of post inoculation
EC	: Exotic collection
ICTV	: International committee on taxonomy of viruses
Kb	: Kilobases
kDa	: Kilodalton
LA	: Luria Agar
LB	: Luria-Bertani Broth
mRNA	: Messenger RNA
MW	: Molecular Weight
MYMV	: Mungbean yellow mosaic virus
NCBI	: National centre for biotechnology information
NCR	: Non coding region
NGS	: Next generation sequencing
CP	: Coat Protein
Dpi	: Days of post inoculation
NLS	: Nuclear localization signal
nm	: Nano meter
NOS	: Nopaline synthase
Nt	: Nucleotide
OCS	: Octopine synthase

OD	: Optical density
ORF	: Open Reading Frame
PAGE	: Polyacrylamide gel electrophoresis
PVP	: Polyvinyl pyrrolidone
RdRp	: RNA dependent RNA polymerase
RT-PCR	: Reverse transcription polymerase chain reaction
RT-PCR	: Real time polymerase chain reaction
SAM	:S-adenosyl- methionine
SAMDC1	:S-adenosyl-methio- nine decarboxylase
TbCSV	: Tobacco curly shoot virus
TGMV	: Tomato golden mosaic virus
TGS	: Transcriptional gene silencing
TNA	: Total nucleic acid
ToLCBaV	: Tomato leaf curl Bangalore virus
ToLCD	: Tomato leaf curl disease
ToLCGuV	: Tomato leaf curl Gujarat virus
ToLCJoV	: Tomato leaf curl Joydebpur virus
ToLCKaV	: Tomato leaf curl Karnataka virus
ToLCKeV	: Tomato leaf curl Kerala virus
ToLCNDV	: Tomato leaf curl New Delhi virus
ToLCPaV	: Tomato leaf curl Patna virus
ToLCPV	: Tomato leaf curl Pune virus
ToLCRaV	: Tomato leaf curl Rajasthan virus
ToLCTyPV	: Tomato leaf curl Ty Pusa virus
TrAP	: Transcriptional activator protein
TYLCV	: Tomato yellow leaf curl virus

CHAPTER 1 INTRODUCTION

Whitefly (*Bemisia tabaci*) transmitted begomoviruses, under the family Geminiviridae, possess ssDNA in their genome and infect dicot plants. They have emerged as a major threat to the productivity of many important vegetables, food and fibre crops in India, like Tomato, Potato, Chilli, Okra, Cotton, Mungbean, Black gram, French bean etc. (Verma *et al*., 2011). Tomato yellow leaf curl disease (TYLCD) and tomato leaf curl disease (ToLCD) are the most important viral diseases infecting tomato worldwide. More than 13 species of begomoviruses are reported to be associated with TYLCD while more than 80 species of begomoviruses are associated with ToLCD throughout the world. Amongst them the most prolific species is tomato yellow leaf curl virus (TYLCV), a monopartite (2.7kb) begomovirus distributed throughout the world, however, in Indian sub-continent till now there is no report of this virus. In India, tomato leaf curl disease was first time identified in northern India by Vasudeva and Samraj (1948). Amongst the 14 begomoviruses reported to be associated with leaf curl disease of tomato in India (Kumar *et al.,* 2018), tomato leaf curl New Delhi virus (ToLCNDV) is the most predominant begomovirus (Malathi *et al.,* 2017). ToLCNDV is a bipartite begomovirus transmitted by Whiteflies (Padidam *et al.,* 1995).

Regardless of the efforts to manage TYLCD and ToLCD, the disease continue persists in tomato, entering newer areas every year (Cohen and Lapidot, 2007, Moriones and Navas-Castillo, 2000). Popular means to manage the disease in field condition mainly rely on control of the whitefly (*Bemisia tabaci*) vector population through the application of insecticides, which in short term could be quite satisfactory, however, non-judicious and prolong intensive application of such insecticides have resulted in the development of resistant *B. tabaci* populations (Cahill *et al.,* 1996a, Cahill *et al.,* 1996b). This calls for engaging more efforts on developing virus resistant cultivars through breeding. Although no resistance to the disease has been recorded in the domesticated tomato (*Solanum. lycopersicum*), several sources of resistance with atleast six resistance loci (*Ty*-1 *to Ty*-6) have been described in wild relatives of tomato that showed resistance against TYLCV and could be used to introgress those resistance genes to breed resistant cultivars (Ji *et al.,* 2007). The involvement of *Ty*-genes in resistance against tomato leaf curl virus was first identified with the discovery of *Ty*-1 gene on the chromosome 6 of wild tomato species *Lycopersicon chilense* by Zamir and his co-workers in 1994. Among the 6 resistance genes, only two have been biochemically characterized where *Ty*-2 codes for a NBS-LRR protein (Yamaguchi *et al.,* 2018). It has been demonstrated that *Ty*-1 and *Ty*-3 are allelic (present on the long arm of chromosome 6) and code, in part, for an RNA dependent RNA polymerase (RDR) of the atypical RDRγ type having a DFDGD catalytic domain instead of the well-known DLDGD domain (Verlaan *et al.,* 2013). Several pre-breeding

lines were generated through introgression of resistance loci in the cultivated tomato and are being utilized by the plant breeders to develop resistant cultivars. These genes have different origins. *Ty*-1, *Ty*-3, *Ty*-4 and *Ty*-6 all introgressed from different *S. chilense* accessions, *Ty*-2 from *S. habrochaites*, *Ty*-5 from *S. peruvianum* (Zamir *et al.,* 1994; Hanson *et al.*, Hutton *et al*., 2012; 2006; Ji *et al*., 2007; Ji *et al*., 2009; Gill *et al.*, 2019). Although cultivars or the pre-breeding lines containing *Ty*-gene(s) are called resistant, they do not show absolute or complete resistance against begomoviruses. Instead, they express variable level of tolerance against the viruses and produce different symptoms as the virus replication is not completely inhibited. One of the reasons behind this variation is maybe the occurrence of mixed-infection. But the exact reason is still unknown. In contrast with classical R-genes none of the resistances to TYLCV described so far are associated with a HR. Amongst them genotypes with *Ty*-1 and *Ty*-2 showed considerable resistance against TYLCV but is not very much successful to inhibit ToLCNDV. In Indian context, genotypes with *Ty*-3 allele alone or pyramided with *Ty*-1 or *Ty*-2 gave major success to impart resistance against ToLCNDV (Prasanna *et al.,* 2015). Besides *Ty*-3 and *Ty*-2, combination of *Ty*-5 and *Ty*-6 also showed considerable resistance against the monopartite tomato leaf curl Bangalore virus (ToLCBaV) (Prabhandakavi *et al.,* 2020).

With respect to evaluation of resistance in *Ty*-gene containing genotypes, limited efforts were carried out in India. Prabhandakavi *et al*.., 2020 demonstrated evaluation of inbred lines of tomato carrying different *Ty*-gene combination against a monopartite begomovirus, tomato leaf curl Karnataka virus (ToLCKV) and a bipartite begomovirus, ToLCNDV. However, in majority of the cases such donor lines carrying the resistance loci have neither been evaluated thoroughly for determining their disease reaction nor the occurrence of viruses in those line have been documented properly.

Research Gaps:

i. The resistance behaviour of *Ty*-gene containing donor lines of tomato is not clear
ii. Virus indexing has not been done for different naturally occurring begomoviruses in these donor lines.

Title of the research:

"Evaluation of Resistance Behaviour of *Ty*-gene containing Donor Lines of Tomato and Indexing of Naturally Occurring Begomoviruses Infecting Them".

Objectives:

i. To evaluate the resistance behaviour of *Ty*-gene(s) containing donor lines of tomato.
ii. To index the naturally occurring begomovirus species infecting different *Ty*-gene containing donor lines.

2.1. Tomato

Tomato (*Solanum lycopersicum* L syn. *Lycopersicon esculentum* Mill, Family-*Solanaceae*) is the second most important vegetable crop and among the most important processed vegetables in the world. It is especially famous for salad as a good source of vitamin A, C and K along with minerals like Potassium (K), Calcium (Ca) and Magnesium (Mg). It is also very rich in antioxidant lycopene with a preventive effect on heart diseases and cancer (Rao *et al.,* 2000). *S. lycopersicum* is a native of western South America and Central America (Knapp *et al.,* 2016). Its worldwide introduction was facilitated by European colonizers during 16th century (Medina *et al.*,2017). Tomato is cultivated in 5.30 Mha area worldwide with a production of 180.76 MT (FAO, 2019). India is the second largest producer of tomato after China with 19.00 MT total production in 0.78 Mha area (FAO, 2019). In India, Andhra Pradesh is the leading state in tomato production followed by Madhya Pradesh and Karnataka.

2.2. Viral diseases of tomato

Just like other crops, tomato cultivation is threatened by a number of diseases caused by many fungi, bacteria as well as viruses. About 146 viruses belonging to 33 genera are reported to infect tomato around the world (Dhaliwal *et al.,* 2019). The major important viral diseases of tomato are tomato leaf curl (several species of *Begomovirus; Geminiviridae*), tomato mosaic (*Tobacco mosaic virus, Tomato mosaic virus; Tobamovirus; Virgaviridae),* tomato necrosis *(Alfalfa mosaic virus; Alfamovirus; Bromoviridae),* tomato spotted wilt (*Tomato spotted wilt virus; Tospovirus; Tospoviridae),* tomato bushy stunt (*Tomato bushy stunt virus; Tombusvirus; Tombusviridae),* curly top (*Beet curly top virus*; *Curtovirus; Geminiviridae*), pseudo curly top (*Tomato pseudo curly top virus; Topcuvirus; Geminiviridae),* tomato mottle (*Tomato mottle virus; Begomovirus; Geminiviridae*) etc. (ICTV, 2020)

2.3. Leaf curl disease of tomato: etiology and distribution

Among the viral diseases of tomato, leaf curl has become the most important emerging disease in recent years. In India, Vasudeva and Sam Raj (1948) first time reported leaf curl disease of tomato as one of the major constrains. It causes yield loss of 17.6% to 99.7% depending on the time and stage of infection, disease incidence and severity (Butter and Rataul,1981; Kalloo,1996). Saikia and Muniyappa (1989) estimated a yield loss of 52.5-100% in winter planted and 6.4-52.2% in summer planted tomato crop. According to the report by Sastry and Singh (1973), when tomato plants get infected by leaf curl disease at 2, 4, 6, 10 weeks after planting, the estimated yield loss was about 94.90%, 90.00%, 78.00% and 10.80% respectively.

The diagnostic symptoms of tomato leaf curl disease include leaf curling, puckering of leaves, smalling of leaves, vein yellowing, enation, pale to deep yellowing of leaves and, stunting. Severe infection may lead to extreme distortion and complete sterility of the plant. Singh and Lal (1964) observed marginal upward rolling of leaves, vein banding, twisting and formation of golden island on leaves (Fig. 1).

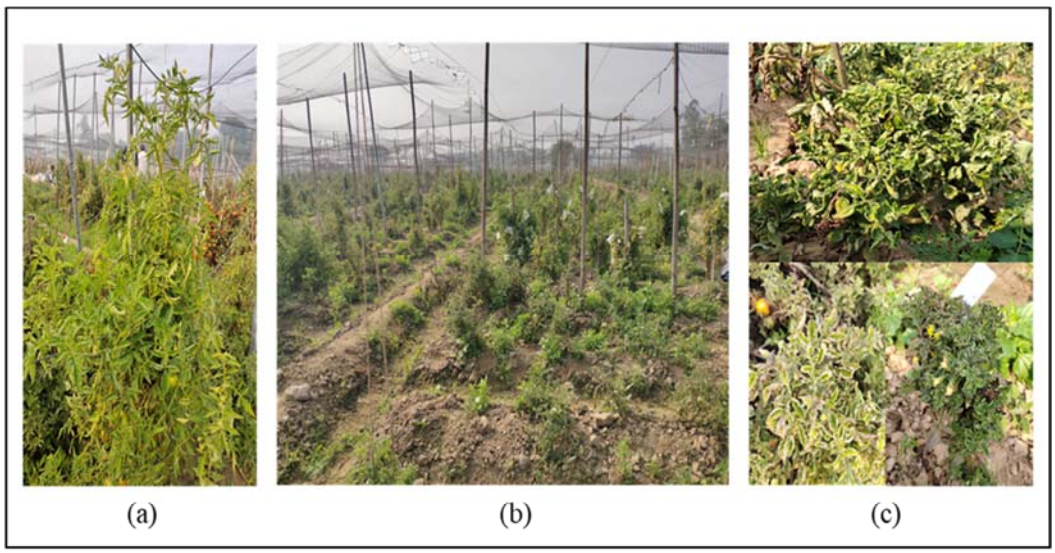


Fig. 1: Occurrence of leaf curl disease in tomato genotypes in IARI experimental field

(a) Asymptomatic tomato plant
(b) Leaf curl disease infected tomato field
(c) Severe leaf curl symptoms in susceptible tomato var. Pusa Ruby

The causal agents of leaf curl disease of tomato are begomoviruses, a group of viruses belonging to the largest plant DNA virus family *Geminiviridae*, which is characterized by circular single stranded DNA (ssDNA) as genomic component, encapsidated in a twinned icosahedral capsid (Goodman, 1981). The genus *Begomovirus* is dicot infecting and transmitted by Whitefly (*Bemisia tabaci*) (Mayo and Martelli, 1993). The begomovirus genome can be either bipartite (two genomic components *i.e* DNA-A and DNA-B, each of ~2.7 kb) or monopartite (only one genomic component which is DNA-A homolog). DNA-A and B contains six (AC1, AC2, AC3, AC4, AV1, AV2) and two (BC1, BV1) ORFs, respectively (Fig. 2). Each genomic component is encapsidated in a twinned icosahedron separately (Stanley, 1985). Sometimes, sub-genomic satellite DNAs like alpha- and beta-satellites are also reported to be associated with the disease and modify disease severity and host adaptability (Briddon *et al.*,2004).

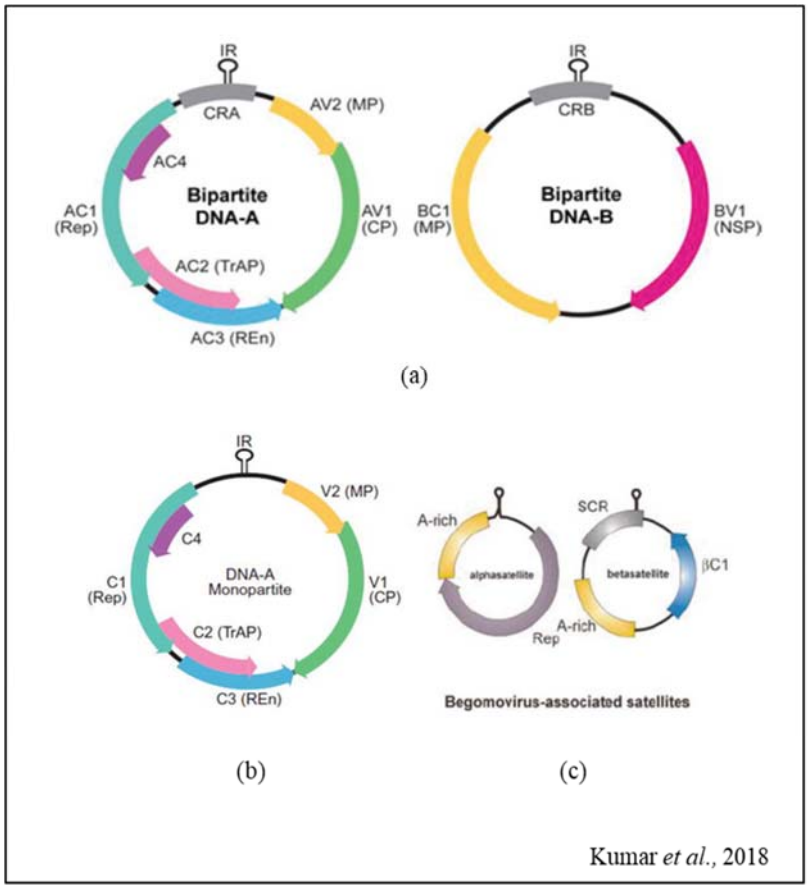


Fig 2: Genome organization of begomoviruses and associated satellites.

(a) Bipartite begomovirus with two genomic components (DNA-A & DNA-B)
(b) Monopartite begomovirus with single genomic component
(c) Begomovirus associated satellites

ORF-AV1/V1: Coat protein (CP)
ORF-AV2/V2: Pre-coat protein
ORF-AC1/C1: Replication initiator protein (Rep)
ORF-AC2/C2: Transcription activator protein (TrAP)
ORF-AC3/C3: Replication enhancer protein (Ren)
ORF-AC4/C4: AC4/C4 protein
ORF-BV1 : Nuclear shuttle protein (NSP)
ORF-BC1 : Movement protein (MP)
ORF- βC1 : RNA silencing suppressor protein

Tomato yellow leaf curl disease (TYLCD) is the most widely distributed phenotype of leaf curl disease of tomato. About 13 species of begomoviruses have been reported to be associated with it (ICTV, 2020), distributed in Africa, America, parts of Europe and Asia. Amongst them, *Tomato yellow leaf curl virus* (TYLCV) is most important which is a monopartite begomovirus. TYLCV, however, has not been reported yet from India. On the

other hand, at least 80 species are associated with another disease phenotype called as tomato leaf curl disease (ToLCD) (ICTV, 2020) which is distributed in the Indian sub-continent, China, Europe, Taiwan, Panama, Oman and many other places exhibiting mild to severe leaf curling symptom.

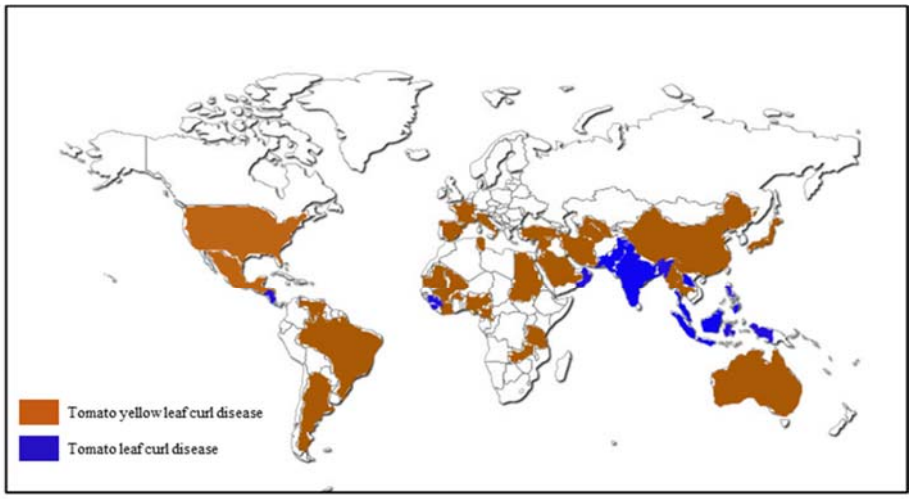


Fig. 3: Worldwide distribution of leaf curl disease of tomato.

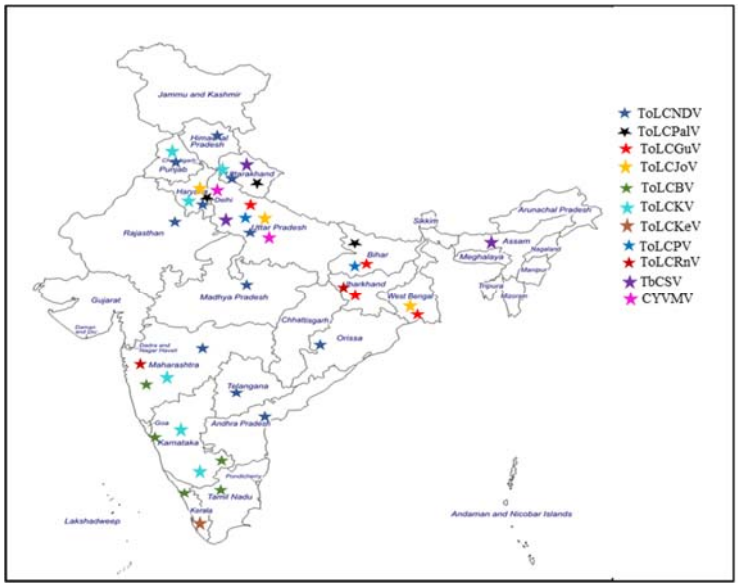


Fig.4: Distribution of different *Begomovirus* species causing leaf curl of tomato in India.

2.4. Begomoviruses associated with leaf curl disease of tomato in India

At least 14 other species of *Begomovirus* have been reported to be associated with leaf curl of tomato (Table 1, Fig. 4). They are *Tomato leaf curl New Delhi virus* (ToLCNDV), *Tomato leaf curl Palampur virus* (ToLCPalV), *Tomato leaf curl Bangalore virus* (ToLCBaV),

Tomato leaf curl Karnataka virus (ToLCKaV), *Tomato leaf curl Joydebpur virus* (ToLCJoV), *Tomato leaf curl Gujarat virus* (ToLCGuV), *Tomato leaf curl Kerala virus* (ToLCKeV), *Tomato leaf curl Patna virus* (ToLCPaV), *Tomato leaf curl Rajasthan virus* (ToLCRaV), *Tomato leaf curl Ranchi virus* (ToLCRnV), *Tomato leaf curl Pune virus* (ToLCPV), *Tobacco curly shoot virus* (TbCSV), *Ageratum enation virus* (AEV), *Croton yellow vein mosaic virus* (CYVMV) (Kumar *et al.*,2018). Amongst them, *Tomato leaf curl New Delhi virus* (ToLCNDV) is the most prevalent and widely distributed begomovirus infecting tomato, potato, and cucurbits.

Table 1. Begomoviruses associated with leaf curl disease of tomato in India

Virus species	Acronym	Genome	Reference
Tomato leaf curl New Delhi virus	ToLCNDV	Bipartite	Srivastava *et al.*,1993
Tomato leaf curl Palampur virus	ToLCPalV	Bipartite	Kumar *et al.,* 2008
Tomato leaf curl Gujarat virus	ToLCGuV	Bipartite	Chakraborty *et al.,* 2003
Tomato leaf curl Bangalore virus	ToLCBaV	Monopartite	Muniyappa *et al.,* 2000
Tomato leaf curl Pune virus	ToLCPuV	Monopartite	Reddy *et al.,* 2004
Tomato leaf curl Joydebpur virus	ToLCJoV	Monopartite	Tiwari *et al.,* 2013
Tomato leaf curl Karnataka virus	ToLCKaV	Monopartite	Chatchawankanphanich *et al.,* 2002
Tomato leaf curl Kerala virus	ToLCKeV	Monopartite	Pasumarthy *et al.,* 2011
Tomato leaf curl Patna virus	ToLCPaV	Monopartite	Kumari *et al.,* 2010
Tomato leaf curl Ranchi virus	ToLCRnV	Monopartite	Kumari *et al.,* 2010
Tomato leaf curl Rajasthan virus	ToLCRaV	Monopartite	Sivalingam, 2005
Tobacco curly shoot virus	TbCSV	Monopartite	Swarnalatha *et al.,* 2013
Ageratum enation virus	AEV	Monopartite	Swarnalatha *et al.,* 2013
Croton yellow vein mosaic virus	CYVMV	Monopartite	Khan *et al,* 2014

2.5. Management of tomato leaf curl disease

Like other viral diseases, management of tomato leaf curl disease is a quite difficult task. The most commonly practiced method by the growers is use of insecticides for controlling the whitefly vectors. But it is not very much effective and also not good for environment along with the risk of insecticide resistance development. The common methods for management of plant viral diseases including ToLCD are-

a) Crop rotation

Crop rotation with non-host crops prevents the establishment of large populations of the whitefly vector and also set up a host-free periods by naturally breaking the life cycles of the disease, insect vectors and weeds (Kumar *et al.,* 2018).

b) Eradication of source plants

Studies on the epidemiology of leaf curl of tomato have confirmed a positive correlation between the level of virus spread and population size of *B. tabaci*. Destruction of alternate hosts harboring viruliferous whiteflies reduces the amount of primary inoculum thus restricting the disease spread to a significant level (Ucko *et al.,* 1998; Cohen *et al.,* 1988). In Cyprus, the spread of TYLCV was effectively controlled by eradicating the primary inoculum sources in overwintered spring tomato, prior emergence of adult whiteflies. 3 consecutive years of practice of this measure was undertaken. It almost completely prevented primary virus spread to spring plants, while further secondary spread was below 5% to summer plantings, compared to 40–50% in previous years (Ioannou, 1987).

c) Bait crops

Studies have reported that planting alternate rows of tomatoes and cucumbers (planted 30 days before tomato transplantation) significantly delayed the spread of leaf curl in the tomatoes (Al-Musa, 1982). Similarly, tomato was protected from leaf curl disease by using squash as a bait plant (Schuster, 2004). The probable controlling mechanism of the bait plants is due to the cumulative effects of (1) the huge difference between the canopy volumes and (2) baits are much more preferred host for whiteflies and once they land on this bait, they do not leave it as long as the plants remain fresh (Cohen, 1988).

d) Reflective mulches

The use of yellow mulches for controlling whitefly-borne viruses including leaf curl has been reported (Cohen & Berlinger, 1986; Cohen & Melamed Madjar, 1974, 1978; Nitzany *et al.,* 1964). The whiteflies are attracted to the yellow color of the mulches and subsequently killed by the reflected heat. Later studies showed that, tomatoes were better protected from leaf curl by aluminum and orange plastic mulches compared to yellow mulches (Csizinsky *et al.,* 1995). Based on research, it is suggested that the protection effect of the yellow and silver soil mulches is linked with their relative high reflections at 560 nm. It is hypothesized that when contrast between the plant foliage and soil background is high, the plant image becomes more visible to the whitefly eye. In other words, plants which grow over the yellow or reflective mulches are camouflaged from the insect eye by the relatively high reflections of the background, resulting in a lower number of insect landing (Yehezkel Antignus, 2007).

e) Biological control

Use of biocontrol agents like plant growth promoting rhizobacteria (PGPR), entomopathogenic fungi, parasitoids, predators etc. represent a potentially attractive alternative approach for disease management. Biopesticides based on *Beauveria bassiana, Paecilomyces fumosoroseus, Encarsia bimaculate*, *Pseudomonas sp., Isaria javanica,* ladybirds, green lacewings, minute pirate bugs etc. showed significant effectiveness in controlling whitefly vector (Kumar *et al.,*2018). Artificial application of *B. bassiana* as endophyte inside tomato plants can be an important component in the integrated control of tomato whiteflies. It may reduce insect survival on inoculated plants. The potential insecticidal action of *B. bassiana* includes toxic principles production especially Beauvercin, chitin degradation, lectin binding and volatile organic compounds release such as sesquiterpene and di-isopropyl naphthalene (El-Deeb *et al.,* 2012). Recent study showed that the application of *Pseudomonas sp*. in combination with chitosan successfully reduces the severity of the tomato leaf curl disease by 80.33–90.33 per cent. Higher levels of phenolics, enzymes like phenylalanine ammonia lyase (PAL), peroxidase and enhanced chitinase activity were also observed in rhizobacteria-treated plants (Mishra *et al.,* 2014).

f) Chemical control

Use of insecticides is easy and most preferred method by the farmers for controlling the vectors of viral diseases including leaf curl disease of tomato. Commonly used insecticides against whitefly are imidacloprid 17.8% SL (@100 ml/ha), acetamiprid 20% SP (@50 g/ha), thiamethoxam 25 WG (@100 g/ha), nitenpyram 10% WSG (@100 g/ha), diafenthiuron 20% WG (@50 g/ha) etc (Bacci *et al.,* 2007). Alternatively, foliar spray of botanical formulations like neem oil, garlic oil, datura, eucalyptus extract also proved to be effective in controlling whitefly population (Khan *et al.,* 2013).

g) Next-Generation Approaches

Transgenic approaches have shown very promising results in controlling viral infections. However, commercialization of genetically modified organisms (GMOs) has been limited in developing countries like India due to low public acceptance. Pathogen derived resistance (PDR) strategy (Antignus *et al.,* 2004; Yang *et al.,* 2004), RNA interference (RNAi) technology (e Ammara *et al.* 2015), plantibody production (Reyes *et al.,*2013), CRISPR mediated gene editing (Mehta *et al.,* 2019), marker assisted breeding (Nevame *et al.,* 2018) etc. have shown a new and innovative way of plant viral disease management. But, field application of these techniques needs more research and improvisation.

h) Use of resistant varieties

Use of disease resistant cultivars is the easiest, most effective and durable way to

manage the plant diseases especially those caused by viruses as no other method is available for killing the viruses directly. Classical resistance breeding program involves many typical steps screening and confirmation of resistance in source plants, discovery of genes/alleles and their mapping onto chromosome, transfer of the resistance gene(s) to popular cultivated varieties and field screening of the introgressed lines (Singh *et al.*,2018). In 2003–2004, three resistant open-pollinated varieties of tomato (Sankranthi, Nandi, Vaibhav) were developed against leaf curl disease and released officially in India (Kumar *et al.*,2018).

2.6. Resistance sources of tomato against leaf curl disease

All the commercially available tomato cultivars are susceptible to ToLCD. Resistance against ToLCD has not been reported from the domesticated *S. lycopersicum.* But, many wild relatives of *S. lycopersicum* like *S. peruvianum, S. chilense, S. habrochaites* show resistance against the disease. Till-date six independently inherited genes/QTLs (*Ty-1, Ty-2, Ty-3, Ty-4, ty*-5 and *Ty-6*) have been identified from different wild germplasm resources showing varying levels of resistance against leaf curl disease of tomato (Welegama *et al.*,2021). Prabhandakavi *et al.* (2020) observed a direct correlation between the effectiveness of resistance to begomovirueses and the number of Ty gene(s) present.In 1988, the first commercial TYLCV resistant hybrid, 'TY 20' carrying resistance derived from *S. peruvianum* 'PI 126935′ was developed by Pilowsky and Cohen (1990).

2.6.1. *Ty-1* locus

Among the six resistant/tolerant loci against TYLCV, *Ty-1* was the first to be identified and mapped on the centromeric side of chromosome 6 in *S. chilense* accession LA1969 by Zamir *et al.* (1994). Marker TG97 and TG297 was found to be linked with *Ty-1* locus at 3.1 and 5.5 cM respectively. *Ty-1* a major incompletely dominant locus and with two modifier gene on chromosome 3 and 7. It is the most widely used resistance gene in worldwide breeding programs against TYLCV. Subsequently, a new CAPS marker, JB-1, was found to be more tightly linked to the *Ty-1* locus (de Castro *et al.*,2007). An extensive study by Verlaan *et al.* (2011) demonstrated that the chromosomal inversions between *S. lycopersicum* and *S. chilense* 'LA 1969′ hampered precise mapping of *Ty-1*. Also, they precisely determined the actual map position on the long arm of chromosome 6 between the marker MSc05732-4 and MSc05732-14. *Ty-1* codes for a DFDGD class RNA dependent RNA polymerase (RDR) (Verlaan *et al.*,2014). Butterbach *et al.* (2014) reported that, resistance by *Ty-1* involves siRNA mediated transcriptional gene silencing and increased cytosine methylation of viral genome. Though *Ty-1* shows effective resistance against TYLCV, in India it is not very much successful against ToLCD especially against those caused by bipartite begomoviruses. *Ty-1* lines showed promising result against monopartite ToLCBaV but were relatively less effective against bipartite ToLCNDV and are ineffective against bipartite ToLCPalV (Prasanna *et al.,* 2015).

2.6.2. *Ty-2* locus

Hanson *et al.* (2006) first time reported *Ty-2* resistant locus on short arm of chromosome 11 (near TG36 region) of *S. habrochaites* derived line H24. Further molecular marker analysis of recombinants and evaluation of progeny for TYLCV resistance from these recombinants localized Ty-2 to an approxmiate 300,000-bp interval between two markers UP8 (51.344 Mb) and M1 (51.645 Mb) on chromosome 11w (Yang *et al.*,2014). *Ty-2* is a dominant gene and provided high degree of resistance against many variants of TYLCV. But it is not very much effective against bipartite begomoviruses, and the resistance could be overwhelmed by some strains of TYLCV (Ji *et al.* 2007). In India, *Ty-2* remain ineffective against monopartite ToLCBaV (Kushal *et al.*,2020) and also against bipartite ToLCNDV and ToLCPalV (Prasanna *et al.*,2015). The *Ty-2* gene acts by restricting virus multiplication and accumulation. Candidate genes in the *Ty-2* region include five putative ORFs codes for CC-NBS-LRR proteins (Yamaguchi *et al.*,2018). Positive correlation was observed between concentration of the virus and severity of the disease in genotypes with *Ty-2* gene (Barbieri *et al.,* 2010).

2.6.3. *Ty-3* locus

In India, *Ty-3* showed most promising result in controlling ToLCD and most importantly against those caused by bipartite begomoviruses. It is a partially dominant major gene, derived from 'LA 2779′ and 'LA 1932′ lines of *S. chilense and* was mapped in the long arm of chromosome 6 (Ji *et al.,* 2007). Subsequently, the name *Ty-3a* was assigned to the resistance locus from 'LA 1932' (Koeda *et al.*,2020). This gene is flanked by CAPS markers cLEG-31-P16 and T1079, separated by 7 cM distance. It was found that *Ty-3* introgression derived from 'LA 2779′ was longer and linked to the *Ty-1* locus. *Ty-1* mapped to the long arm of chromosome 6 and flanked by MSc05732-4 and MSc05732-14 markers, an interval which is overlapped with the previously reported map position of *Ty-3*. This finding confirmed that both *Ty-1* and *Ty-3* are the alleles of the same locus (Verlaan *et al.,* 2011). It is possible that *Ty-1* is a truncated fragment of *Ty-3*. Verlan *et al.* (2013) also reported that *Ty-3* locus consists five candidate genes and both *Ty-1* and *Ty-3* encode for DFDGD class RNA dependent RNA polymerase (RDR). Prasanna *et al.* (2015) found that, though *Ty-1* and *Ty-3* are allelic, but *Ty-1* and *Ty-3* alleles showed differential response in genotypes through ToLCV in the field screening and challenge inoculation with ToLCPalV. *Ty-3* locus though exhibited less dominance but tomato genotypes carrying *Ty-3* locus exhibited highly resistant response against both the monopartite ToLCBaV and bipartite viruses (ToLCNDV and ToLCPalV) (Prasanna *et al.,* 2015). Thus, It has been assumed that *Ty-3* exhibits a higher level and wider spectrum of resistance than the *Ty-1* and *Ty-2*. Moreover, highly effective and durable resistance/tolerance to ToLCV was achieved by pyramiding both *Ty-2* and *Ty-3*. It was also observed that, although tomato lines carrying both *Ty-2* and *Ty-3* loci did not exclude the virus

completely, yet symptom expression and level of virus accumulation were almost negligible in those pyramided lines (Lata *et al.,*2019; Kushal *et al.,*2020; Prasanna *et al.,*2015; Tabein *et al.,*2017; Sadashiva *et al.,*2017).

2.6.4. *Ty-4* locus

Use of advanced breeding lines derived from 'LA 1932′ of *S. chilense*, a TYLCV resistance locus termed *Ty-4*, was identified and mapped to the 2.3 cM marker interval located between C2_At4g17300 and C2_At5g60160 onto the long arm of chromosome 3 (Ji *et al.,*2009). While variance in TYLCV resistance was found to be in the tune of 60% with respect to *Ty-3* locus, it was only 16% for *Ty-4* locus. Hence, it is regarded as minor gene for having relatively lesser effect on resistance. Therefore, *Ty-4,* if used independently, would be ineffective under high disease pressure (Ji *et al.,*2009). Depending on ease of scoring, strength and consistency of amplification, Prasanna *et al.* (2015) identified marker C2_At5g63460 for genotyping assays of *Ty-4*. It is effective against TYLCV, but very few reports are available on bipartite begomoviruses. However, inbreds with *Ty-3* and *Ty-4* showed greater resistance than lines with *Ty-3* alone in Guatemala (D.P. Maxwell, unpublished data), suggesting the effectiveness of *Ty-4* against multiple (up to seven) bipartite begomoviruses reported there (Nakhla *et al.,* 2005).

2.6.5. *Ty-5* locus

The resistance locus, *Ty-5,* was discovered recently in the breeding line TY172 of *S. peruvianum*. This locus was mapped on chromosome 4 near the marker SlNAC1 (Anbinder *et al.,* 2009). Later, marker analysis revealed that SlNAC1 marker co-segregates with a resistance allele which is derived from the cultivar Tyking. The present study clearly shows that this allele is recessive as mean disease severity of plants heterozygous at *ty-5* locus was no different from mean disease severity of plants homozygous-susceptible. Data again suggest that there may be involvement of an additional resistance gene, because homozygosity of *ty-5* allele did not show on average equal parental level of resistance, and also absence of *Ty-5* allele did not cause on average equal parental level of susceptibility (Hutton *et al.,*2012). Recent finding on fine mapping and characterization of *ty-5* gene suggested that the origin of this gene may not be *S. peruvianum* (Lapidot *et al.,* 2015). Lapidot and his co-workers also identified a tomato homolog of the messenger RNA surveillance factor Pelota (pelo) as the controlling factor of *ty-5* resistance.

2.6.6. *Ty-6* locus

In 2014, Hutton and Scott first time identified a major resistance gene on chr. 10 of *S. chilense* line Fla. 8383 closely linked to the marker SLM-10-46. It was found that *Ty-6* shows incomplete dominance in heterozygous condition. Also, further analysis on segregating

populations for *Ty-6* locus along with *Ty-3* or *ty-5* locus showed that, when *Ty-6* was combined with the other resistance alleles, it gave maximum resistance against TYLCV. *Ty-6* is proved effective against both monopartite (*Tomato yellow leaf curl virus*) as well as bipartite (*Tomato mottle virus*) tomato infecting begomoviruses. (Gill *et al.,* 2019). In India, combination of *Ty-6* with other resistance genes like *Ty-5, Ty-2* showed considerable effectiveness against begomoviruses like *Tomato leaf curl New Delhi virus (*ToLCNDV) and *Tomato leaf curl Bangalore virus* (ToLCBaV) (Kaushal *et al.,* 2020).

2.7. Evaluation of tomato genotypes against leaf curl disease

Evaluation of tomato genotypes derived from different wild tomato species showed that, a number of accessions from *S. arcanum*, *S. chilense*, *S. chmielewskii*, *S. corneliomulleri*, *S. habrochaites*, *S. huaylasense*, *S. neorickii*, *S. pennellii* and *S. peruvianum* remained symptomless even after inoculation with TYLCD. On the other hand, most of the accessions from *S. pimpinellifolium* and *S. lycopersicum* were lacking effective resistance with severe symptoms like yellowing, purple vein, leaf curling, stunting, and reduced leaflet size etc. (Azizi *et al.,* 2008; Yan *et al.,* 2018). Also, highly resistant to highly susceptible disease reactions were observed in different wild species derived tomato genotypes against ToLCD (Ponselvakumari *et al.,* 2020). Assessment of resistance of different cultivated varieties, hybrids and wild accessions against TYLCD/ToLCD based on either percent disease incidence or disease severity also resulted similar findings with variable resistance response of the domesticated/wild genotypes (Muniyappa *et al.,* 1991; Picó *et al.* 1998; Maruthi *et al.,*2003; Reddy *et al.,*2008; Camara *et al.,*2012; Singh, 2014; Singh *et al.,* 2015; Nadkarni *et al.,*2017; Kumar *et al.* 2019; Mandali *et al.,*2020). Similarly, evaluation of many wild tomato genotypes containing *Ty*-genes highlighted superiority of *Ty-3, Ty-5* genes with best performance in combination with other *Ty*-genes like *Ty-1, Ty-2* etc (Elbaz *et al.,*2016; Al-Shihi *et al.,*2018; Prabhandakavi *et al.,* 2020).

2.8. PCR based detection of begomoviruses

PCR or polymerase chain reaction is one of the most widely used methods of detection of plant viruses. Specific primers for different begomovirus species were used to amplify a specific region of the genome. Sequencing of the PCR amplicon and comparative sequence analysis gave the confirmatory evidence for detection of particular species (Garg *et al.,*2015; Kumar *et al.,*2018; Prabhandakavi *et al.,* 2018). Genetic diversity and distribution of begomoviruese were assessed following the PCR based detection approach (Ribeiro *et al.,*2002; Reddy *et al.,*2005; Shohrab, 2020).

3.1. Development of a disease scoring scale

A scoring scale was developed for quantitative estimation of tomato leaf curl disease as no such scale was available for it. Extent of leaf curl, leaf smalling, stunting of the plant and number of fruits were considered as the parameters for the disease scoring scale. To understand the developmental stages of tomato leaf curl disease, 20 plants of a susceptible check variety of tomato (cv. Pusa Ruby) were artificially inoculated with a well characterized isolate of tomato leaf curl New Delhi virus (ToLCNDV, MW429271) using viruliferous whiteflies @ 5 per plant with 24 hours of acquisition access period and inoculation access period each. Inoculation was done 7 days after transplanting of the seedlings. The disease development was recorded.

To measure the qualitative parameters quantitatively, they were assigned with numerical values according to their contribution towards disease development. The scale was developed following a method earlier used by Bag *et al.* (2014) and others in different viral diseases (Table 2), where coefficient of infection (CI) was considered as the parameter for deciding the ultimate disease reaction and CI was calculated by multiplying the percent disease incidence with response value (RV). The response value (RV) is actually quantitative transformed value of percent severity range in 0 to 1 scale and the percent severity range was calculated based on cumulative value of all the disease evaluation parameters.

$$\text{Percent disease incidence} = \frac{\text{No. of leaf curl infected plants in a genotype}}{\text{Total no. of plants of that genotype}} \times 100$$

CI = Percent disease incidence x Response value (RV)

Table 2: Scale for classifying disease reaction under field conditions.

Percent severity range	Response value	Coefficient of infection (CI)	Disease reaction
0	0.0	0-4.0	Highly resistant (HR)
>0-25%	0.25	>4.0-9.0	Resistant (R)
>25-50%	0.50	>9.0-19.0	Moderately resistant (MR)
>50-75%	0.75	>19.0-39.0	Moderately susceptible (MS)
>75%	1.0	>39.0-69.0	Susceptible (S)
		>69.0	Highly susceptible (HS)

3.2. Disease phenotyping and scoring of tomato genotypes

Phenotypic screening was done for 17 *Ty*-gene(s) containing donor genotypes of tomato along with a susceptible check (cv. Pusa Ruby) (Table 3). These *Ty*-gene(s) containing donor lines are actually breeding lines in F8-9 generation, developed by selection from exotic lines. They were procured from AVRDC, Taiwan as TYLCV tolerant sources. Seedlings of these genotypes were transplanted during last week of September, 2020 at experimental field of Division of vegetable science, IARI-New Delhi following randomized complete block design (RCBD) method with 3 replications. A spacing of 75 cm (plant to plant) and 90 cm (row to row) was followed with standard fertilizer application @ 150 Kg N, 60 Kg P and 60 Kg K per hectare. For disease scoring, 5 leaves from each disease infected plant of a genotype were given the score for leaf curl and leaf smalling. The cumulative average score of both leaf curl and leaf smalling parameters of individual plant were then summed with the scores for stunting and fruiting parameters to get the disease severity score of that plant.

$$\text{Severity score} = \left[\frac{\sum_{s=A}^{B} L_{1s}+\sum_{s=A}^{B} L_{2s}+\sum_{s=A}^{B} L_{3s}+\cdots+\sum_{s=A}^{B} L_{ns}}{T_N} + C + D\right]$$

Where, L_1, L_2, L_3, … L_n = number of affected leaves on a plant ranging from 1 to n; s = score of a diseased leaf varying from A (leaf curl) to B (leaf smalling); L_{is} = total score on diseased leaf of a plant in a particular stage/period (i = 1/2/3/.../n); C=score for stunting parameter, D= score for fruiting parameter); T_N = total number of leaves studied on infected plant.

Table 3: List of *Ty*-gene containing donor genotypes of tomato used in the study along with resistance gene(s) present in them

Sl. No.	Tomato genotypes	Selection from	Ty-gene(s) present
1.	ToLCD Tol -1	EC904113	Ty-3
2.	ToLCD Tol -2	EC814907	Ty-2
3.	ToLCD Tol -3	EC814906	Ty-1
4.	ToLCD Tol -4	EC814914	Ty-2, Ty-3a
5.	ToLCD Tol -5	EC814916	Ty-2, Ty-3
6.	ToLCD Tol -6	EC814918	Ty-5, Ty-6
7.	ToLCD Tol -7	EC814913	Ty-3a
8.	ToLCD Tol -8	EC2901	Ty-2, Ty-3
9.	ToLCD Tol -9	EC814915	Ty-2, Ty-3
10.	ToLCD Tol -10	EC814911	Ty-5
11.	ToLCD Tol -11	EC814917	Ty-2, Ty-3
12.	Pusa Ruby (susceptible check)	Pusa Ruby (susceptible check)	-
13.	ToLCD Tol -12	EC904111	Ty-2, Ty-3
14.	ToLCD Tol -13	EC904115	Ty-2, Ty-3
15.	ToLCD Tol -14	EC751804	Ty-2, Ty-3
16.	ToLCD Tol -15	EC904117	Ty-2, Ty-3
17.	ToLCD Tol -16	EC751802	Ty-2, Ty-3
18.	ToLCD Tol -17	EC904114	Ty-2, Ty-3

3.3. Glasshouse evaluation of selected tomato genotypes

Tomato genotypes with highly resistant response under field condition were identified and subjected to artificial inoculation with tomato leaf curl New Delhi virus (ToLCNDV, MW429271) using viruliferous whiteflies @ 5 per plant, to confirm their resistance/tolerance by challenge inoculation under controlled condition. An acquisition access period and inoculation access period of 24 hours each were given. Inoculation was done after 7 days of transplanting of the seedlings and symptom progression was recorded periodically.

3.4. Electron microscopy of collected leaf samples

For electron microscopic analysis, 20 leaf samples were collected randomly from all the tomato genotypes. Collected samples were then subjected to microscopic analysis using JEM 1011 Transmission Electron Microscope (TEM) following leaf-dip method at Advanced Centre for Plant Virology, IARI-New Delhi. 2% uranyl acetate was used as negative stain.

3.4.1. Preparation of carbon coated grid

Copper grids of diameter 3 mm (400 mesh) were cleaned using acetic acid and loaded on a clean filter paper, placed at the bottom of a sterilized petridish containing sterile distilled water. A clean slide was coated with a thin film of carbon using a vacuum coating unit and then brought gently in contact with water of the petridish containing the grids. The carbon film was then gently allowed to drift off on the water. The filter paper was softly lifted with the grids together and raised off with the floating carbon film. In this process the grids will be coated with thin carbon film. The carbon coated grids were then air dried in dark at room temperature for about 12-24 hours or in an oven at 37^0C for about 30 minutes.

3.4.2. Selection of tissues

In different parts of the plant, virus particles generally occur in varying concentrations. Young leaves usually contain high concentration of the virus. Corkborer was used to cut the diseased leaf bits from the infected young leaves. The cut leaf bits contained both veins and interveinal portions.

3.4.3. Preparation of virus extract from leaf tissues

Selected leaf bits were then macerated on a precleaned glass slide with a glass rod along with 2-3 drops of phosphate buffer (0.07 M, pH 6.5) and the finely homogenized material was allowed to settle down for a few seconds. The clear supernatant was taken with the help of a fine glass capillary and then used for mounting the grids.

3.4.4. Mounting

Carbon coated grid was then mounted with few drops of homogenized leaf extract. After

one minute, the extract was sucked off from the grid surface by touching the filter paper on one side of the grid. The grid was then washed with 5 to 6 drops of sterile distilled water before staining with 2 drops of uranyl acetate (2% aqueous, pH 4.2), which was used as negative stain. Excess stain was removed using a strip of filter paper by touching the edge of the grid. The grid was kept for air drying for 1-2 minutes and finally examined under EM.

3.4.5. Grid examination for measurement of particles

Negatively stained grid was loaded to the grid holder of the EM and scanned. Virus particles for their morphology and size were studied with good contrast. The EM photographs of virus particles were captured with 80000X magnification. The dimensions of the particles were measured in nm by using a formula,

$$\text{Size of virus particles (nm)} = \frac{\text{Diameter of the particle in mm}}{\text{Magnification}} \text{ X } 10^6$$

3.5. Isolation of total nucleic acid (TNA) from leaf samples

The total nucleic acid (TNA) was extracted from the tomato leaf samples following CTAB method (Doyle and Doyle, 1987). 0.1 gm of leaf sample was weighed and ground in pre-chilled mortar and pestle using liquid nitrogen till a fine powder was obtained. The powder was transferred to a 2 ml polypropylene micro centrifuge tube containing 1 ml of warm extraction buffer, using a spatula. The tube was then vortexed well and incubated at 65°C for 1 hr with intermittent mixing after every 10-15 min by inverting the tubes few times. Equal volume (3 ml) of Phenol : Chloroform : isoamyl alcohol (25:24:1) mixture was then added and was mixed well by inverting the tube. The reaction mixture was then centrifuged for 10 min at 12000 rpm at room temperature. Then a wide bore pipette was used to transfer the upper aqueous phase to a clean tube. 0.6 volume of ice cooled isopropanol was added to the aquas solution and mixed gently. The reaction mixture was centrifuged at 12000 rpm for 10 minutes at room temperature and the supernatant was discarded leaving the nucleic acid (NA) pellet. The NA pellet was then washed with 70% ethanol (0.5-1 ml) by centrifuging at 12000 rpm for 10 min at 4°C. The supernatant was discarded carefully and the pellet was dissolved in 50 µl nuclease free water. TNA were isolated from 5 leaf samples (symptomatic and asymptomatic) collected from each tomato genotype. Then, equal amount from those 5 TNA solutions were mixed to get a pooled TNA sample. A total of 18 pooled TNA samples were made for 18 different tomato genotypes. The quantity and quality of NA was measured by agarose gel (0.8%) electrophoresis.

Recipe of DNA extraction buffer

Reagents	Working concentration	Working volume (ml)
1 M Tris-HCl (pH-8)	100 mM	10
5 M NaCl	1.4 M	28
0.5 M EDTA	20 mM	4
10% CTAB	2%	20
Beta-mercaptoethanol	0.2%	0.2
Distilled water		37.8
Total volume		100

3.6. PCR amplification with specific primers

The pooled TNA samples were then used as templates for PCR amplification using a universal begomovirus primer and seven different begomovirus species specific primers (Table 4). PCR master mix was prepared in a micro centrifuge tube by mixing all the components (except the template) in appropriate amount followed by a quick spinning to collect them at the bottom of the tube. The TNA templates were taken in separate PCR tubes. Then required amount of master mix was added to each tube. The PCR tubes were tightly capped and spinned quickly before placing in the thermal cycler. The temperature profile and PCR programme used for amplification are given below. The PCR amplified product was then analysed by 1% agarose gel electrophoresis followed by gel documentation under UV.

Recipe of PCR reaction mixture

Reagents	Working volume (µl)	Final volume (µl) (22X)
10X Dream*Taq* DNA pol. buffer	2.5	55
dNTP mixture (10mM)	0.5	11
Forward primer (10µM/µl)	0.5	11
Reverse primer (10µM/µl)	0.5	11
Dream*Taq* DNA polymerase (5U/µl)	0.125	2.75
Template (~100 ng/ µl)	1	-
Nuclease-free water	19.875	437.25
Total	25	528

Table 4: List of primers used for indexing of begomoviruses

Sl. No	Primer name	Primer sequences* (5'-3')	Primer location	Annealing temperature (°C)	Amplicon size (bp)	Primer specific for-
1	BM783F BM784R	CCCCTGTGCGTGAATCCGT SDVTBCMGCTGCGCGGCC	AC1	58	538	*Begomovirus*
2	BM794F BM795R	CCTTGTAAGGTGCAGTCC AACCCAGGTCCTTAAGTACC	AV1	56	702	*Tomato Leaf Curl New Delhi Virus* (ToLCNDV)
3	BM798F BM799R	AGACTTGCTCACCAAGC GAAATCTTGTGGCGCAC	AV2/AV1	56	647	*Tomato Leaf Curl Palampur Virus* (ToLCPalV)
4	BM800F BM801R	GAAGCGACCAGCAGATATGC GTGTTGGTGTGGTTCTTCAC	AV2/AV1	60	396	*Tomato Leaf Curl Gujrat Virus* (ToLCGuV)
5	BM802F BM803R	AGAATGTGCATCGTGACAGG TCGTCGCGTTGACCTGGAC	AV1	62	732	*Tomato Leaf Curl Joydevpur Virus* (ToLCJoV)
6	BM796F BM797R	CACGGATTCAGGTGTATGCTT ACAGCAGCACGCTTGCTG	AV2	62	215	*Tomato Leaf Curl Bangalore Virus* (ToLCBV)
7	BM861F BM862R	GAGTCTAGACACGATGTA CATCAGAGCATTCTCACT	AV1	56	453	*Chilli Leaf Curl Virus* (ChiLCV)
8	BM605F BM606R	TCAGAGCTTAATTGTTTTGTGGT GACAAATAACCTTACCCATGTGA	AV2/AV1	56	498	*Croton Yellow Vein Mosaic Virus* (CYVMV)

*S = G /C; D = A/T/G; V = A/C/G; B = C/G/T; M = A/C

Temperature profile for PCR amplification

Initial denaturation- 3 min. at 95°C
Denaturation: 30 sec. at 95°C
Annealing: 45 sec. at-
52°C for ChiLCV specific primers
56°C for ToLCNDV, CYVMV,ToLCPalV specific primers
58°C for Universal begomovirus specific primers
60°C for ToLCGuV specific primers
62°C for ToLCJoV, ToLCBaV specific primers
Extension: at 72°C for-
30 sec for *Begomovirus*,ToLCGuV, ToLCBaV, ChiLC, CYVMV specific primers
1 min for ToLCNDV, ToLCPalV, ToLCJoV specific primers
Final extension: 10 min. at 72°C
Store: 4°C

3.7. Agarose gel electrophoresis

The PCR amplified products were analyzed using 1% agarose gel prepared in 1X Tris-acetate-EDTA (TAE) buffer (Appendix). For preparing 1% agarose gel, 1 g agarose (Agarose Plus, PRONASTAR, Spain) was dissolved in 100 ml 1X TAE buffer by boiling it for 2-3 minutes in microwave oven and cooled down such that gel does not set. 5.0 µl GoodView™ Nucleic Acid Stain (BR Biochem Life Sc.) was added and mixed carefully so that no bubble is formed. Now, the melted gel was poured into gel casting tray fixed with comb. After solidification of gel the tray was transferred into the gel running tray containing 1X TAE buffer and the comb was removed carefully without disturbing the gel. The PCR products, mixed with 6X gel loading dye (Thermo Fisher Scientific, USA), was loaded into wells of the gel and electrophoresed at 70-80V for 30-45 minutes. An aliquot (0.1 µg/µl) of 100 bp plus DNA ladder (Thermo Fisher Scientific, USA) was simultaneously electrophoresed as marker to measure the size of amplified DNA. After electrophoresis, the PCR amplified DNA fragments were visualized using UV gel documentation system (PERKINELMER, Geliance 200 imaging system).

3.8. Purification of PCR amplified DNA

The representative PCR amplicons obtained from each begomovirus specific primer were purified from the agarose gel using Wizard® SV Gel and PCR clean-up kit (Promega Corporation) following the manufacturer's protocol.

a) Dissolving gel slice

The DNA band containing portion of the gel was excised and placed in a 2 ml microcentrifuge tube. The weight of the gel was recorded. Equal amount of Membrane Binding Solution (10 µl of Membrane Binding Solution for 10 mg of gel slice) was then added and

vortexed and then incubated at 60°C with intermittent shaking until the gel slice dissolved completely.

b) Purification of DNA

The dissolved gel mixture was transferred to a minicolumn (upto 800 µl at once) provided by the company and incubated at room temperature for one minute. The column was centrifuged in a tabletop centrifuge at 14000 rpm for 1 min at room temperature. The flow through collected at the bottom of the collection tube was discarded and column was placed back. 700 µl of Membrane Wash Solution (diluted with 95% ethanol) was then added and centrifuged at 14000 rpm for 1 min at room temperature. The flow through was discarded again. This washing step was repeated using 500 µl Membrane Wash Solution. One empty spin was given at 14000 rpm for 1 min to remove any residual ethanol present in the column filter. The column was then placed in fresh autoclaved 1.5 ml Eppendorf tube and 25 µl of Nuclease-Free Water (pre-warmed at 70^0C for 5 minutes) was added to the column and incubated for 1 minute at room temperature. This was then centrifuged at 14000 rpm for 1 minute at room temperature. The last step was repeated with 25 µl of Nuclease-Free Water. The column was then thrown and the tube containing the purified DNA was stored at -20^0C for further use.

3.9. Sequencing of PCR amplified DNA

The gel purified PCR amplicons were then sent for sequencing using the forward primer for the corresponding begomovirus. Sanger sequencing was done using Applied Biosystems™ 3730 DNA Analyzer.

3.10. Analysis of sequences

The nucleotide sequences of PCR amplicons were then used for NCBI nucleotide BLAST analysis to find their related sequences and those sequences were used for ClustalW multiple sequence alignment using BioEdit 7.2 software (Hall, 1999). The alignment data and sequence identity matrix were recorded.

3.11. Cloning of viral genome

Complete genome of begomoviruses detected in tomato genotype ToLCD Tol-7 and ToLCD Tol-1 were cloned using pUC18 cloning vector (Fig 5).

3.11.1. Rolling circle amplification (RCA)

RCA is used to amplify any circular DNA molecule. For performing RCA of begomovirus genome, 3 µl of TNA sample from genotype ToLCD Tol-7 and ToLCD Tol-1 was mixed separately with 1 µl of exo-resistant random primer (500 µM, 1.1 µg/µl), 2 µl of 10X reaction buffer and 10.2 µl of nuclease free water. The reaction mixture was incubated at 95°C for 5 min and then immediately chilled on ice for 2 min. After that, 2 µl of 10 mM dNTP

mix, 1.6 µl of phi-29 DNA polymerase (10 U/µl) and 0.2 µl of inorganic pyrophosphatase (0.1 U/µl) were to the reaction mixture and incubated at 30°C for 18 hrs for amplification. The amplification reaction was then stopped by heating it at 65°C for 10 min. Amplification of viral genome was confirmed by agarose gel electrophoresis of the RCA product. The RCA product was stored at 4°C for further use.

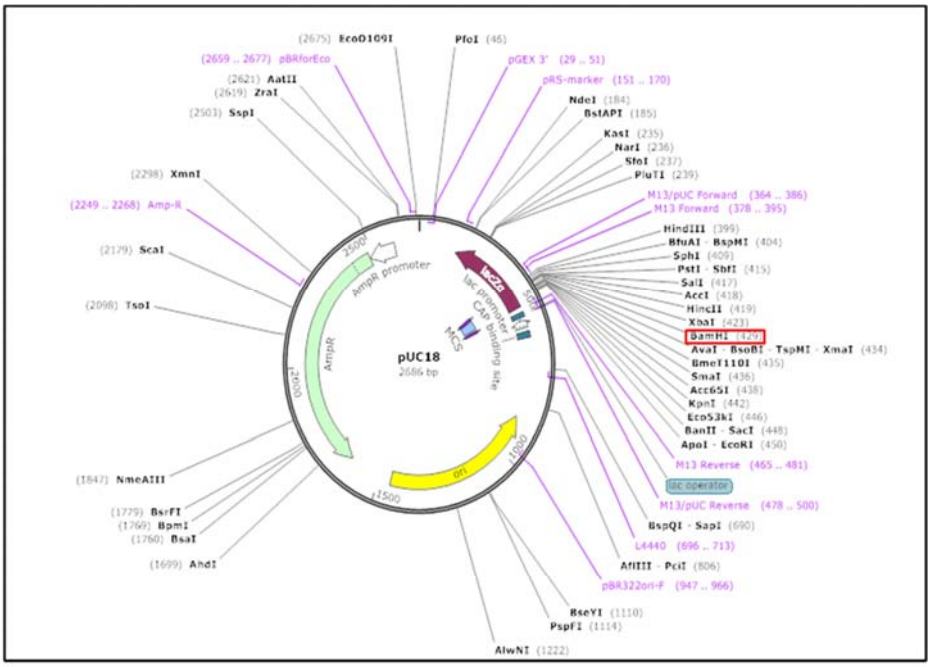


Fig. 5: Genome map of pUC18 cloning vector showing *Bam*HI restriction site (marked with red box) used to clone complete genome of begomoviruses and recombinant colonies were selected based on ampicillin resistance (marked as green arrow)

Components of rolling circle amplification

Reagents	Working volume (µl)
Template	3
Exo-resistant random primer (1.1 µg/µl)	1
10X buffer	2
dNTPs (10 mM)	2
phi-29 DNA polymerase (10 U/µl)	1.6
Pyrophosphatase, inorganic (0.1 U/µl)	0.2
Nuclease free water	10.2
Total	20

3.11.2. Restriction digestion of RCA amplified product and vector

pUC18 cloning vector (2686 bp, Thermo Fisher Scientific) was used as the vector for cloning the viral genome. Restriction enzyme *Bam*HI was used to linearize and to produce steaky ends with 5' overhang in both RCA amplified insert DNA and vector. For restriction digestion of RCA amplified product, 3 µl of RCA product was mixed with 2 µl of 10X FastDigest® buffer, 1 µl of FastDigest® *Bam*HI enzyme (Thermo Fisher Scientific, 10 U/µl) and 14 µl of nuclease free water. The reaction mixture was incubated at 37°C for 30 min for the enzyme to work. For restriction digestion of vector, 2 µl of pUC18 vector (0.5 µg/µl) was mixed with 1 µl of FastDigest® *Bam*HI, 1 µl of alkaline phosphatase (FastAP, 1 U/µl), 2 µl 10X FastDigest® buffer and 14 µl of nuclease free water. After incubation, the digested product was visualized by 0.5% agarose gel electrophoresis followed by UV transillumination.

Recipe for restriction digestion of RCA amplified product

Reagents	Working volume (µl)
RCA amplified product	3
FastDigest® *Bam*HI (10 U/µl)	2
10X FastDigest® buffer	1
Nuclease free water	14
Total	20

Recipe for restriction digestion of pUC18 vector

Reagents	Working volume (µl)
pUC18 vector (0.5 µg/µl)	3
FastDigest® *Bam*HI (10 U/µl)	2
FastAP (1 U/µl)	1
10X FastDigest® buffer	1
Nuclease free water	13
Total	20

3.11.3. Purification of linearized RCA product and pUC18 vector

The *Bam*HI digested linearized RCA products and pUC18 vector were then purified from agarose gel following the same procedure described earlier (section 3.8).

3.11.4. Ligation of linearized viral genome with pUC18 vector

The purified linearized RCA products were ligated with linearized pUC18 vector by mixing the following reagents and keeping it at 4°C overnight.

Composition of ligation reaction mixture

Reagents	Working volume (µl)
Linearized pUC18 vector (~50 ng/µl)	1.0
Linearized RCA product (~25 ng/µl)	7.5
T4 ligase (1 FDU/µl)	0.5
10X T4 ligase buffer	1.0
Total	10.0

3.11.5. Preparation of competent cells of *Escherichia coli* strain DH5α

$CaCl_2$ method was followed to prepare the competent cells of *E. coli* DH5α strain (Mandel and Higa, 1970). A single colony was picked from the streaked plate of *E. coli* DH5α strain and inoculated in a glass vial having 5 ml sterile Luria-Bertani broth (LB) medium. The bacterial culture was then incubated for overnight in a shaker incubator at 37°C (Lab Shaker, Adolf Kuhner AG, Schweiz) with 200 rpm. 1ml bacterial culture from the overnight grown culture was taken and inoculated in a conical flask containing 50ml of LB. The culture was then incubated at 37°C in an incubator shaker with 200 rpm for about one and half hour until the $O.D_{600}$ value of 0.6-0.8 was obtained. Then the bacterial culture was distributed equally in two 50 ml sterilized, ice-cold Oakridge tubes (25 ml each) under aseptic condition of laminar air flow. The cultures were then kept on ice for 10-15 minutes followed by centrifugation for 10 minutes at 4100 rpm at 4°C. The supernatant was discarded and traces of LB media was drained away by keeping the tubes in an inverted position in a stand on a pad of tissue paper. 12 ml of 0.1M $MgCl_2$ and 3ml of 0.1M $CaCl_2$ were taken in a falcon centrifuge tube and mixed. 7.5 ml of the mixture was transferred into each Oakridge tube containing bacterial pellet. The pellet was suspended through vortexing and the suspension was re-centrifuged at 4100 rpm for 10 minutes at 4°C. Supernatant was discarded and the pellet was dissolved in a mixture containing 500 µl of 0.1 M $CaCl_2$ and 500 µl of 50% glycerol, by gentle pipetting. Then the solution was equally divided into 1.5 ml micro centrifuge tubes with 100 µl in each tube and subjected to instant freezing by keeping in liquid nitrogen (-196°C) for about five minutes and then stored at -80°C.

3.11.6. Transformation of recombinant plasmid into *E. coli* DH5α strain

Heat-shock method was followed to transform *E. coli* DH5α cells with recombinant pUC18 vector. The ligation products (10 µl) were added to 100 µl aliquot of *E. coli* DH5α competent cells, mixed lightly and incubated in ice for 30 minutes in laminar air flow chamber. Then heat-shock was given to the mixture at 42^0C for 50 seconds followed by keeping in ice for 30 seconds and again at 42^0C for 30 seconds followed by keeping in ice for 2 minutes. The

mixture was then kept for 5 minutes at room temperature and 900 µl of LB media was added to it. The culture was then incubated at 37^0C in an incubator shaker with 120 rpm for 1.5 hours. After that, transformed bacterial cells were pelletized by centrifugation at 8000 rpm for 2 minutes at room temperature. 900 µl of supernatant was discarded and the pellet was resuspended in remaining 100 Luria-Bertani broth (LB) media. The suspension was then carefully spread on Luria-Bertani agar (LA) plate containing Ampicillin (100 mg /ml stock conc.) as selection antibiotic. The plate was incubated overnight at 37^0C in an incubator. Master plate (having Ampicillin @ 100 mg /ml as selection) was prepared from overnight grown single colonies and incubated overnight at 37^0C. In this way, two recombinant clones, TY-6 (from ToLCD Tol-7) and Ty-4A (from ToLCD Tol-1), were obtained.

3.11.7. Confirmation of transformed colonies by colony PCR and restriction digestion of isolated plasmids

Recombinant colonies from Ty-6 and Ty-4A clones were confirmed by colony PCR using Croton yellow vein mosaic virus (CYVMV) specific and universal begomovirus primers, respectively (Table 4). The recipe for PCR reaction mixture and the temperature profiles are mentioned earlier. The PCR amplified products were analyzed by 1% agarose gel electrophoresis followed by visualization through UV transilluminator. The clones were further confirmed by restriction digestion of isolated plasmids from the confirmed colonies using *Bam*HI and *Bgl*I enzymes followed by agarose gel (0.8%) electrophoresis of restriction digested products and UV transillumination. By this, two confirmed clones Ty-6.1 and Ty-4A.2 were obtained.

Recipe for restriction digestion of isolated plasmid

Reagents	Working volume (µl)
Plasmid (~50 ng/µl)	3
FastDigest® *Bam*HI (10U/ µl)	1
FastDigest® *Bgl*I (10U/ µl)	1
10X FastDigest® buffer	2
Nuclease free water	13
Total	20

3.13 Sequencing of clones and sequence analysis

The confirmed clones, Ty-6.1 and Ty-4A.2 were sequenced following Sanger sequencing method accompanied by primer walking using Applied Biosystems 3037 DNA analyzer. After initial sequence editing the assembling using BioEdit Sequence Alignment Editor version 7.2 (Hall, 1999), the ORFs of the assembled complete genome of the

begomoviruses were predicted through NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) and the position of the ORFs were mapped into the respective genome of the begomoviruses from both the clones using SnapGene Viewer version 5.3.1 (from Insightful Science; available at snapgene.com) tool. The amino acid sequence of the predicted ORFs were deduced through ExPasy Translate server (https://web.expasy.org/translate/). Presence of multiple functional motifs in the deduced amino acid sequence of the ORFs were predicted using Motif Scan tool (https://myhits.sib.swiss/cgi-bin/motif_scan). Also, nuclear localization signals within first 60 amino acid region of the protein, were predicted using cNLS mapper tool (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) with a cut off score of 3. MEGA-X version 10.2.6 (Kumar et al., 2018) and BioEdit Sequence Alignment Editor version 7.2 (Hall, 1999) tools were used for multiple sequence alignment analysis of both the clone sequences with other sequences of related begomovirus species, retrieved from NCBI. Pairwise global alignment of these sequences was performed through MUSCLE algorithm and a Neighbor-Joining method was used in MEGA-X version 10.2.3 tool (Kumar et al., 2018), with 1000 bootstrap replicates to identify the phylogenetic relationship of the clone sequences. Phylogenetic conflict analysis was also performed using SplitsTree version 4.7.1 tool (Huson and Briyant, 2006) with default parameters. Seven different algorithms (RDP, GENECONV, BootScan, MaxChi, Chimera, SiScan) integrated in RDP version 4.101 (Martin et al., 2015) tool were used for statistical prediction of recombination event in the clone sequences. Arrangement of Rep binding iterons as well as other promoter elements were also identified in the intergenic region (IR) of the clone sequences using PlantCare tool (Rombauts *et al*., 1999).

3.14 Development of partial tandem repeat (PTR) construct

A partial tandem repeat (PTR) construct of begomovirus clone Ty-6.1 was developed (Fig. 6). In the first step, a partial fragment (0.5 kb) of viral genome containing the IR was released from pTy-6.1 plasmid using *Bam*HI-*Sac*I digestion and ligated with *Bam*HI-*Sac*I digested linearized pCambia2300 binary vector and transformed in *E. coli* DH5α cells. Kanamycin was used as selection antibiotic. The recombinant pCambia2300 was confirmed by restriction digestion using *Bam*HI and *Sac*I. In the second step, complete genome of the virus (2.7 kb) was released from pTy-6.1 using *Bam*HI-*Sca*I digestion and ligated with *Bam*HI digested linearized recombinant plasmid of pCambia2300 harbouring the partial (0.5 kb) fragment of the virus in a tandem orientation and transformed in *E. coli* DH5α cells to generate a 1.2 mer partial tandem repeat construct of begomovirus clone Ty-6.1. The orientation was confirmed by restriction digestion using *Sac*I enzyme.

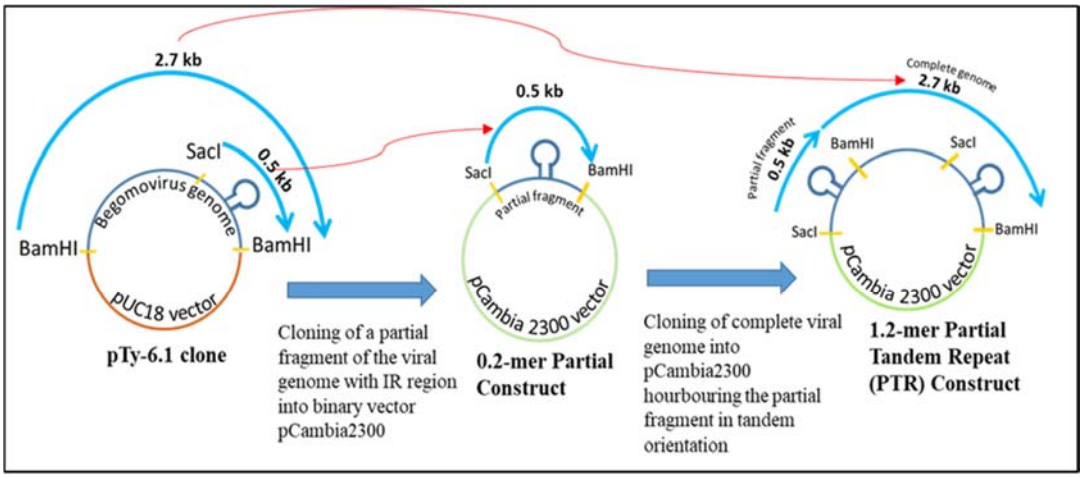


Fig 6. Schematic representation of the process of development of 1.2-mer partial tandem repeat construct of begomovirus clone Ty-6.1 in binary vector pCambia2300.

Recipe for *Bam*HI- *Sac*I digestion of pTy-6.1 clone

Reagents (stock conc.)	**Working volume(µl)**
pTy-6.1 clone (~50 ng/µl)	10
FastDigest® *Bam*HI (10U/ µl)	1
FastDigest® *Sac*I(10U/ µl)	1
10X FastDigest® buffer	3
Nuclease free water	15
Total	30

Recipe for *Bam*HI- *Sac*I digestion of pCAMBIA2300

Reagents (stock conc.)	**Working volume(µl)**
pCAMBIA2300(0.5 µg/µl)	7
FastDigest® *Bam*HI (10U/ µl)	1
FastDigest® *Sac*I(10U/ µl)	1
10X FastDigest® buffer	3
Nuclease free water	18
Total	30

Recipe for ligation mixture of 0.2-mer partial construct

Reagents (stock conc.)	Working volume(µl)
*Bam*HI- *Sac*I digested 0.5 kb fragment of pTy-6.1	2
*Bam*HI- *Sac*I linearized pCambia2300 vector (50 ng/	3
T4 DNA ligase (1 FDU/µl)	0.5
Buffer	0.7
Nuclease free water	0.8
Total	7

Recipe for *Bam*HI- *Sac*I digestion of 0.2-mer partial construct

Reagents (stock conc.)	Working volume(µl)
0.2-mer partial construct (~50 ng/µl)	3
FastDigest® *Bam*HI (10U/ µl)	0.3
FastDigest® *Sac*I (10U/ µl)	0.3
10X Buffer	2
Nuclease free water	14.4
Total	20

Recipe for *Bam*HI- *Sca*I digestion of pTy-6.1 clone

Reagents (stock conc.)	Working volume(µl)
pTy-6.1 clone (~50 ng/µl)	10
FastDigest® *Bam*HI (10U/ µl)	1
FastDigest® *Sca*I (10U/ µl)	1
10X Buffer	3
Nuclease free water	15
Total	30

Recipe for *Bam*HI digestion of 0.2-mer partial construct

Reagents (stock conc.)	Working volume(µl)
0.2-mer partial construct (~50 ng/µl)	7
FastDigest® *Bam*HI (10U/ µl)	1
FastAP (1 U/µl)	1
10X Buffer	3
Nuclease free water	18
Total	30

Recipe for ligation mixture of 1.2-mer PTR construct

Reagents (stock conc.)	Working volume(µl)
*Bam*HI- *Sca*I digested 2.7 kb fragment of pTy-6.1	2
*Bam*HI linearized 0.2-mer partial construct (~50 ng/µl)	1.5
T4 DNA ligase (1 FDU/µl)	0.5
Buffer	0.5
Nuclease free water	0.5
Total	5

Recipe for *Sac*I digestion of 1.2-mer PTR construct

Reagents (stock conc.)	Working volume(µl)
1.2-mer PTR construct (~100 ng/µl)	3
FastDigest® *Sac*I (10U/ µl)	0.5
Buffer	2
Nuclease free water	14.5
Total	20

3.12. Standardization of reverse transcriptase PCR (RT- PCR) for *Ty-3* gene

3.12.1. Isolation of total RNA from leaf samples

The total RNA was extracted from the tomato leaf samples following TRIzol method. 0.1 gm of leaf sample was weighed and ground in prechilled mortar and pestle using liquid nitrogen till a fine powder was obtained. The powder was transferred to a 2 ml polypropylene centrifuge. 1 ml of ice cold TRIzol extraction buffer was added to the tube and mixed well. The mixture was centrifuged at 12000 rpm for 10 minutes at room temperature. The supernatant was collected in a separate tube and incubated at room temperature for 5 min. 1 ml of TRIzol extraction buffer was again added to the supernatant and mixed well by shaking vigorously by hand for 15 sec. The mixture was then incubated at 15-30^{0}C for 2-3 min followed by centrifugation at 12000 rpm for 10 min at room temperature. The colourless upper phase obtained after centrifugation was collected in a new tube. Precipitation of RNA was done by mixing the colourless solution with 0.5 ml ice cold isopropanol followed by incubating at -20^{0}C for 30-60 min. after incubation, the mixture was centrifuged at 12000 rpm for 10 minutes at 4^{0}C. The supernatant was discarded carefully and the RNA pellet was washed using 1 ml 75% ethanol by centrifuging at 8000 rpm for 5 min at 4^{0}C. Excess ethanol was removed and RNA pellet was air dried. Finally, the pellet was dissolved into 50 µl nuclease free water and stored at 4^{0}C for further use. The quality and quantity of RNA was checked by agarose gel (0.8%) electrophoresis.

3.12.2. Standardization of PCR primers

A *Ty-3* gene specific primer (SCAR1 marker) (Table 5) was standardized for confirmation of *Ty-3* gene in the tomato genotypes. For that, PCR amplification was carried out at 53^0C, 55^0C and 56^0C annealing temperatures. Similarly, a qRT-PCR primer, ACY for *Ty-3* gene was also standardized by PCR amplification at 56^0C, 57^0C, 58^0C annealing temperatures. Complete temperature profile of PCR amplification is given below. The PCR amplified products were analyzed using 1.5% (for SCAR1) and 2% (for ACY) agarose gel electrophoresis followed by gel documentation.

Temperature profile for primer standardization

Initial denaturation- 3 min. at 95°C

Denaturation: 30 sec. at 95°C

Annealing: 45 sec. at-

53^0C, 55^0C and 56^0C for SCAR1

56^0C, 57^0C, 58^0C for ACY

Extension: 30 sec at 72°C

Final extension: 10 min. at 72°C

Table 5: List of primers used for standardization of qRT-PCR

Name of the primer	**Sequence (5'-3')**
SCAR1-F	GCTCAGCATCACCTGAGACA
SCAR1-R	TGCAGGAACAGAATGATAGAAA
ACY-F	CCTTATGATGTCTCGTGAAAGG
ACY-R	GAAGCACAGATTGAAGAAAACC

4.1. To evaluate the resistance behavoiur of *Ty*-gene(s) containing donor lines of tomato

4.1.1. Disease progression and symptom category in tomato cv. Pusa Ruby for developing a scoring scale

After inoculation of a pure culture of an isolate of tomato leaf curl New Delhi virus (ToLCNDV, MW429271), maintained in susceptible cv. Pusa Ruby, into 20 plants of the same cultivar through viruliferous whitefly; symptoms started appearing from 10 dpi. Progression of disease development was monitored upto 30 dpi and two distinct symptom phenotypes, *viz.* leaf curl and smalling of leaves were noticed in the leaves of inoculated plants along with stunting of the whole plants (Fig. 7). These symptoms appeared exclusively in some plants where in some others more than one symptom phenotypes were noticed in different combinations. Furthermore, within these symptom phenotypes, different grades were observed based on their severity (Fig. 7). Impact of the disease in the fruit numbers was also considered for developing a disease severity score. Based on the severity and their impact on overall disease phenotype different grades of symptoms were assigned with numerical values mentioned in Table 6.

The deduced average severity score of a particular genotype was further categorized into four response value (RV) category, where response value score of 0, 0.25, 0.5, 0.75 and 1 represent 0%, >0 to 25%, >25 to 50%, >50 to 75%, >75 to 100% severity score, respectively. The response value of individual genotype was multiplied with the percent average disease incidence to measure the coefficient of infection (CI), which always presented within 0-100 scale. The disease reaction categories for individual genotypes were deduced from a standard scale as mentioned in materials and method section.

4.1.2. Disease phenotyping and scoring of tomato genotypes

Evaluation of 17 donor line of tomato and a susceptible check (Pusa Ruby) under field condition against leaf curl disease using the developed scoring scale showed that 5 genotypes (ToLCD Tol-5, ToLCD Tol-8, ToLCD Tol-12, ToLCD Tol-14, ToLCD Tol-15), all containing both *Ty-2* and *Ty-3* gene, were expressing highly resistant (HR) disease reaction while 6 genotypes (ToLCD Tol-1, ToLCD Tol-4, ToLCD Tol-9, ToLCD Tol-11, ToLCD Tol-6, ToLCD Tol-16) showed moderate resistance (MR), 4 genotypes (ToLCD Tol-3, ToLCD Tol-10, ToLCD Tol-17, ToLCD Tol-13) showed moderate susceptibility (MS) and ToLCD Tol-1, ToLCD Tol-2, Pusa Ruby showed resistant (R), susceptible (S) and highly susceptible (HS) disease reaction, respectively (Table 7). The complete data of field evaluation of tomato genotypes is shown in Table 8.

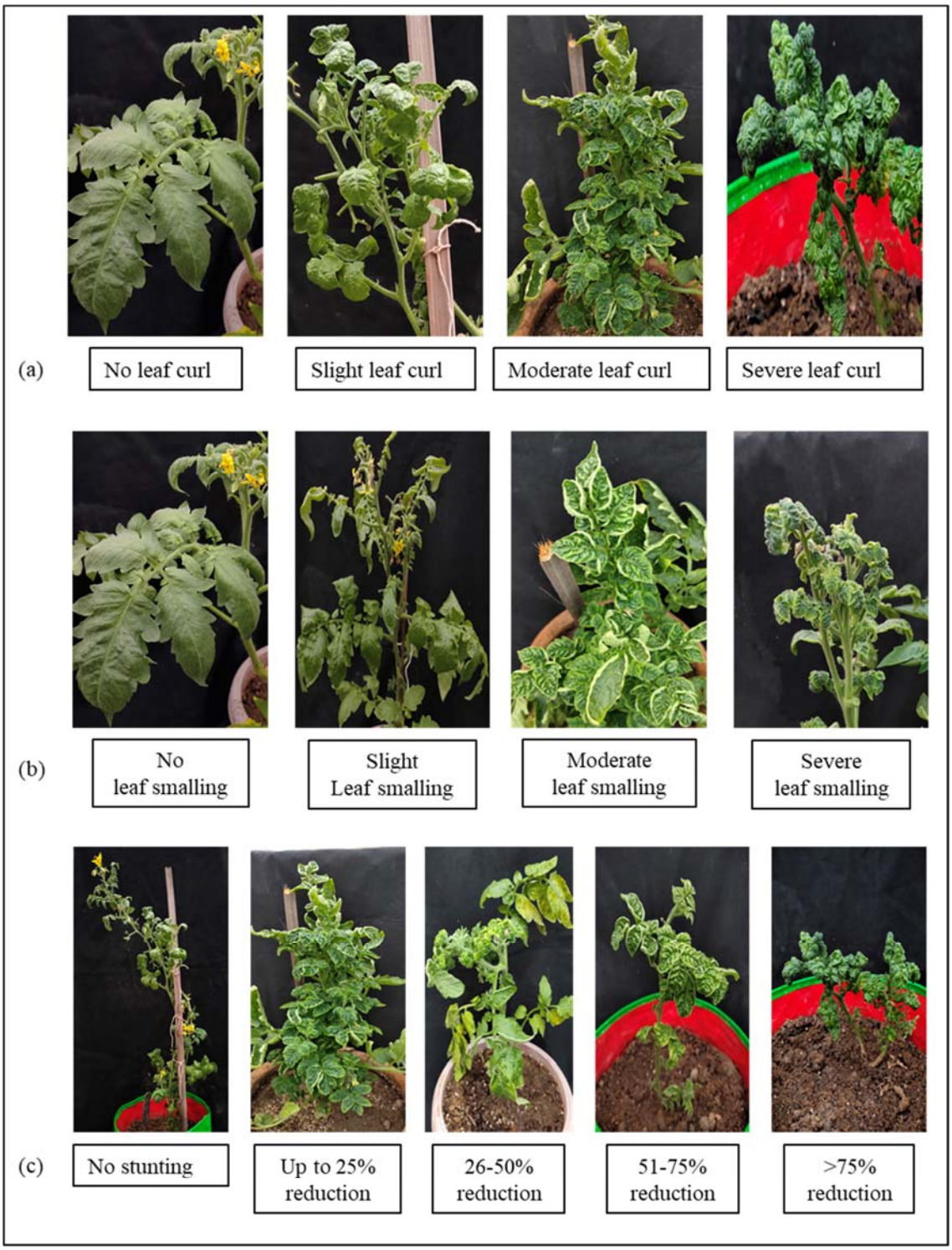


Fig. 7: Parameters used for scoring of tomato leaf curl disease based on leaf curl, leaf smalling, stunting symptom phenotypes.

(a) Categories of leaf curl
(b) Categories of leaf smalling
(c) Categories of whole plant stunting

Table 6: List of parameters used for scoring of tomato leaf curl disease and corresponding numerical values assigned to them for development of a quantitative scoring scale

Parameters	Categories	Values
(A) Leaf curl	No curling	0
	Slight curling	5
	Moderate curling	10
	Severe curling	15
(B) Smalling of leaves	No smalling	0
	Slight smalling	5
	Moderate smalling	10
	Severe smalling	15
(C) Stunting	No stunting	0
	Up to 25%	10
	26-50%	20
	51-75%	25
	>75%	30
(D) No. of fruits	No reduction	0
	Up to 25% reduction	10
	26-50% reduction	20
	51-75% reduction	30
	>75% reduction	40

Table 7: Field response of tomato genotypes against leaf curl disease

Tomato genotypes	Disease reaction under field condition
ToLCD Tol-5 (*Ty-2+Ty-3*), **ToLCD Tol-8**(*Ty-2+Ty-3*), **ToLCD Tol-12**(*Ty-2+Ty-3*), **ToLCD Tol-14**(*Ty-2+Ty-3*), **ToLCD Tol-15**(*Ty-2+Ty-3*)	Highly resistant (HR)
ToLCD Tol-7 (*Ty-3a*)	Resistant (R)
ToLCD Tol-1 (Ty-3), **ToLCD Tol-4** (Ty-2+Ty-3a), **ToLCD Tol-9** (Ty-2+Ty-3), **ToLCD Tol-11**(*Ty-2+Ty-3*), **ToLCD Tol-6** (Ty-5+Ty-6), **ToLCD Tol-16**(*Ty-2+Ty-3*)	Moderately resistant (MR)
ToLCD Tol-3 (Ty-1), **ToLCD Tol-10** (Ty-5), **ToLCD Tol-17** (*Ty-2+Ty-3*), **ToLCD Tol-13** (*Ty-2+Ty-3*)	Moderately susceptible (MS)
ToLCD Tol-2 (Ty-2)	Susceptible (S)
Pusa Ruby (susceptible check)	Highly susceptible (HS)

Table 8: Disease response of *Ty*-gene containing donor genotypes of tomato based on coefficient of infection

Sl. No	Tomato genotypes	*Ty*-gene(s) present	Total no. of plants[1]			No. of diseased plants[2]			Disease incidence (%)[3]			Severity Score[4]			Avg. Severity Score±SE	Avg. Response Value[5]±SE	Avg. CI[6]±SE	Disease Reaction
			R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3				
1	ToLCD Tol -1	*Ty-3*	18	20	20	2	4	3	11.11	20	15	73	71.33	70	71.44±0.71	0.75±0	11.53±1.56[b]	MR
2	ToLCD Tol -2	*Ty-2*	15	18	12	7	9	5	46.67	50	41.67	90.4	83.5	85	86.30±1.71	1±0	46.11±1.98[b]	S
3	ToLCD Tol -3	*Ty-1*	16	12	14	6	5	5	37.5	41.67	35.71	51	56.5	54.4	53.97±1.31	0.75±0	28.72±1.08[b]	MS
4	ToLCD Tol -4	*Ty-3a, Ty-2*	17	19	20	2	5	4	11.77	26.32	20	49.4	62.33	57.5	56.41±3.08	0.75±0.07	14.52±3.32[b]	MR
5	ToLCD Tol -5	*Ty-2, Ty-3*	34	37	31	1	3	0	2.94	8.11	0	23.33	26.5	0	16.61±6.82	0.25±0.12	0.92±1.02[b]	HR
6	ToLCD Tol -6	*Ty-5, Ty-6*	20	17	18	4	3	4	20	17.65	22.22	73	72.4	72.25	72.55±0.19	0.75±0	14.97±0.81[b]	MR
7	ToLCD Tol -7	*Ty-3a*	15	18	20	2	1	2	13.33	5.56	10	57.5	54.25	62.5	58.08±1.96	0.75±0	7.22±1.38[b]	R
8	ToLCD Tol -8	*Ty-2, Ty-3*	20	14	16	2	0	1	10	0	6.25	42	0	30	24.00±10.20	0.25±0.14	1.35±1.19[b]	HR
9	ToLCD Tol -9	*Ty-2, Ty-3*	40	36	38	11	9	9	27.5	25	23.68	55	50	57.55	54.19±1.81	0.75±0.07	19.05±1.94[b]	MS
10	ToLCD Tol -10	*Ty-5*	16	14	13	7	5	5	43.75	35.71	38.46	70.25	62.4	65	65.88±1.89	0.75±0	29.48±1.44[b]	MS
11	ToLCD Tol -11	*Ty-2, Ty-3*	10	8	9	2	1	2	20	12.5	22.22	36.5	32	38.5	35.67±1.57	0.5±0	9.12±1.20[b]	MR
12	Pusa Ruby	-	9	10	7	9	8	5	100	80	71.43	94.33	89.75	91.33	91.80±1.10	1±0	83.81±6.91[a]	HS
13	ToLCD Tol -12	*Ty-2, Ty-3*	44	41	42	2	0	1	4.54	0	2.38	24	0	22	25.25±1.34	0.5±0.07	1.97±0.48[b]	HR
14	ToLCD Tol -13	*Ty-2, Ty-3*	8	5	6	4	2	4	50	40	66.67	46.25	45.4	49.75	47.13±1.09	0.5±0	26.11±3.18[b]	MS
15	ToLCD Tol -14	*Ty-2, Ty-3*	20	15	19	2	0	0	10	0	0	21.5	0	0	07.16±5.85	0.25±0.07	0.83±0.68[b]	HR
16	ToLCD Tol -15	*Ty-2, Ty-3*	16	14	12	1	1	0	6.25	7.14	0	22	23.4	0	15.13±6.19	0.25±0.07	1.12±0.46[b]	HR
17	ToLCD Tol -16	*Ty-2, Ty-3*	7	6	3	3	1	1	42.86	16.67	33.33	49.5	31.5	40	40.33±4.24	0.5±0	15.48±3.12[b]	MR
18	ToLCD Tol -17	*Ty-2, Ty-3*	14	16	19	3	5	5	21.43	31.25	26.32	52	55.33	54.4	53.91±0.81	0.75±0	19.75±1.74[b]	MS

R1, R2. R3 represent three replications

[3]Disease incidence = [2]No. of diseased plants/[1]Total no. of plants

[4]Severity score = $[\frac{\sum_{S=A}^{B} L_{1s} + \sum_{S=A}^{B} L_{2s} + \sum_{S=A}^{B} L_{3s} + \cdots + \sum_{S=A}^{B} L_{ns}}{T_N} + C + D]$

L_1, L_2, L_3, L_n = number of affected leaves on a plant ranging from 1 to n; s = score of a diseased leaf varying from A to B;

L_{is} = total score on diseased leaf of a plant in a particular stage/period (i = 1/2/3/.../n); C= stunting , D= no. of fruits); T_N = total number of leaves on infected individual plant.

[5]Response value= transformed severity score where response value score of 0, 0.25, 0.5, 0.75, 1 represent 0%, >0 to 25%, >25 to 50%, >50 to 75%, >75 to 100% severity score, respectively

[6]CI = [5]Response value x [3]Disease incidence

Different alphabets represent significant difference as calculated by paired t-test assuming equal variances and $p \leq 0.05$

HR: Highly resistant; R: Resistant; MR: Moderately resistant; MS: Moderately susceptible; S: Susceptible; HS: Highly susceptible

4.1.3. Confirmation of resistance of three highly resistant genotypes through viruliferous whitefly inoculation

Resistance of three tomato genotypes *viz*. ToLCD Tol-5, ToLCD Tol-15 and ToLCD Tol-14 with highly resistant (HR) disease response as evaluated under field were further evaluated for confirmation of their resistance along with the susceptible check cv. Pusa Ruby in the glasshouse through viruliferous whitefly carrying ToLCNDV. After 10 dpi with plants of cv. Pusa Ruby started showing typical leaf curl symptom. But, all other plants of resistant genotypes did not show any symptom. Even after 20 dpi when all the plants of Pusa Ruby variety were showing severe leaf curl symptom due to ToLCNDV infection, plants of resistant genotypes remained free from any symptom (Fig. 8).

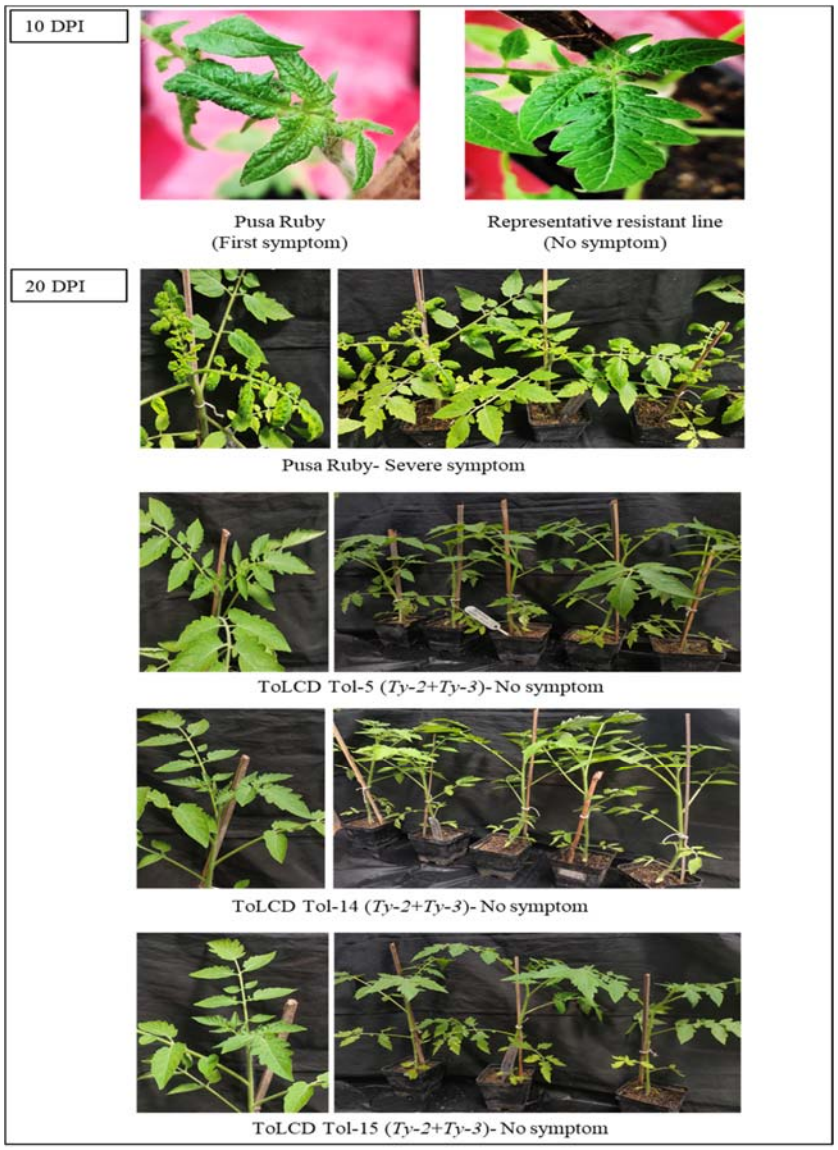


Fig. 8: Tomato leaf curl symptom phenotype of selected tomato genotypes upon inoculation with ToLCNDV using whitefly. (DPI: Days Post Inoculation)

4.2. Indexing the naturally occurring *Begomovirus* species infecting different *Ty*-gene(s) containing donor lines of tomato

4.2.1. Presence of begomovirus particles in the majority of the samples tested as revealed by transmission electron microscopy

Transmission electron microscopy (TEM) of 20 randomly collected leaf samples showed geminate particle (~30x20 nm) in 12 samples, both geminate particle (~30x20 nm) and flexuous particle (~700x11 nm) in 3 samples, and only flexuous particle (~700x11 nm) in 3 samples.

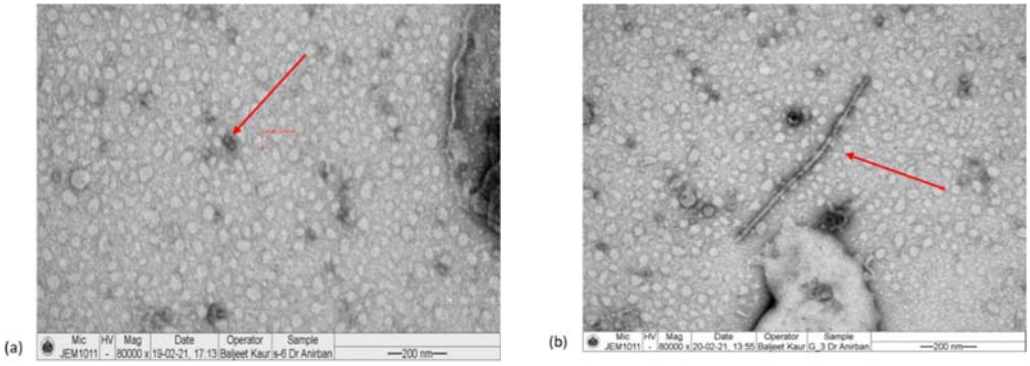


Fig. 9: Representative transmission electron micrograph using 2% uranyl acetate (negative stain) of infected leaf samples under 80000X magnification showing presence of –
(a) Geminate particle (~30 x 20 nm)
(b) Flexuous particle (~700 x 11 nm)

4.2.2. Mixed infection of different begomoviruses in the genotypes under study as revealed through PCR amplification with specific primers for begomoviruses

Total nucleic acid (TNA) was isolated separately from 5 leaf samples collected from each genotype. Equal amount of these 5 TNA samples were mixed to make a pooled TNA sample. Thus, 18 pooled TNA sample ware made for 18 tomato genotypes and used as templates in PCR analysis. Quantity and quality of the TNA samples were analyzed with both agarose gel (0.8%) electrophoresis (Fig. 10) as well as NanoDrop spectrophotometer. PCR amplification using *Begomovirus* genus specific primer yielded amplicon in 17 tomato genotypes. Only one genotype, ToLCD Tol-15, did not give amplification with this primer. ToLCNDV specific primer showed amplification only in 7 tomato genotypes. On the other hand, the primer specific to CYVMV, gave expected amplification in 13 genotypes. Similarly, the primers specific to ToLCPalV, ToLCGuV, ToLCJoV, and ChiLCV gave expected amplification in 11, 08, 02 and 04 genotypes, respectively (Fig. 11). ToLCBaV specific primer did not give amplification in any sample (Table 9). Presence of more than one begomovirus was detected in most of the genotypes (Table 10 and 11), with the maximum 6 numbers in the pooled TNA isolated from ToLCD Tol-10. Interestingly, a genotype ToLCD Tol-1, which was tested positive for begomovirus infection (gave amplification with universal begomovirus primer primer), did not yield any amplicon with any of the species specific primers used in the study.

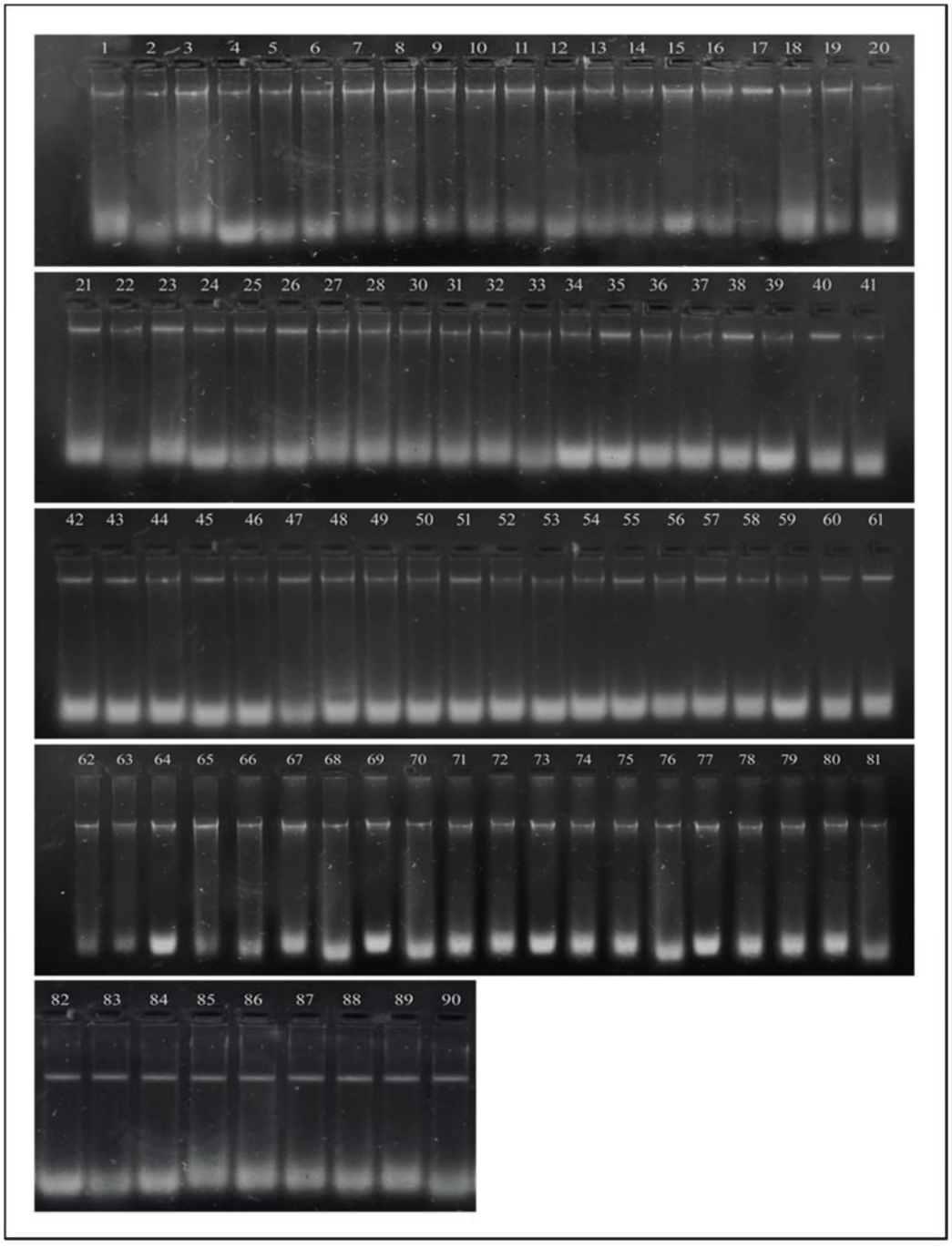
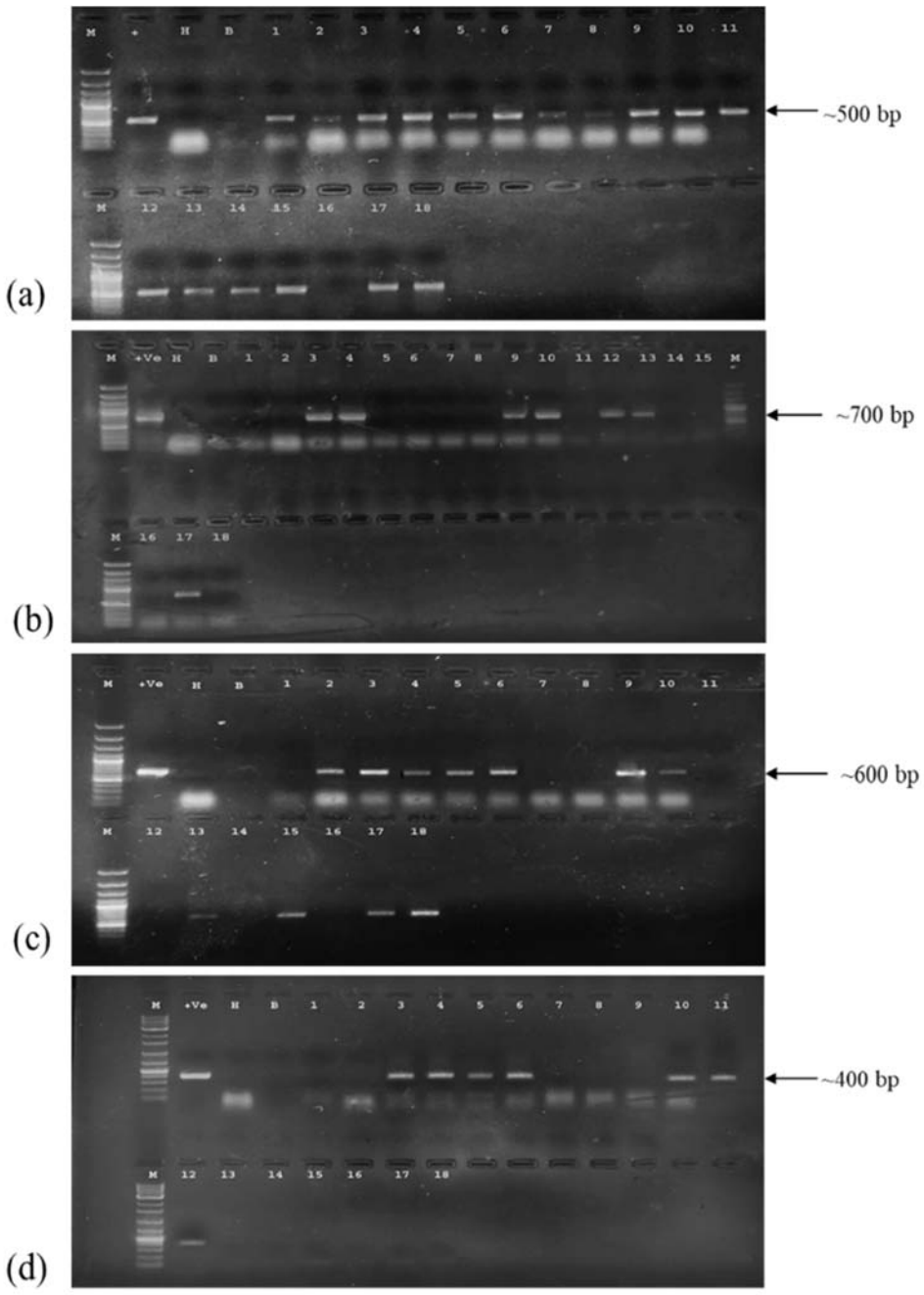


Fig. 10: Agarose gel (0.8%) electrophoresis of total nucleic acid (TNA) isolated from leaf samples (symptomatic and asymptomatic) of 17 tomato genotypes with Ty- genes and a susceptible cultivar (Pusa Ruby) (5 samples from each genotype)

Lane 1: ToLCD Tol-1; Lane 2: ToLCD Tol-2; Lane 3: ToLCD Tol-3; Lane 4: ToLCD Tol-4; Lane 5: ToLCD Tol-5; Lane 6: ToLCD Tol-6; Lane 7: ToLCD Tol-7; Lane 8: ToLCD Tol-8; Lane 9: ToLCD Tol-9; Lane 10: ToLCD Tol-10; Lane 11: ToLCD Tol-11; Lane 12: Pusa Ruby; Lane 13: ToLCD Tol-12; Lane 14: ToLCD Tol-13; Lane 15: ToLCD Tol-14; Lane 16: ToLCD Tol-15; Lane 17: ToLCD Tol-16; Lane 18: ToLCD Tol-17


M + H B 1 2 3 4 5 6 7 8 9 10 11
M 12 13 14 15 16 17 18
~500 bp
(a)
M +Ve H B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
M 16 17 18
~700 bp
(b)
M +Ve H B 1 2 3 4 5 6 7 8 9 10 11
M 12 13 14 15 16 17 18
~600 bp
(c)
M +Ve H B 1 2 3 4 5 6 7 8 9 10 11
M 12 13 14 15 16 17 18
~400 bp
(d)

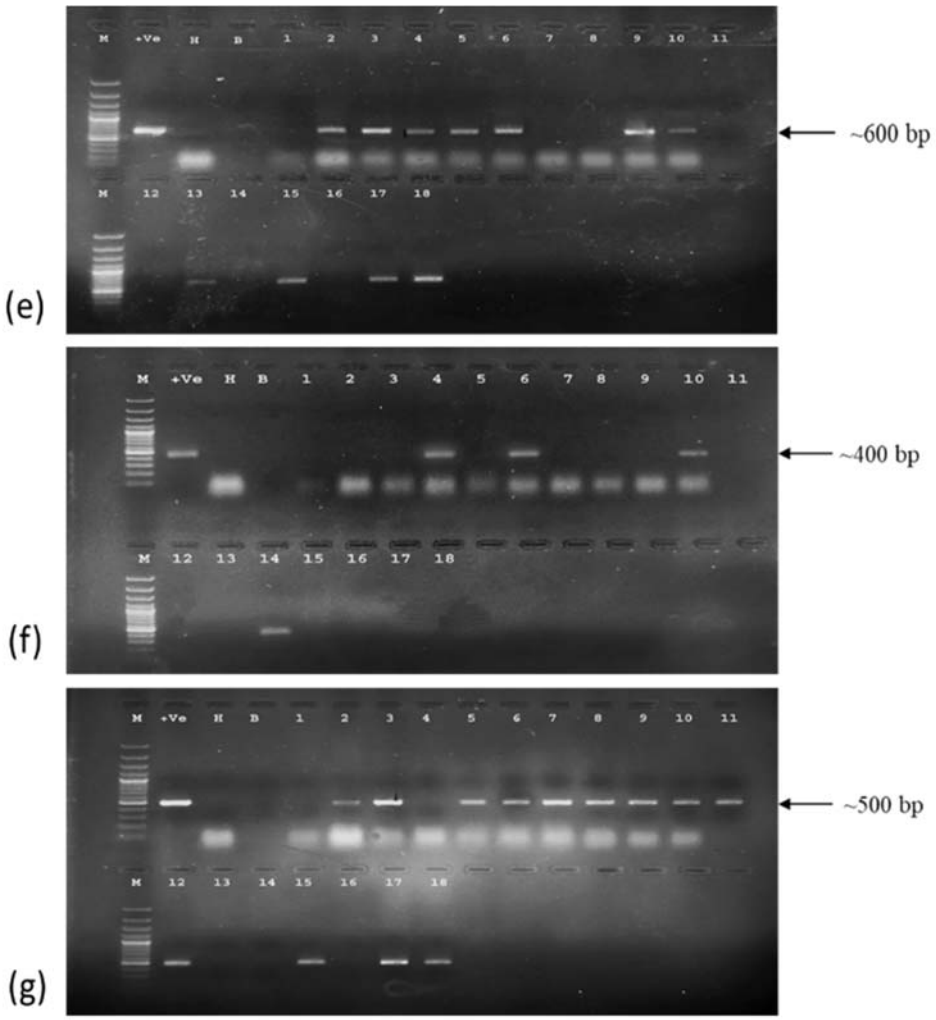


Fig. 11: Agarose gel (1%) electrophoresis of PCR amplified products using pooled total nucleic acid of different leaf samples from individual genotype as template and a-

(a) universal begomovirus genome (DNA-A/homolog) specific primer, which amplified ca. 500 bp amplicon
(b) ToLCNDV specific primer, which amplified ca. 700 bp amplicon
(c) ToLCPalV specific primer, which amplified ca. 600 bp amplicon
(d) ToLCGuV specific primer, which amplified ca. 400 bp amplicon
(e) ToLCJoV specific primer, which amplified ca. 600 bp amplicon
(f) ChiLCV specific primer, which amplified ca. 400 bp amplicon
(g) CYVMV specific primer, which amplified ca. 500 bp amplicon

M: Molecular weight marker (100 bp plus DNA ladder)
+: Positive control
H: Negative control using total nucleic acid from healthy plant as template
B: Reagent control without template DNA
Lane 1 to 18: PCR amplified product obtained using pooled TNA from 18 tomato as template genotypes. Lane 1: ToLCD Tol-1; Lane 2: ToLCD Tol-2; Lane 3: ToLCD Tol-3; Lane 4: ToLCD Tol-4; Lane 5: ToLCD Tol-4; Lane 6: ToLCD Tol-6; Lane 7: ToLCD Tol-7; Lane 8: ToLCD Tol-8; Lane 9: ToLCD Tol-9; Lane 10: ToLCD Tol-10; Lane 11: ToLCD Tol-11; Lane 12: Pusa Ruby; Lane 13: ToLCD Tol-12; Lane 14: ToLCD Tol-13; Lane 15: ToLCD Tol-14; Lane 16: ToLCD Tol-15; Lane 17: ToLCD Tol-16; Lane 18: ToLCD Tol-17

Table 9: Association of different begomoviruses with tomato genotypes under study as revealed by PCR amplification using specific primers

Name of the virus	No. of PCR positive genotypes (out of 18)
Begomovirus	17
Tomato leaf curl New Delhi virus (ToLCNDV)	7
Tomato leaf curl Palampur virus (ToLCPalV)	11
Tomato leaf curl Gujarat virus (ToLCGuV)	8
Tomato leaf curl Joydebpur virus (ToLCJoV)	2
Tomato leaf curl Bangalore virus (ToLCBaV)	0
Chilli leaf curl virus (ChiLCV)	4
Croton yellow vein mosaic virus (CYVMV)	13

Table 10: Number of begomovirus(es) detected in different tomato genotypes

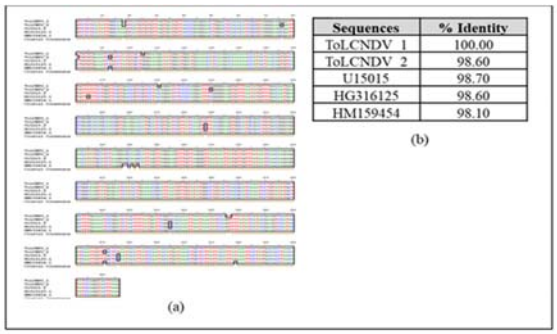
No. of begomovirus(es) detected	No. of positive genotypes	Name of the genotypes
1	3	ToLCD Tol-7, ToLCD Tol-8, ToLCD Tol-13
2	5	ToLCD Tol-2, ToLCD Tol-11, ToLCD Tol-12, ToLCD Tol-14, ToLCD Tol-17
3	3	Pusa Ruby, ToLCD Tol-5, ToLCD Tol-16
4	3	ToLCD Tol-4, ToLCD Tol-6, ToLCD Tol-9
5	1	ToLCD Tol-3
6	1	ToLCD Tol-10

Table 11: Details of mixed infection by begomoviruses in different tomato genotypes

Viruses in single or mixed infection	No. of genotypes	Name of the genotypes
ToLCNDV+ToLCPalV+ToLCGuV+ToLCJoV+ChiLCV+CYVMV	1	ToLCD Tol-10
ToLCNDV+ToLCPalV+ToLCGuV+ToLCJoV+CYVMV	1	ToLCD Tol-3
ToLCNDV+ToLCPalV+ToLCGuV+ChiLCV	1	ToLCD Tol-4
ToLCNDV+ToLCPalV+ToLCGuV+CYVMV	1	ToLCD Tol-9
ToLCPalV+ToLCGuV+ChiLCV+CYVMV	1	ToLCD Tol-6
ToLCNDV+ToLCPalV+CYVMV	1	ToLCD Tol-16
ToLCNDV+ToLCGuV+CYVMV	1	Pusa Ruby
ToLCPalV+ToLCGuV+CYVMV	1	ToLCD Tol-5
ToLCNDV+ToLCPalV	1	ToLCD Tol-12
ToLCPalV+CYVMV	3	ToLCD Tol-2 ToLCD Tol-14 ToLCD Tol-17
ToLCGuV+CYVMV	1	ToLCD Tol-11
CYVMV	2	ToLCD Tol-7 ToLCD Tol-8
ChiLCV	1	ToLCD Tol-13

4.2.3. Sequencing of PCR amplified DNA and sequence analysis confirmed the identity of the begomovirus species

Sequencing, followed by sequence analysis (pairwise sequence analysis using ClustalW) of representative PCR amplicons obtained using different begomovirus species specific primers showed >96% sequence identity with the reference sequences of the respective begomoviruses retrieved from NCBI (Fig. 12 to Fig. 16), except the ChiLCV specific primers whose amplified product showed more sequence identity with ToLCKV, (94%) than ChiLCV (88.4%) (Fig. 17).



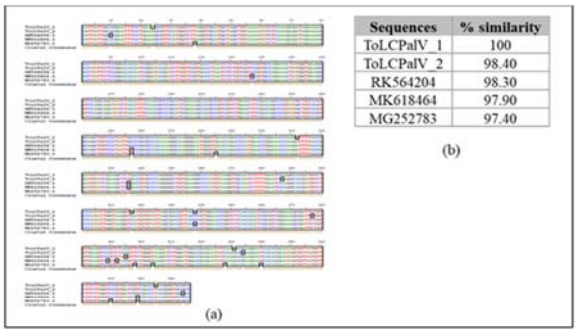
Sequences	% Identity
ToLCNDV_1	100.00
ToLCNDV_2	98.60
U15015	98.70
HG316125	98.60
HM159454	98.10

(b)

(a)

Fig. 12: Comparative sequence analysis of sequence obtained from PCR amplified product using ToLCNDV specific primer and other homologous sequences

(a) Multiple global sequence alignment using ClustalW
(b) Sequence identity matrix
ToLCNDV_1, ToLCNDV_2: Sequences of PCR amplified product using ToLCNDV specific primers.
U15015, HG316125, HM159454: ToLCNDV reference sequences



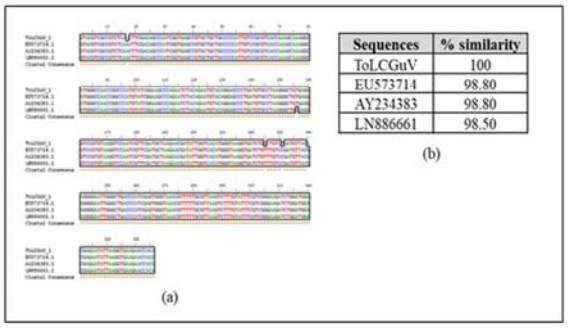
Sequences	% similarity
ToLCPalV_1	100
ToLCPalV_2	98.40
RK564204	98.30
MK618464	97.90
MG252783	97.40

(b)

(a)

Fig. 13: Comparative sequence analysis of sequence obtained from PCR amplified product using ToLCPalV specific primer and other homologous sequences

(a) Multiple global sequence alignment using ClustalW
(b) Sequence identity matrix
ToLCPalV_1, ToLCPalV_2: Sequences of PCR amplified product using ToLCPalV specific primers.
RK564204.1, MK618464.1, MG252783.1 : ToLCPalV reference sequences



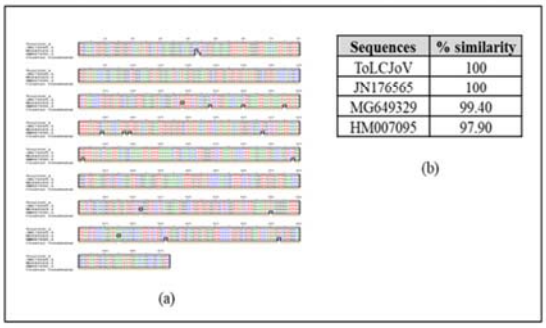
Sequences	% similarity
ToLCGuV	100
EU573714	98.80
AY234383	98.80
LN886661	98.50

(b)

(a)

Fig. 14: Comparative sequence analysis of sequence obtained from PCR amplified product using ToLCGuV specific primer and other homologous sequences

(a) Multiple global sequence alignment using ClustalW
(b) Sequence identity matrix
ToLCGuV: Sequence of PCR amplified product using ToLCGuV specific primers.
EU573714, AY234383, LN886661: ToLCGuV reference sequences.



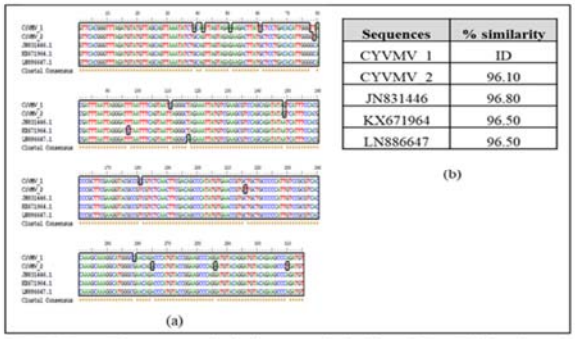
Sequences	% similarity
ToLCJoV	100
JN176565	100
MG649329	99.40
HM007095	97.90

(b)

(a)

Fig. 15: Comparative sequence analysis of sequence obtained from PCR amplified product using ToLCJoV specific primer and other homologous sequences

(a) Multiple global sequence alignment using ClustalW
(b) Sequence identity matrix
ToLCJoV: Sequence of PCR amplified product using ToLCJoV specific primers.
JN176565, MG649329, HM007095: ToLCJoV reference sequences.



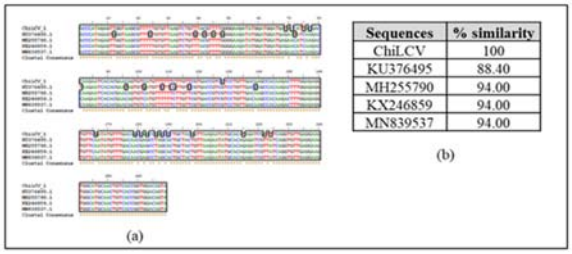
Sequences	% similarity
CYVMV_1	ID
CYVMV_2	96.10
JN831446	96.80
KX671964	96.50
LN886647	96.50

(b)

(a)

Fig. 16: Comparative sequence analysis of sequence obtained from PCR amplified product using CYVMV specific primer and other homologous sequences

(a) Multiple global sequence alignment using ClustalW
(b) Sequence identity matrix
CYVMV_1, CYVMV_2: Sequence of PCR amplified product using CYVMV specific primers.
JN831446, KX671964, LN886647: CYVMV reference sequences.



Sequences	% similarity
ChiLCV	100
KU376495	88.40
MH255790	94.00
KX246859	94.00
MN839537	94.00

(b)

(a)

Fig. 17: Comparative sequence analysis of sequence obtained from PCR amplified product using ChiLCV specific primer and other homologous sequences

(a) Multiple global sequence alignment using ClustalW
(b) Sequence identity matrix
ChiLCV: Sequence of PCR amplified product using ChiLCV specific primers.
KU376495: ChiLCV virus reference sequence.
MH255790, KX246859, MN839537: ToLCKV reference sequences.

4.2.4. Characterization of complete genome of begomoviruses

4.2.4.1. Cloning of the begomovirus genome

Indexing of naturally occurring begomoviruses in different Ty-gene containing donor lines of tomato highlighted two interesting findings. First, CYVMV was detected in maximum number of genotypes and second, genotype ToLCD Tol-1 was positive for presence of begomovirus but it did not give amplification with any of the begomovirus species specific primers tested. Simultaneously, two questions also arose with these findings. First, whether it was really the weed infecting CYVMV? and second, which begomovirus is actually present in ToLCD Tol-1 genotype? Complete genome characterization of this two begomoviruses was needed to answer the questions.

RCA generated high molecular weight concatemeric DNA obtained from TNA of infected ToLCD Tol-7 and ToLCD Tol-1 (Fig. 18) yielded ca. 2.7 kb linearized fragments upon digestion with *Bam*HI (Fig. 19). The linearized RCA products were ligated to the *Bam*HI digested pUC18 vector and transformed into *E. coli* DH5α cells to obtain recombinant clone Ty-6 (from ToLCD Tol-7) and Ty-4A (from ToLCD Tol-1). Recombinant colonies were confirmed by colony PCR using CYVMV specific primer (for clone Ty-6) and universal begomovirus primer (for clone Ty-4A) (Fig. 20), followed by restriction digestion of isolated recombinant plasmids using *Bam*HI and *Bgl*I (Fig. 21). Begomovirus genome in Ty-6.1 colony has no *Bgl*I site but Ty-4A.2 has one *Bgl*I site as evidenced by releasing out extra DNA fragment. Two confirmed recombinant plasmids *i.e.* Ty-6.1 and Ty-4A.2 were sequenced through commercial facility.

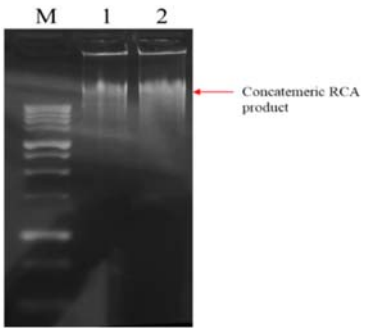


Fig. 18: Agarose gel (0.8%) electrophoresis of amplified product from rolling circle amplification (RCA) using total nucleic acid from ToLCD Tol-7 and ToLCD Tol-1 genotypes as template

M: Molecular weight marker (100 bp plus DNA ladder)
Lane 1: RCA product obtained from TNA of ToLCD Tol-7 genotype
Lane 2: RCA product obtained from TNA of ToLCD Tol-1 genotype

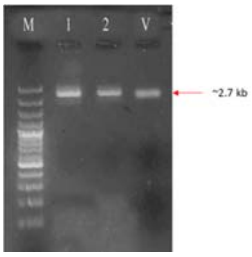


Fig. 19: Agarose gel (0.8%) electrophoresis of linearized RCA product and pUC18 vector after *Bam*HI digestion
M: Molecular weight marker (100 bp plus DNA marker)
Lane 1: *Bam*HI digested RCA product obtained from ToLCD Tol-7 genotype yielding begomovirus genome specific amplicon (~2.7 kb)
Lane 2: *Bam*HI digested RCA product obtained from ToLCD Tol-1 genotype yielding begomovirus genome specific amplicon (~2.7 kb)
Lane V: *Bam*HI digested linearized pUC18 vector (~2.7 kb)

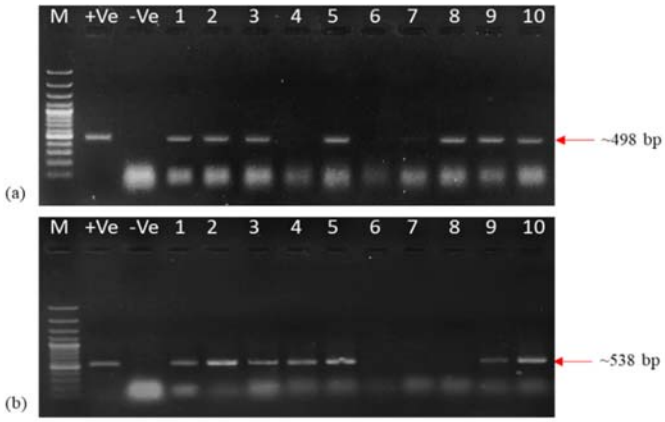


Fig. 20: Agarose gel (1%) electrophoresis of colony PCR amplified products of *E. coli* DH5α recombinant colonies transformed with Ty-6 and Ty-4A recombinant plasmids
M : Molecular weight marker (100 bp plus DNA marker)
+ve : Positive control using known plasmid
-ve : Negative control (reagent control)
Lane 1 to 10: PCR products using CYVMV specific primer (a) and universal

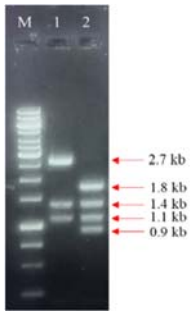


Fig. 21: Agarose gel electrophoresis of recombinant plasmid Ty-6.1 and Ty-4A.2 digested with *Bam*HI and *Bgl*I restriction enzyme
M : Molecular weight marker (1 kb DNA ladder)
Lane 1 : Recombinant plasmid with CYVMV clone Ty-6.1
Lane 2 : Recombinant plasmid with unknown begomovirus clone Ty-4A.2

4.2.4.2. Sequence analysis of recombinant plasmid Ty-6.1 and Ty-4A.2

(a) Construction of complete genome map

Sequence of begomoviruses in the recombinant plasmid Ty-6.1 and Ty-4A.2 were 2765 bp and 2760 bp, respectively. Construction of complete genome map of both the clones using SnapGene Viewer tool version 5.3.1 showed presence of all six typical begomovirus specific ORFs (Fig 22 and 23).

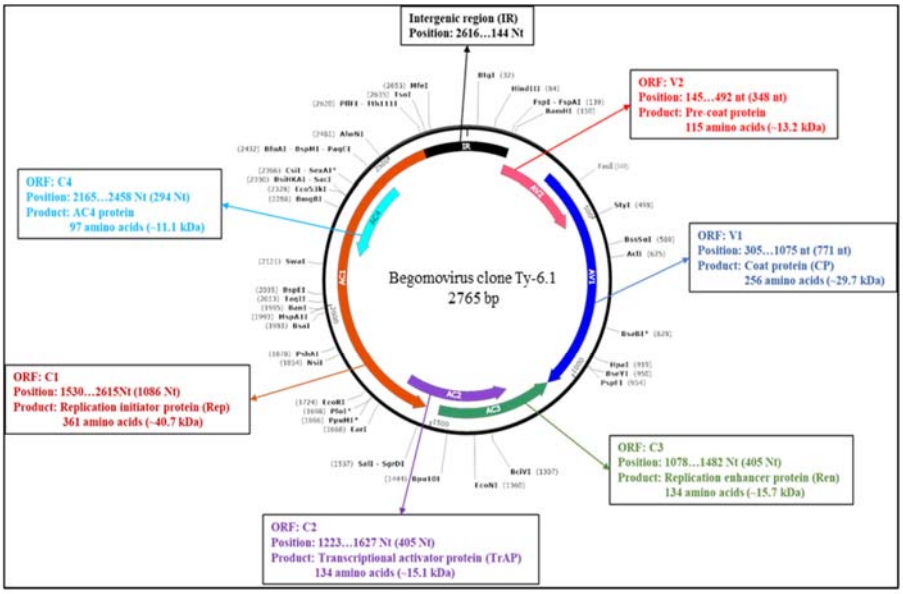


Fig. 22: Annotated complete genome map of begomovirus clone Ty-6.1 displaying viral ORFs and intergenic region with respective nucleotide co-ordinates and unique restriction enzymes spanning the genome

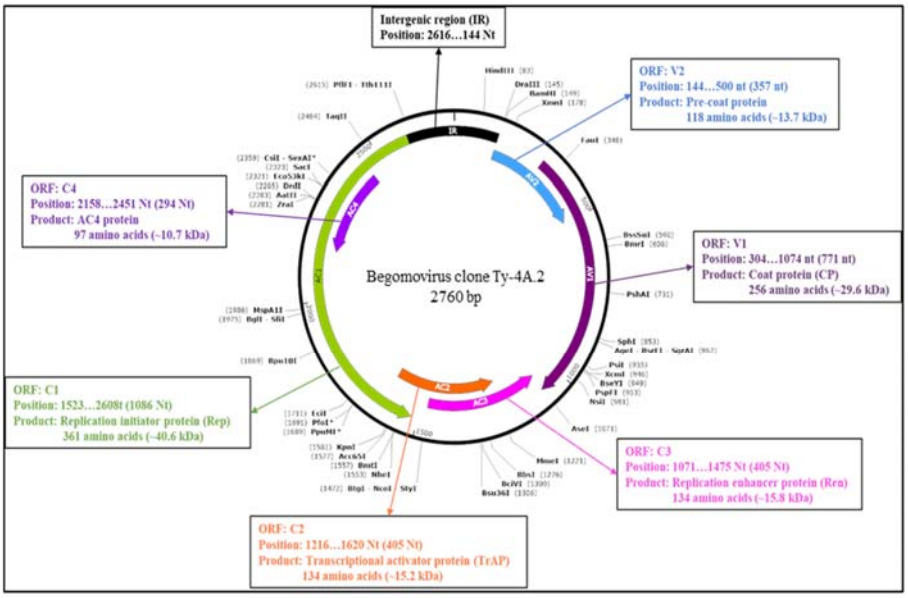


Fig. 23: Annotated complete genome map of begomovirus clone Ty-4A.2 displaying viral ORFs and intergenic region with respective nucleotide co-ordinates and unique restriction enzymes spanning the genome

(b) Prediction of functional motifs in amino acid sequences of different ORFs

Presence of different functional motifs and patterns were detected in the amino acid sequence of all the ORFs in both begomovirus clone Ty-6.1 (Table 12) and Ty-4A.2 (Table 13), using Motif scan tool. Kinase motifs were found in all proteins which indicated the involvement of these proteins in signal transduction pathway and they can be activated by phosphorylation and also can activate other proteins through phosphorylation. Amino acid sequence of C1 protein showed presence of both ATP-binding motif and ATPase motif which are required for nicking and ligation of origin of replication of the viral genome.

Table 12: List of functional motifs identified in amino acid sequence of different ORFs in begomovirus clone Ty-6.1 (identified by Motif Scan tool)

ORFs	Motifs	Position in amino acid sequence
V1	Amidation site	105-108
	N-glycosylation motif	130-133; 221-224
	cAMP and cGMP dependent protein kinase phosphorylation site	143-146
	Casein kinase II phosphorylation site	148-151
	Protein kinase C-phosphorylation site	2-4; 102-104; 167-169; 243-245
	Tyrosine kinase phosphorylation site	20-27
V2	Protein kinase C-phosphorylation site	58-60
	Cystein rich region profile	81-89
	WCCH motif	79-102
C1	N-glycosylation motif	308-311; 330-333; 345-348
	ATP/GTP binding site	221-228
	Casein kinase II phosphorylation site	22-27; 96-99; 102-105; 129-132; 142-145; 167-170; 187-190; 310-313
	Protein kinase C-phosphorylation site	39-41; 122-124; 225-227; 245-249; 310-312
	Tyrosine kinase phosphorylation site	95-103
	Nebuline repeat profile	311-320
C2	Casein kinase II phosphorylation site	61-64; 102-105; 107-110; 124-127
	Protein kinase C-phosphorylation site	52-54
C3	Protein kinase C-phosphorylation site	79-81; 128-130
C4	Casein kinase II phosphorylation site	38-41; 66-69

Table 13: List of functional motifs identified in amino acid sequence of different ORFs in begomovirus clone Ty-4A.2 (identified by Motif Scan tool)

ORFs	Motifs	Position in amino acid sequence
V1	N-glycosylation motif	130-133; 221-224
	Casein kinase II phosphorylation site	78-82
	Protein kinase C-phosphorylation site	2-4; 102-104; 167-169; 243-245
	Tyrosine kinase phosphorylation site	20-27
V2	Casein kinase II phosphorylation site	66-69
	Protein kinase C-phosphorylation site	58-60
	Cystein rich region profile	81-89
	WCCH motif	79-102
	Acid shock protein repeat	91-111
C1	N-glycosylation motif	245-248; 345-348
	ATP/GTP binding site	221-228
	Casein kinase II phosphorylation site	22-27; 96-99; 102-105; 129-132; 142-145; 167-170; 187-190; 310-313
	Protein kinase C-phosphorylation site	39-41; 122-124; 225-227; 245-249; 310-312
	Tyrosine kinase phosphorylation site	95-103
	Nebuline repeat profile	308-320
C2	Casein kinase II phosphorylation site	61-64; 102-105; 107-110; 124-127
	Protein kinase C-phosphorylation site	52-54; 61-63
C3	Protein kinase C-phosphorylation site	79-81; 128-130
C4	Casein kinase II phosphorylation site	11-14; 38-41; 66-69
	Protein kinase C-phosphorylation site	11-13; 17-19; 35-37; 38-40; 51-53
	Serine rich repeats	9-26

(c) Prediction of Nuclear localization signal (NLS)

cNLS Mapper tool predicted nuclear localization signal (NLS) with cut-off value 3.0 within first 60 amino acid region of V1, C1 and C2 proteins of both the clones (Table 14).

Table 14: Identification of nuclear localization signal (NLS) in different ORFs of begomovirus clone Ty-6.1 and Ty-4A.2 (identified by cNLS Mapper tool)

Name of the clone	ORFs	Position in amino acid sequence	Amino acid sequence	Score
Begomovirus clone Ty-6.1	V1	1	MSKRPADIVISTPASKVRRR	9.2
	C1	8	RINAKNYFLTYPHCSLTKEEAL SQLKNLETP	4
	C2	6	PSKAHSTQVPIKVQHKIAQNGI RRRRVDLP	4.3
Begomovirus clone Ty-4A.2	V1	1	MSKRPADIIISTPASKVRRR	9.2
	C1	17	TYPKCSLTKEEALSQLLNLQTP TSKKFIRI	6.4
	C2	6	PSKAHSTQVPIKVKHRLAKKG NRRRRVDLP	3.7

(d) Multiple sequence alignment

Multiple sequence alignment analysis followed by development of sequence identity matrix of begomovirus clone Ty-6.1 sequence with 29 other sequences of related begomovirus species, retrieved from NCBI, revealed that the complete nucleotide sequence of begomovirus in clone Ty-6.1 was showing maximum 89.7% sequence identity with *Tobacco curly shoot virus* (TbCSV) isolate (Acc. No- KM383754) and only 87.5% sequence identity with *Croton yellow vein mosaic virus* (CYVMV) isolate (Acc. No- FN645898) which is quite less than the ICTV approved species demarcation criteria of bgomoviruses (91%). Considering this, begomovirus clone Ty-6.1 was proposed to be a new species of begomovirus and named as *Tomato leaf curl Ty Pusa virus* clone Ty-6.1 (ToLCTyPV clone Ty-6.1) according to its place of identification (Pusa, New Delhi) and source (*Ty* resistance gene containing tomato line). Similarly, multiple sequence alignments were done for both nucleotide and amino acid sequences of individual ORF. It revealed that ORF V1, V2, C2 and right intergenic region (right-IR) of ToLCTyPV were showing maximum sequence identity with CYVMV isolates, ORF C3 was showing maximum sequence identity with Papaya leaf curl virus (PaLCuV) isolate and ORF C1, C4 and left intergenic region (left-IR) were showing maximum sequence identity with TbCSV isolates (89.7%) (Table 15).

The sequence of begomovirus in clone Ty-4A.2 revealed that the complete nucleotide sequence as well as individual ORF (nucleotide and amino acid) and IR sequence of begomovirus in clone Ty-4A.2 were showing >99% sequence identity with isolates of Tomato leaf curl Karnataka virus (ToLCKV) (Table 16). So, the begomovirus in clone Ty-4A.2 was believed to be an isolate of ToLCKV and named as Tomato leaf curl Karnataka virus clone Ty-4A.2 (ToLCKV clone Ty-4A.2).

Table 15: Sequence identity of the complete genome, different ORFs at nucleotide (Nt) and amino acid (aa) level and intergenic region of begomovirus clone Ty-6.1 with other related begomoviruses (analyzed by MEGA-X version 10.2.6 and BioEdit version 7.2) (value in bold indicates highest value)

Sequence ID	Host	Location	Percent sequence identity														
			Complete genome	V1		V2		C1		C2		C3		C4		Left IR	Right IR
			Nt	Nt	aa	Nt	aa	Nt	aa	Nt	aa	Nt	aa	Nt	aa	Nt	Nt
KM383754_TbCSV-CN[BD:Raj]	Tomato *(Solanum lycopersicum L.)*	Rjshahi, Bangladesh	**89.7**	79.3	82.8	**95.6**	93	**97.1**	**96.9**	89.8	82	90.8	89.5	**100**	**100**	94	80
KF551584_TbCSV-TC240	Tomato *(Solanum lycopersicum L.)*	Punjab, India	88.8	79.3	81.2	90.1	84.7	95.6	96.3	90.3	83.5	90.1	85.8	99.6	98.9	**95.3**	76.5
JN387045_TbCSV-To-Ag-1	Tomato *(Solanum lycopersicum L.)*	Tripura, India	83.3	78.5	80	89.9	84.7	87.9	89.7	86.4	75.3	86.9	84.3	63.8	57.1	78.7	66.4
MK087120_PaLCuV-Cal-1	*Calendula sp.*	Karnataka, India	88.7	94.9	96	95.5	**94**	81.9	84.2	96.5	92.5	94	92.5	60.9	37.1	76.3	92.4
HQ407395_TbCSV-WSF1	Wild sunflower *(Helianthus sp.)*	India	88.4	79.6	81.6	94.2	89.5	94.2	85.7	91.8	83.5	90.6	88	99.6	**100**	**95.3**	73.6
FJ593629_PaLCuV	Papaya *(Carica papaya)*	India	87	95	**97.2**	94.9	**94**	78.7	78.1	96.7	93.2	**97.5**	**97.7**	57.2	29.8	59.7	94.5
JN663850_CYVMV-Bhubaneswar	Chilli *(Capsicum annum)*	Odisa, India	87	94.9	96.8	94.9	**94**	78.6	77.8	96.5	93.2	97.2	97.7	57.2	29.8	59.7	**95.1**
MH577030_ToLCKV-pBamA4	Tomato *(Solanum lycopersicum L.)*	Gujarat, India	88.1	94.1	96.4	91	88.9	86.3	88.3	90.1	80.5	90.8	88	78.4	67.8	77.8	74.4
KP195263_ToLCKV-TC253	Tomato *(Solanum lycopersicum L.)*	Maharastra, India	87.9	92.2	96	92.4	92.3	86.7	88.9	91.3	82	92	89.5	91.1	80.4	77.2	75.6
LN886647_CYVMV-RM162	*Croton sparsiflorus"*	Punjab, Pakistan	86.8	**95.5**	**97.2**	95.2	**94**	79	78.3	97.2	94.7	95.3	94.7	57.2	30.9	60.8	92.4
FN645898_CYVMV-9b-RCA-AI-F	*Acalypha sp.*	Hrayana, India	87.5	**95.5**	**97.2**	94.9	**94**	60.4	57.6	**97.7**	**94.7**	95.5	94.7	57.5	29.8	74.8	92.3
JN831446_CYVMV-Bang-Cr2	*Croton bonplandianus*	India	86.5	95.2	**97.2**	95.5	**94**	77.9	79.7	97.2	**94.7**	95	93.2	57	26.7	62.1	95.1
AJ507777_CYVMV	*Croton bonplandianus*	India	86.4	95.4	**97.2**	94.6	92.3	77.9	78.1	97.2	94	94.8	92.5	58.2	31.9	62.1	94.4
JN817516_CYVMV-Del-Cr	*Croton bonplandianus*	India	86.4	95.4	**97.2**	94.6	92.3	78.4	78.1	97.5	94	95.3	94	56.5	28.8	58.4	89.7
KF888655_CYVMV-Del-Turnip	Turnip *(Brassica rapa subsp. rapa)*	New Delhi, India	86.4	95.2	96.8	95.2	**94**	78.1	78.1	97.5	94	95.5	94.7	56.9	29.8	62.5	91.7
JN817517_PaLCuV	*Croton bonplandianus*	India	86.3	93.3	72	94.9	**94**	79.8	78.1	96.5	92.5	95	95.5	58.2	31.9	62.1	88.3
KJ747958_CYVMV-Del-Crambe	*Crambe abyssinica*	New Delhi, India	86.2	94.8	96	95.2	94	77.9	77.5	97.5	94	95.5	94.7	56.9	29.8	62.5	92.4
JX270684_CYVMV-M4A	*Brassica rapa*	New Delhi, India	86.5	94.9	97.2	95.2	**94**	78	77.2	97	94	94.3	92.5	57	29.8	59	90.9
KM383753_TbCSV-CN[BD:Chi]	Tomato *(Solanum lycopersicum L.)*	Chittagong, Bangladesh	85.9	78.5	82	91	87.2	93	93.6	85.4	73.1	85.9	82	97.6	95.8	86.1	69.3
LN886525_CYVMV-15AA3-1	Tomato *(Solanum lycopersicum L.)*	Lahore, Pakistan	85.9	92.9	94.5	91.5	88.9	77.7	77	96.7	93.2	91.9	88.2	57.2	29.8	73.5	90.3
KF307208_PaLCuV-Pap:ND:13	Papaya *(Carica papaya)*	India	85.3	94.8	96.8	88.7	86.4	79.4	77.8	96.2	91.7	96.5	95.5	58.9	34	63.2	73.7
KM383757_TbCSV-CN[BD:Cox:]	Tomato *(Solanum lycopersicum L.)*	Coxbazar, Bangladesh	85.2	78.8	82.8	91.3	87.2	91	91.6	89.3	82	89.6	87.3	95.5	89.6	81.8	63.4
MN481120_TbCSV-YN3346-80	*Tomato (Solanum lycopersicum L.)*	Unnan, China	85.3	79.1	82	93.9	90.4	90.2	91.9	86.9	75.3	88.1	84.3	90.3	80.8	87.5	67.8
LN794214_ToLCGuV-SAZ-94	Cotton (*Gossypium hirsutum)*	Pakistan	84.6	85	92.9	90.8	87.8	87.6	90.8	91.3	82	84.4	81.3	91.8	82.4	68	76.3
AY234383_ToLCGuV-[Nepal]	Tomato *(Solanum lycopersicum L.)*	Panchkhal, Nepal	84.6	85.6	93.7	91.6	87.8	86.7	89.7	91.1	82	84.1	79.8	92.1	82.4	66.8	75.6
KP164862_ToLCGuV-TC153	Tomato *(Solanum lycopersicum L.)*	Maharastra, India	84.5	85.8	93.7	91.9	87.8	87.2	89.4	90.1	79.8	83.7	79.1	91.4	80.4	67.4	76.3
HM625838_ToLCGuV-[Pune:2008]	Tomato *(Solanum lycopersicum L.)*	Maharastra, India	84.6	86.1	93.7	91.3	86	87.5	90.5	90.3	81.3	83.7	79.1	92.1	82.4	67.2	73.9
EU573714_ToLCGuV-[Dhanbad]	Tomato *(Solanum lycopersicum L.)*	Jharkhand, India	84.5	85.6	93.7	91.3	87.8	87.5	90	91.3	82.8	84.4	80.5	92.1	82.4	66.8	74.3
MN698280_MYMIV-Raichur	*Vigna stipulacea*	Karnataka, India	64.7	68.6	73.9	57.8	42.2	71.2	73.2	57.4	44	60.8	42.6	76.2	59.5	42.8	42.4

Table 16: Sequence identity of the complete genome, different ORFs at nucleotide (Nt) and amino acid (aa) level and intergenic region of begomovirus clone Ty-4A.2 with other related begomoviruses (analyzed by MEGA-X version 10.2.6 and BioEdit version 7.2) (value in bold indicates highest value)

Sequence ID	Host	Location	Percent sequence identity													
			Complete genome	V1		V2		C1		C2		C3		C4		IR
			Nt	Nt	aa	Nt	aa	Nt	aa	Nt	aa	Nt	aa	Nt	aa	Nt
KX246859_ToLCKV	Pigeon pea (*Cajanas cajan*)	India	**99.6**	**99.8**	**100**	99.7	99.1	**99.6**	**99.4**	**99.7**	**99.2**	**99.5**	99.2	99.6	98.9	**98.6**
MH255790_ToLCKV	Radish (Raphanus sativus)	India	99.5	99.8	99.6	**100**	**100**	**99.6**	**99.4**	99.5	**99.2**	99.2	98.5	**100**	**100**	**98.6**
MN839537_ToLCKV-PhL1	Parthenium hysterophorus	Pakistan	99.4	99.7	**100**	99.4	99.1	99.5	99.1	99.7	**99.2**	99.5	99.2	99.3	98.9	97.9
HM803118_ToLCKV-IKH12	Tomato (Solanum lycopersicum)	New Delhi, India	98.6	98.7	**100**	99.1	**100**	98.9	98.6	99	98.5	99.2	98.5	99.6	98.9	96.9
KX219744_ToLCKV	Sunflower (Helianthus annuus)	Andhra Pradesh, India	98	98.9	99.2	98.8	99.1	97.5	95.8	98.2	96.2	99	99.2	97.9	97.9	95.6
HM007094_ToLCKV-pChBanB2	Chilli (Capsicum annum)	Karnataka, India	97.3	98.5	99.6	99.1	**100**	96.7	96.9	98.7	97.7	99.2	98.5	96.5	91.7	93.2
LN878103_ToLCKV-18AA3	Tomato (Solanum lycopersicum)	Lahore, Pakistan	96.8	98.8	99.2	95.7	94.9	96.2	96.1	99	98.5	69.8	68.6	96.2	91.7	92.8
MK965196_ToLCKV-Bhopal	Zinnia elegans	Madhya Pradesh, India	96.4	98.8	99.2	98.5	**100**	96	95.8	95	90.2	97.2	97	96.2	90.7	90.5
KP178731_ToLCKV-TC120	Tomato (Solanum lycopersicum)	Maharastra, India	95.6	98.3	99.2	98	97.4	95.3	93.3	97	95.5	98.7	97.7	96.5	93.8	85
LT556083_ToLCKV		Pakistan	94.8	97.6	97.6	94.3	94	94	95	99.5	**99.2**	99.5	**100**	96.2	91.7	87.3
JX987088_ToLCKV-Lucknow	Zinnia elegans	Uttar Pradesh, India	95	98.3	99.2	98	98.3	92.8	91.4	96	91.7	96	93.2	95.9	91.7	91.5
KX665544_ToLCKV-IH-35	Brassica rapa	Punjab, India	94.7	98	98	97.7	97.4	92.8	93.6	99	98.5	99	97.7	96.2	91.7	87.7
KF551579_ToLCKV-TC255	Tomato (Solanum lycopersicum)	Namil Nadu, India	93.1	80.8	83.5	94.9	90.6	98.1	97.2	97.7	95.5	98.5	97	99.3	98.9	97.2
MN020537_ToLCKV	Tomato (Solanum lycopersicum)	India	92.7	91.1	96	92.9	90.6	93.3	92.5	96	94.7	96.2	94	96.5	95.8	89.8
MH577030_ToLCKV-pBamA4	Tomato (Solanum lycopersicum)	Gujarat, India	92.4	80.5	83.2	95.5	92.3	97.7	96.9	97	94	98	97	85.5	83.9	93.5
KX665541_ToLCKeV-IH-37	Brassica rapa	Punjab, India	90.5	96.3	97.2	96	92.3	84.5	85.5	95.3	94.7	98.5	97.7	72.1	49	83.5
KY926894_ToLCKeV-RM425	Populus alba	Pakistan	88.7	92	95.7	92.7	90.6	84.7	85	94.8	93.2	94.8	90.2	64.9	44.7	82.5
MH973690_ToLCKeV-AAK39	Digera muricata	Pakistan	88.3	90.7	93.7	90.7	88.1	84.3	85.5	95.3	94	95.3	91.7	71.4	47	83.8
LT556075_ToLCKeV		Pakistan	88.4	91.4	94.9	91.5	88.9	84.5	85	94.5	94	94.8	91	64.9	44.7	82.8
KM383758_ToLCBV-[BD:Syl]	Tomato (Solanum lycopersicum)	Sylhet, Bangladesh	88.7	88.1	97.2	95.2	95.7	92.4	92.7	86.6	79.8	86.9	85.8	94.8	89.6	79.8
KM383762_ToLCBV-[BD:Joy]	Tomato (Solanum lycopersicum)	Joydebpur, Bangladesh	88.6	86.6	97.2	90.7	87.6	92.4	93	89.8	83.5	88.3	87.3	94.8	90.7	75.8
EU910140_ToLCKeV-ToLCV-K5	Tomato (Solanum lycopersicum)	India	88	91.9	96.4	96	92.3	84.2	85.8	96.2	94	96.2	94.7	72.6	48.4	77.2
MT048410_ToLCBaV-BT20-1	Country bean	Bangladesh	88	86.6	97.2	90.1	85.9	91.9	92.7	89.3	81.3	87.6	86.5	93.8	88.6	79.1
MN698280_MYMIV-Raichur	*Vigna stipulacea*	Karnataka, India	64.2	69.3	74.7	58.4	41.1	70.3	72.6	56.9	42	61.1	43.3	75	57.5	39

Complete genome sequence of both ToLCTyPV clone Ty-6.1 and ToLCKV clone Ty-4A.2 were submitted to NCBI and accession number of MZ578457 and MZ578458 were obtained, respectively.

(e) Phylogenetic analysis

In phylogenetic analysis using Neighbor-Joining method in MEGA-X version 10.2.3 tool, with 1000 bootstrap replicates, Tomato leaf curl Ty Pusa virus clone Ty-6.1 (ToLCTyPV-Ty-6.1) appeared as a separate branch from other related begomoviruses (Fig. 24). But, Tomato leaf curl Karnataka virus clone Ty-4A.2 (ToLCKV-Ty-4A.2) appeared in the same clade along with other ToLCKV isolates (Fig. 25). As, ToLCTyPV clone Ty-6.1 appeared as a distinct branch in phylogenetic tree and different ORFs of the virus was showing maximum sequence identity with different viruses, possibility of phylogenetic conflict was suspected. Phylogenetic conflict analysis using Neighbor-Net analysis in SplitsTree version 4.7.1 tool showed Tomato leaf curl Ty Pusa virus clone Ty-6.1 as a completely separated out group from other related begomoviruses. Also, presence of rectangular boxes at the base of ToLCTyPV-Ty-6.1 branch clearly evidenced the phylogenetic conflict in ToLCTyPV clone Ty-6.1 sequence (Fig. 26). As, recombination is believed to be the primary reason for phylogenetic conflict, Phi-test analysis was done that found statistically significant evidence of recombination (p=0).

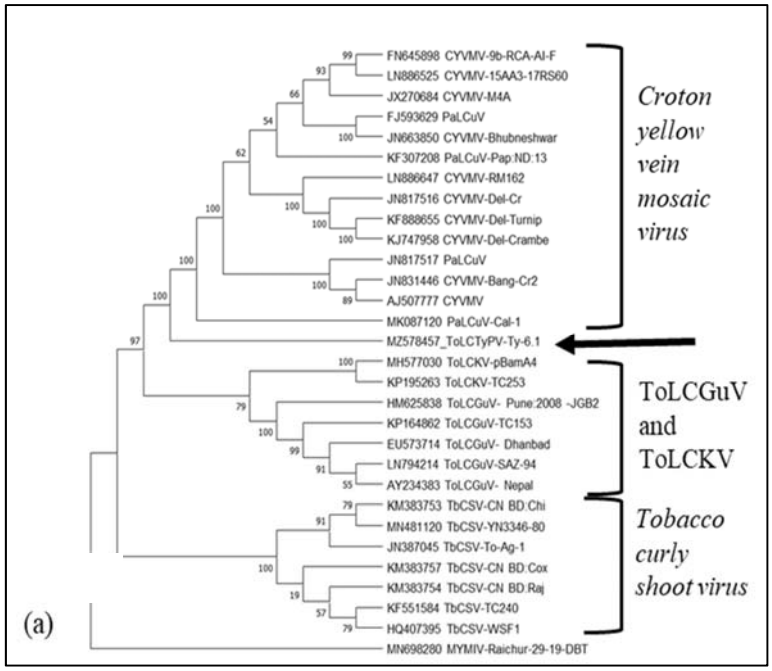


Fig. 24: Phylogenetic relationship of *Tomato leaf curl Ty Pusa virus* clone Ty-6.1 with other related begomoviruses infecting tomato in India. The dendrogram was constructed using Neighbor-Joining method in MEGA-X. A bootstrap analysis with 1000 replicates was performed. Present isolate of the virus was marked by black arrow.

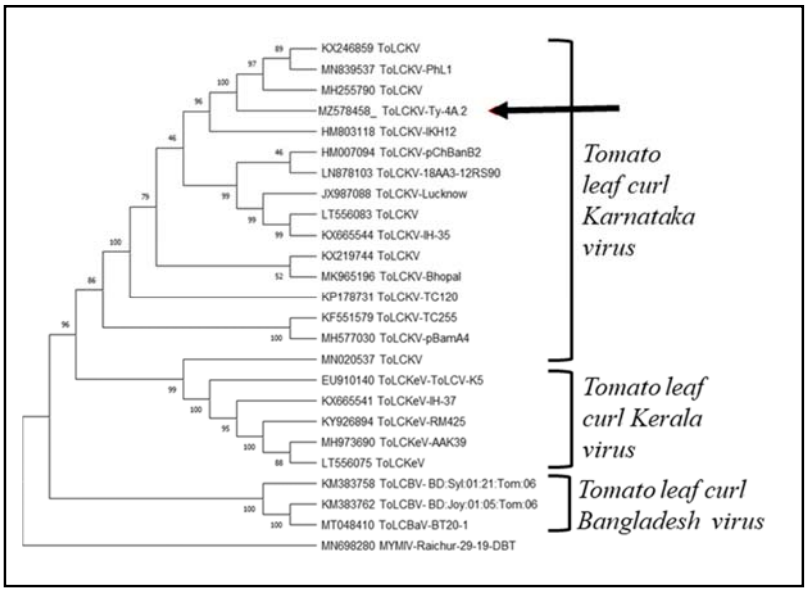


Fig. 25: Phylogenetic relationship of *Tomato leaf curl Karnataka C* clone 4A.2 with other related begomoviruses infecting tomato in India. The dendrogram was constructed using Neighbor-Joining method in MEGA-X. A bootstrap analysis with 1000 replicated was performed. Present isolate of the virus was marked in black arrow.

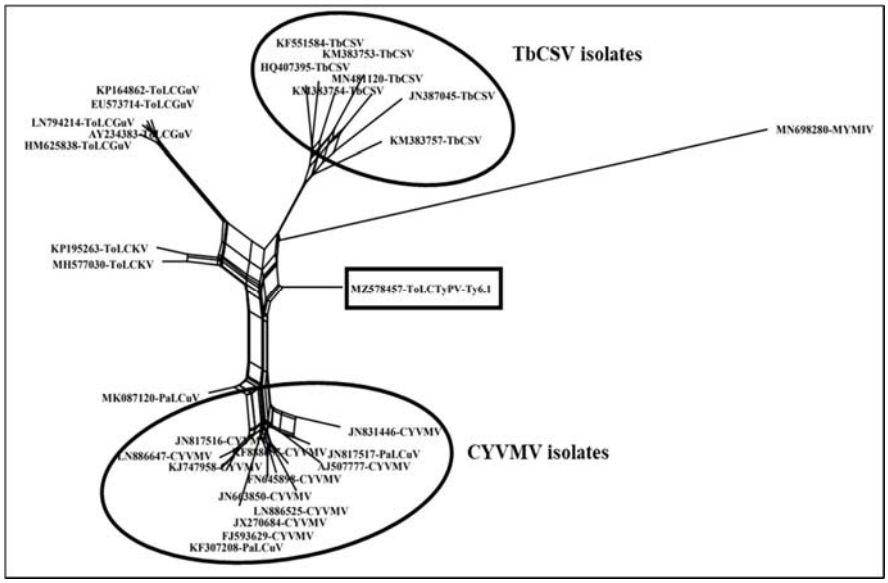


Fig 26: Phylogenetic conflict analysis using SplitsTree version 4.7.1 tool showing phylogenetic conflict in ToLCTyPV clone Ty-6.1 sequence (marked in black box)

(f) Recombination analysis

Probable occurrence of recombination in the genome of Tomato leaf curl Ty Pusa virus clone Ty-6.1 was further confirmed by analyzing with default parameters of RDP version 4.101 tool, which revealed two recombination breakpoints at 1836 bp and 2709 bp in ToLCTyPV clone Ty-6.1 genome enclosing major portion of ORF-C1, complete ORF-C4 and part of left intergenic region (left-IR) (Fig 27). All the seven methods used in RDP4.101 tool (RDP, GENECONV, BootScan, MaxChi, Chimera, SiScan, 3Seq) detected statistically significant recombination event between these two points with average p-value of $1.166x10^{-46}$, $7.750x10^{-51}$, $2.228x10^{-44}$, $4.174x10^{-28}$, $1.227x10^{-31}$, $1.190x10^{-45}$, $3.637x10^{-13}$, respectively. They also predicted Tomato leaf curl Ty Pusa virus clone Ty-6.1 as a daughter of interspecific recombination between Croton yellow vein mosaic virus (CYVMV, Acc. no. JN663850) as major parent and Tobacco curly shoot virus (TbCSV, Acc. no. KM383754) as minor parent (Table 17).

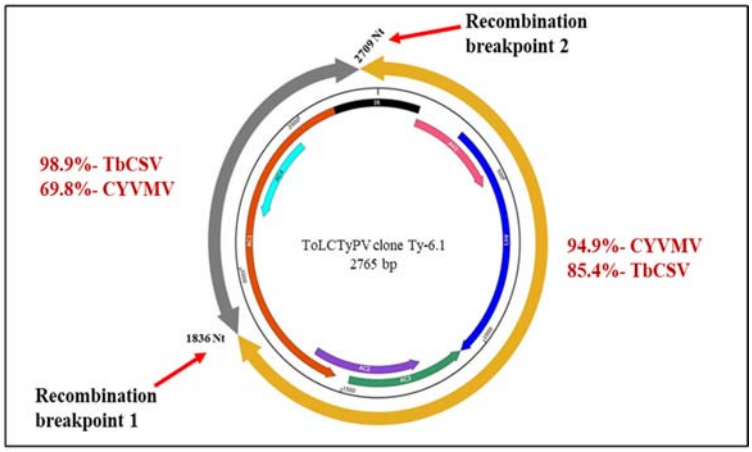


Fig. 27: Contribution of major and minor parents of *Tomato leaf curl Ty Pusa virus* clone Ty-6.1 displaying corresponding nucleotide sequence identities and recombination breakpoint as deduced from RDP analysis

Table 17: Recombination analysis of Tomato leaf curl Ty Pusa virus clone Ty-6.1 using RDP4.101 showing interspecific recombination between Croton yellow vein mosaic virus (CYVMV) as major parent and Tobacco curly shoot virus (TbCSV) as minor parent

Breakpoints	Position in genome	Avg. p-value of the recombination event	Major parent	Minor parent
1836, 2709	C1, C4, left-IR	RDP ($1.166x10^{-46}$), GENECONV ($7.750x10^{-51}$), BootScan ($2.228x10^{-44}$), MaxChi ($4.174x10^{-28}$), Chimera ($1.227x10^{-31}$), SiScan ($1.190x10^{-45}$), 3Seq ($3.637x10^{-13}$)	JN663850_ **CYVMV**-Bhubaneswar	KM383754_ **TbCSV**-CN-BD-Raj

(g) Analysis of intergenic region

Sequence analysis as well as recombination analysis showed that replication initiator protein (Rep) of ToLCTyPV clone Ty-6.1 is closely related with Rep of Tobacco curly shoot virus (TbCSV). So, the positions of Rep binding iterons in the sequence of ToLCTyPV clone Ty-6.1 must be similar to that of TbCSV for effective binding of Rep to the origin of replication of the viral genome. Critical analysis of intergenic regions of ToLCTyPV clone Ty-6.1, TbCSV, CYVMV and ToLCKV clone Ty-4A.2 revealed that, arrangement of iterons and all the promoter elements of ToLCTyPV clone Ty-6.1 is same to that of TbCSV but different from that of CYVMV, ToLCKV clone Ty-4A.2 (Fig. 28).

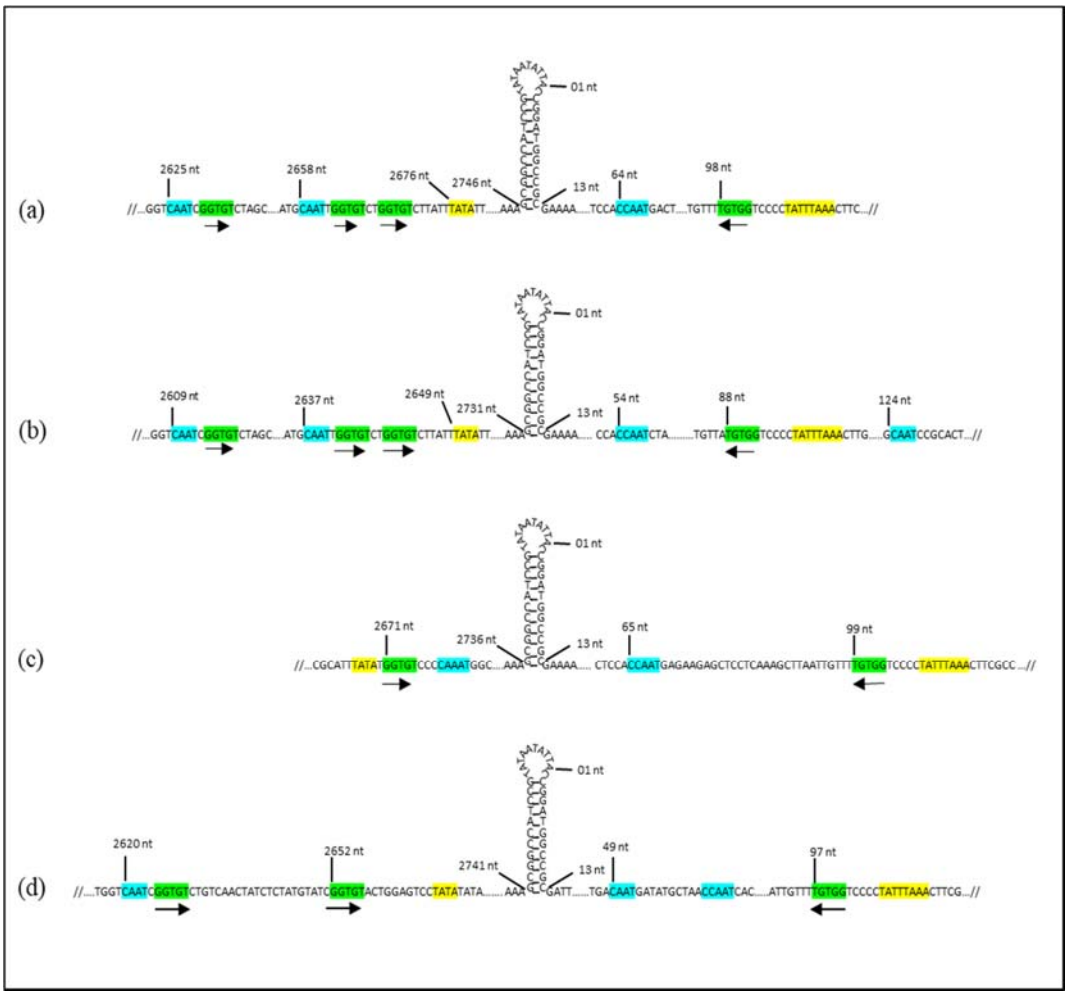


Fig. 28: Location of Replication initiator protein (Rep) binding iterons and different cis- acting regulatory elements in the intergenic region of (a) ToLCTyPV clone Ty-6.1 (recombinant), (b) KM383754_TbCSV (minor parent of ToLCTyPV clone Ty-6.1) and (c) JN6638_CYVMV (major parent of ToLCTyPV clone Ty-6.1) and (d) ToLCKV clone Ty-4A.2 genome

Green markings indicate Rep binding iterons; Yellow markings indicate TATA box; Blue markings indicate CAAT boxes; Arrows indicate orientation of the sequence

(h) Development of partial tandem repeat (PTR) construct

Sequence analysis of Tomato leaf curl Ty Pusa virus clone Ty-6.1 indicated it as a recombinant begomovirus. For confirming its infectivity, a partial tandem repeat (PTR) construct of ToLCTyPV clone Ty-6.1 was developed. ~0.5 kb fragment of viral genome containing the IR was ligated with linearized pCambia2300 binary vector *Bam*HI-*Sac*I digestion (Fig. 29). Restriction digestion using of recombinant pCambia2300 *Bam*HI and *Sac*I yielded ~0.5 kb DNA fragment (Fig. 30). Restriction digestion of pTy-6.1 using *Bam*HI-*Sca*I separated the complete viral genome of ~2.7 kb (Fig. 31) which was ligated with *Bam*HI digested linearized recombinant plasmid of pCambia2300 harbouring the partial (~0.5 kb) fragment of the virus in a tandem orientation to generate a 1.2-mer partial tandem repeat construct of ToLCTyPV clone Ty-6.1. Restriction digestion of 1.2-mer construct using *Sac*I enzyme confirmed the orientation (Fig. 32).

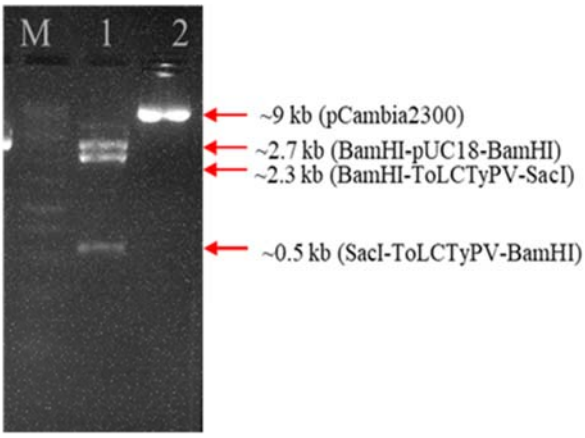


Fig. 29: Agarose gel (0.8%) electrophoresis of *Bam*HI and *Sac*I digested pTy-6.1 clone and pCambia2300 vector
M : 1 kb DNA ladder
Lane 1: *Bam*HI-*Sac*I digested pTy-6.1 clone
Lane 2: *Bam*HI-*Sac*I digested pCambia vector

Fig. 30: Agarose gel (0.8%) electrophoresis of *Bam*HI and *Sac*I digested 0.2-mer partial construct

M: 1 kb DNA ladder
Lane 1 to 4: *Bam*HI and *Sac*I digested 0.2-mer partial construct

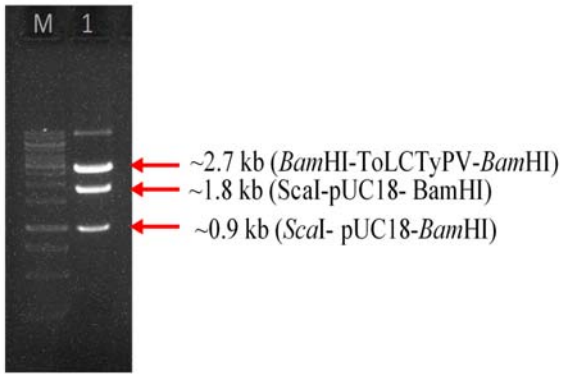


Fig. 31: Agarose gel (0.8%) electrophoresis of BamHI and ScaI digested pTy-6.1 clone

M: 1 kb DNA ladder
Lane 1: BamHI and *ScaI* digested pTy-6.1 clone

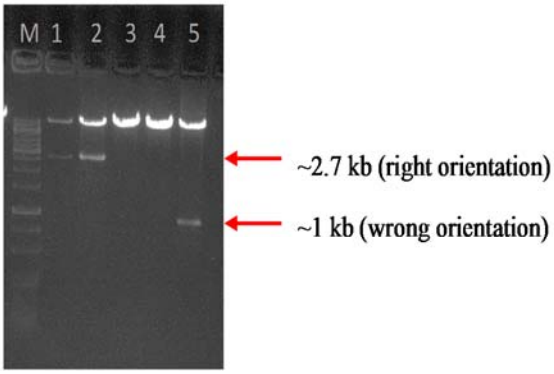


Fig. 32: Agarose gel (0.8%) electrophoresis of *Sac*I digested 1.2-mer PTR construct

M: 1 kb DNA ladder
Lane 1 -5: *Sac*I digested PTR construct

4.2.5. Amplification of a portion of *Ty-3* gene

Total RNA from the leaf samples of *Ty-3* gene containing genotypes yielded good quality and quantity of RNA (Fig. 33). PCR amplification using *Ty-3* gene specific primer (SCAR1 marker) generated amplicon of ca. 519 bp and ca. 219 bp in *Ty-3* resistance gene containing genotype and susceptible genotype, respectively (Fig. 34). Also, another primer set designed for the qRT-PCR for analysis of the expression profile of *Ty-3* gene (ACY) generated ca. 132 bp amplicon in *Ty-3* gene containing genotype (Fig. 35). But, due to unfortunate lock down (COVID-19), the qRT-PCR analysis could not be completed.

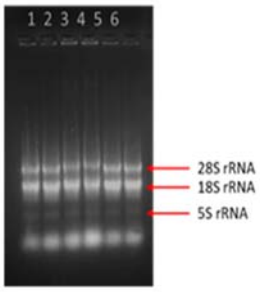


Fig 33: Agarose gel (0.8%) electrophoresis of total RNA isolated from leaf samples of tomato genotypes

Lane 1 to 5: Total RNA isolated from tomato genotypes

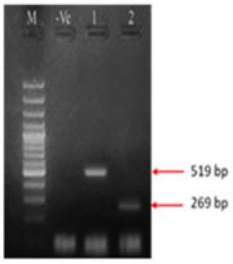


Fig 34: Agarose gel (1.5%) electrophoresis of PCR amplified product obtained using *Ty-3* gene specific primer (SCAR-1)

M : Molecular weight marker (100 bp plus DNA marker)
-Ve : Negative control
Lane 1: PCR amplified product using TNA from *Ty-3* gene containing line
Lane 2: PCR amplified product using TNA from susceptible line

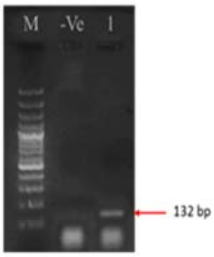


Fig 35: Agarose gel (2%) electrophoresis of PCR amplified product obtained using *Ty-3* gene specific qRT-PCR primer (ACY)

M : Molecular weight marker (100 bp plus DNA marker)
-Ve : Negative control
Lane 1: PCR amplified product using TNA from *Ty-3* gene containing line

CHAPTER 5 DISCUSSION

The current study aimed to understand the resistance behaviour of *Ty* resistance gene containing tomato genotypes, used as donor of *Ty* genes in breeding programs and to identify the begomoviruses infecting these genotypes under natural condition. Although, efforts have been made for assessing the effectiveness of different tomato genotypes against leaf curl disease of tomato but, they were either focused on tomato yellow leaf curl virus or involving other resistant stocks (Kumar *et al.,* 2019; Picó *et al.,* 1998; Prabhandakavi *et al.,* 2020). The present *Ty* gene containing donor genotypes remained to be investigated. Literature on evaluation of resistance in field condition revealed that two major criteria, *viz.* occurrence of disease in the population as measured through percent disease incidence (Azizi *et al*. 2008; Rajasri, and Vijayalakshmi, 2013, Singh, 2014) or symptom severity in individual plant (Reddy *et al*, 2008; Prabhandakavi *et al*., 2020), are considered to categorize the genotypes into different disease reaction category. Camara *et al*., 2013 though considered both disease incidence and disease severity to categorize the genotypes but they were estimated separately. A more robust approach using coefficient of infection (CI), including both disease incidence and severity in same disease scoring scale has been followed by few researchers (Singh *et al*., 2010), however, they used a particular severity grade for an individual plant. But in natural condition different disease symptom phenotypes appear in different plants and even within a plant there is great variation of different grades of one symptom phenotype in different leaves. So, a single severity grade of a plant, is not represented the true impact of a disease in a plant as it overlooks the different symptom grades of different leaves. During this study three distinct symptom phenotypes *viz.* leaf curl, leaf smalling and stunting were noticed in different plants, exclusively or in combinations. Even within a plant some leaf displayed severe leaf curl and some others exhibited mild leaf curl symptoms. Hence, it was realized that in a plant the symptom severity should be calculated as a cumulative severity index taking consideration of different symptom severity grades of different leaves. Thus, a more robust severity grades based on the cumulative disease severity were deduced and such severity grades were used to calculate CI. Thus, such new in depth method for categorizing the disease reaction in different genotypes, is one of the major outputs of this study and could be used to determine the disease response of genotype in breeding population.

Based on the disease scoring scale, developed in the study, 17 genotypes of tomato containing different combinations of *Ty* genes were evaluated and categorized in different groups. It was evident from the result that genotypes which showed highly resistant response had *Ty*-2 and *Ty*-3 resistant loci, though with the same gene combinations some other genotypes exhibited relatively susceptible response. This variation may be attributed due to difference in

the source of the *Ty* genes from where they were introgressed into the cultivated species and differential expression of *Ty*-genes. Earlier studies in this aspect indicated that under Indian context, genotypes with either *Ty*-3 alone or pyramided genotypes with *Ty-2* and *Ty-3* genes showed resistance under field as well as challenge inoculated condition (Prasanna *et al.,* 2015; Lata *et al.,* 2019). However, our study showed that such statement cannot be generalized, because sources of resistance loci for these genes are highly variable and that actually govern the ultimate disease response. Though compared to other *Ty* genes, it is definite that *Ty*-2 and *Ty*-3 gene containing genotypes are exhibiting more resistance response than other gene combinations. Following the *Ty*-2 and *Ty*-3 combinations, considerable resistance was observed in genotypes with *Ty*-5 and *Ty*-6 combination, which supported the findings of Kaushal *et al*., 2020. The field resistance behaviour of few of these highly resistant lines also continued to hold their resistance even under challenged inoculation condition, using viruliferous whiteflies carrying ToLCNDV, the most predominant tomato infecting begomovirus in India. Besides ToLCNDV, there are reports of many begomoviruses infecting tomato in natural condition, so, artificial screening of the genotypes using only one species of begomovirus does not guarantee their resistance against other begomoviruses, that may be the future scope of this study.

As under field condition besides ToLCNDV, there may be possibility of infection by other begomoviruses, it was realized that for proper understanding the disease response in the genotypes, information on the status of occurrence of different begomoviruses under the field condition is necessary. In order to understand that, PCR based indexing of different begomoviruses, for which species specific primers were available in the laboratory, was carried out.

PCR based detection followed by representative sequencing of the PCR amplicons indicated except Chilli leaf curl virus (ChiLCV) specific primers other species specific primers could amplify the targeted virus. ChiLCV specific primers gave an amplification of expected size but the sequence showed closeness to that of tomato leaf curl Karnataka virus (ToLCKV). Such anomaly probably resulted due to very high sequence similarity in the primer binding region (two internal mismatches only). ChiLCV specific primers were designed after analyzing different begomovirus sequences reported in chilli, where except ToLCNDV we did not take other viruses those infect tomato, as under natural condition except ToLCNDV, till now there was no report of other tomato infecting begomoviruses in chilli. Indexing of the begomoviruses clearly demonstrated that mixed infection of different begomoviruses in the genotypes tested was very frequent. In India, extensive and variable agro-climatic conditions coupled with intensive and mixed crop patterns supports perpetuation of whitefly vector round the year through their survival on overlapping host and thus allowed the chances of mixed infection. Mixed infection of different begomoviruses is one of the contributing factors responsible for

widening the host range of begomoviruses (Tripathi and Verma, 2017; Malathi *et al*., 2017), which has modeled a serious risk to many economically important cultivated crop species (Varma and Malathi, 2003). Frequency of mixed infections with different begomoviruses and satellite molecules is very high in begomoviruses, which many times contributed to the emergence of the more aggressive new variants of viruses due to recombination of existing ones (Zhou *et al*.., 1997, García-Andrés *et al*.., 2007, Fiallo-Olivé *et al*.., 2019).

Indexing result also indicated that ToLCNDV, which was supposed to be the most predominant begomovirus species in tomato in India, was detected only from 7 genotypes, irrespective of the disease response category of the genotypes. Interestingly, detection of CYVMV (later analyzed to be new begomovirus species) in 13 out of 18 genotypes tested, clearly revealed that this virus was the predominant begomovirus species in majority of the genotypes carrying *Ty* genes. Though the deployment of resistance gene containing cultivar is the most looked-for mean to devise strategy for managing viral diseases, such concern of altered population of virus species to adapt to the resistant host due to the selection pressure exerted by the resistance genes on the virus population has been raised by many researchers (Fabre *et al*., 2009, Jones, 2009). The widespread use of resistant cultivars has been shown to alter virus diversity and thus allow less predominant virus species becoming more important (Seal *et al*., 2006). In case of TYLCD, it has been shown that *Ty*-gene containing resistant tomato cultivars could allow the emergence of more suitable virus species with capability to overcome resistance (Roossinck, 1997, Harrison, 2002, García-Arenal and McDonald, 2003). It was also demonstrated earlier in Spain, not only the resistant genotypes play a role in selection of viruses; even in absence of resistant genotypes, due to more ecological fitness, the TYLCV replaced more predominant virus species tomato yellow leaf curl Sardenia virus (TYLCSV), originally associated with TYLCD-infections (Sánchez-Campos *et al*.., 1999, Navas-Castillo *et al*.., 1999).

No direct correlation between the disease response of a genotype and number of begomoviruses detected in that genotype could be drawn. As expected, no begomovirus was detected in genotype ToLCD Tol-15 (*Ty*-2+*Ty*-3), which showed highly resistant disease response with no disease symptom in the field. Genotype ToLCD Tol-3 (*Ty*-1) and ToLCD Tol-10 (*Ty*-5), which showed moderate susceptibility under field condition were positive for 5 and 6 number of begomoviruses, respectively. On the contrary, genotype ToLCD Tol-5 (*Ty*-2+*Ty*-3), which showed presence of 3 begomoviruses remained highly resistant with no disease symptom in the field and ToLCD Tol-2 (*Ty*-2) showed susceptible disease reaction even after presence of only 2 begomoviruses. Differential effectiveness of *Ty*-genes as well as variability in the begomovirus species could be the reasons for such findings, which needs to be investigated in future.

Though CYVMV was detected through PCR and partial sequencing in majority of the genotypes under study, but it was realized that the exact identity of the virus could only be possible if complete genome sequence is known. Complete genome of one of the suspected CYVMV isolate along with another begomovirus which did not gave any amplicon with any specific primers were characterized through sequencing. Detailed sequence analysis indicated that the suspected CYVMV isolate is actually a new species of begomovirus probably evolved due to recombination between a TbCSV and CYVMV isolate which supports the relevance of development of recombinant strains of both the parent viruses reported earlier in India (Shilpi *et al.,* 2015; Sinha *et al.,* 2016). The other begomovirus was an isolate of ToLCKV, though it has not yielded any amplicon with ChiLCV primers, which detected ToLCKV from other two genotypes.

Emergence of new begomoviruses or variants of existing begomoviruses due to intra- and interspecific recombinations and mutations in the genomes coupled with introduction of susceptible cultivars of crops and change in climatic conditions resulted into more begomovirus induced diseases in the last two decades (Juárez *et al*., 2019). Recombination is the major driving force for evolution of viruses (Pagán and García-Arenal, 2018, Zaffalon *et al*., 2012) so that they can get adapted to new hosts and environmental conditions (Verma *et al*., 2015). Recombination driven dynamic process of evolution has been frequently encountered for ssDNA viruses especially with respect to begomoviruses (Martin *et al.*, 2011). Emergence of recombinant begomoviruses from plants mixed-infected with different begomoviruses seems to be frequent and as a consequence such new recombinant begomoviruses may have greater fitness to adapt into resistant plants (Monci *et al*., 2002), thus could lead to the breakdown of resistance (García-Arenal and McDonald, 2003, Padidam et al.., 1999, van der Walt et al.., 2009). Emergence of ToLCTyPV in the *Ty*-gene containing resistant genotypes, may not immediately pose any serious threat of resistance breaking as the genotype ToLCD Tol-8 (*Ty-2+Ty-3*) and ToLCD Tol-7 (*Ty-3a*), where only ToLCTyPV was detected, showed highly resistant and resistant responses, respectively in the field, however, frequent occurrence of ToLCTyPV along with other begomoviruses may contributes for susceptibility reaction in other *Ty*-gene containing genotypes tested in the study. The infectivity study of this new recombinant virus alone or in combination with other begomoviruses in the resistant genotypes could focus light on the impact of such new recombinant viruses in the resistance breaking. Resistance genes may contribute for the reduction of ToLCNDV titre in *Ty*-gene containing genotypes as evidenced from the fact that ToLCNDV was detected in fewer genotypes as it was expected but such situation may allow other viruses or the new recombinant viruses to rapidly accumulate in those genotypes. This is in agreement with the earlier studies where it has been demonstrated that exploitation of *Ty-1* gene driven resistance against one virus (Michelson *et al.*, 1994), may

lead to selection of new virus variants with higher accumulation rates in the host (Van den Bosch *et al*., 2006).

The present investigation thus not only evaluated the *Ty* gene containing genotypes for their resistance against begomoviruses, but with the indexing of the viruses it is clear that under natural mixed infection condition, with the emergence of new begomovirus species, breeders have more challenging task ahead to incorporate the resistance in tomato breeding programs.

- *Ty-2* + *Ty-3* genes containing genotypes are more resistant against tomato leaf curl disease than other gene combinations
- ToLCD Tol-15, ToLCD Tol-14, ToLCD Tol-5 are highly resistant both under field and challenge inoculated condition
- No virus was detected in ToLCD Tol-15 using any of the primers tested
- Genotype ToLCD Tol-10 showed presence of six different begomoviruses
- ToLCBaV was not detected in any genotype.
- Many genotypes showed presence of multiple begomoviruses.
- CYVMV specific primers showed amplifications maximum number of genotypes.
- ToLCD Tol-1 did not give amplification with any species specific primer but gave amplification with universal begomovirus primer.
- Full length genome characterization from two showed presence of a new species of begomovirus named as *Tomato leaf curl Ty Pusa virus* (ToLCTyPV) (Acc. no- MZ578457) and an isolate of *Tomato leaf curl Karnataka virus* (ToLCKV) (Acc. no- MZ578458).
- ToLCD Tol-7 (*Ty-3a*, source of ToLCTyPV) and ToLCD Tol-8 (*Ty-2*+*Ty-3*), where only ToLCTyPV was detected, showed resistant and highly resistant phenotype, respectively under field condition
- ToLCTyPV may be evolved due to interspecific recombination between *Croton yellow vein mosaic virus* (CYVMV) and *Tobacco curly shoot virus* (TbCSV) as major and minor parents, respectively.

ABSTRACT

Evaluation of Resistance Behaviour of *Ty*-Gene(s) Containing Donor Lines of Tomato and Indexing of Naturally Occurring Begomoviruses Infecting Them

Tomato leaf curl disease (ToLCD) is caused by a number of begomoviruses. Use of resistant/tolerant varieties is the most feasible strategy for the management of the leaf curl disease. Resistance sources with different *Ty* genes have been identified in different wild relatives of *Solanum lycopersicum*. In this study, seventeen tomato genotypes with different *Ty*-gene combinations, those are used as parental donor lines for incorporating resistance in tomato breeding programme, along with a susceptible check cv. Pusa Ruby were evaluated for understanding their response against tomato leaf curl disease under field condition. PCR based indexing of begomoviruses in those genotypes were also carried out to understand the status of occurrence of different begomoviruses. In order to evaluate the resistance behaviour of these genotypes, a quantitative scoring system for analyzing disease severity was developed based on different grades of symptom phenotypes and their impact in disease development. Such deduced disease severity score was used to develop a disease scoring scale based on the co-efficient of infection that includes both percent disease incidence and severity grade. Based on such disease scoring scale, highly resistance response was observed in five genotypes (ToLCD Tol-5, ToLCD Tol-8, ToLCD Tol-12, ToLCD Tol-14, ToLCD Tol-15) containing both *Ty-2* and *Ty-3* genes. Further evaluation of three highly resistant genotypes under challenged viruliferous whitefly inoculation with a well characterized isolate of tomato leaf curl New Delhi virus, the most prevalent begomovirus in tomato causing leaf curl disease in India, confirmed their resistance behaviour. Indexing of the begomoviruses in all these genotypes through PCR based detection using seven begomovirus species specific primers and one genus specific primer followed by their confirmation through representative sequencing revealed presence of a number of begomovirus species in these tomato genotypes indicating the probable occurrence of mixed infection of two to six different begomoviruses under field condition. While tomato leaf curl New Delhi virus was detected in seven samples, majority of the samples (thirteen) showed association of a Croton yellow vein mosaic virus (CYVMV), alone or in combination with other begomovirus species. One genotype (ToLCD Tol-15), which did not show any symptom, also did not gave amplification with any of the primers. Another genotype (ToLCD Tol-1) gave amplification only with genus specific primers indicating presence of other begomovirus(es) for which we did not had any primers. Complete genome of the begomovirus, from this sample and from another sample where only CYVMV infection was detected, was isolated, cloned and sequenced. A detail sequence analysis of the CYVMV infected sample showed that the virus is actually a new species of begomovirus with less than 91% sequence

identity with any known begomoviruses, while the begomovirus from the other sample, which did not show any amplification with the species specific primers, was a strain of tomato leaf curl Karnataka virus. The name *Tomato leaf curl Ty Pusa virus* (ToLCTyPV) was proposed for the new species, which was probably evolved due to an interspecific recombination between CYVMV as major parent and tobacco curly shoot virus (TbCSV) as minor parent. A partial tandem repeat construct against the ToLCTyPV has been developed. Further agro-inoculation study will help us to know whether this could be a resistance breaking virus in tomato. The study thus clearly showed the resistance response of the tomato genotypes used as parental donor lines in breeding programme and the scenario of the occurrence of the begomoviruses in them.

Keywords: Tomato leaf curl disease, *Ty* resistance genes, *Begomovirus*, PCR, *Croton yellow vein mosaic virus, Tobacco curly shoot virus,* Recombination, *Tomato leaf curl Ty Pusa virus, Tomato leaf curl Karnataka virus*

सार

टमाटर में पाए जाने वाले *Ty*-जीनों के प्रतिरोध व्यवहार का मूल्यांकन एवं उन्हें संक्रमित करने वाले बेगोमोविषाणु का अनुक्रमण

टोमैटो लीफ कर्ल बीमारी (टीओएलसीडी) कई बेगोमोविषाणु के कारण होता है। लीफ कर्ल रोग के प्रबंधन के लिए प्रतिरोधी/सहिष्णु किस्मों का उपयोग सबसे व्यवहार्य रणनीति है। सोलनम लाइकोपर्सिकम के विभिन्न जंगली प्रजातियों में विभिन्न *Ty*-जीन वाले प्रतिरोध स्रोतों की पहचान की गई है। इस अध्ययन में, विभिन्न *Ty*-जीन संयोजनों के साथ सत्रह टमाटर जीनोटाइप, जिन्हें टमाटर प्रजनन कार्यक्रम में प्रतिरोध को शामिल करने के लिए पेरेंट्स की दाता लाइनों के रूप में उपयोग किया जाता है, साथ ही एक अतिसंवेदनशील प्रजाति पूसा रूबी का खेत की स्थिति के तहत टमाटर की पत्ती कर्ल रोग के खिलाफ उनकी प्रतिक्रिया को समझने के लिए मूल्यांकन किया गया था। विभिन्न बेगोमोवायरस की घटना की स्थिति को समझने के लिए उन जीनोटाइप में पीसीआर आधारित बेगोमोविषाणु का अनुक्रमण भी किया गया था। इन जीनोटाइप के प्रतिरोध व्यवहार का मूल्यांकन करने के लिए, रोग की गंभीरता का विश्लेषण करने के लिए एक मात्रात्मक स्कोरिंग प्रणाली विकसित की गई थी जो विभिन्न ग्रेड के लक्षण फेनोटाइप और रोग के विकास में उनके प्रभाव के आधार पर विकसित की गई थी। इस तरह के घटाए गए रोग गंभीरता स्कोर का उपयोग संक्रमण के सह-कुशलता के आधार पर एक रोग स्कोरिंग स्केल विकसित करने के लिए किया गया था जिसमें प्रतिशत रोग घटना और गंभीरता ग्रेड दोनों शामिल हैं। इस तरह के रोग स्कोरिंग पैमाने के आधार पर, Ty-2 और Ty-3 जीन वाले पांच जीनोटाइप (ToLCD Tol-5, ToLCD Tol-8, ToLCD Tol-12, ToLCD Tol-14, ToLCD Tol-15) में अत्यधिक प्रतिरोध प्रतिक्रिया देखी गई। टमाटर लीफ कर्ल विषाणुजनित व्हाइटफ्लाई इनोक्यूलेशन के तहत तीन अत्यधिक प्रतिरोधी जीनोटाइप ने नई दिल्ली वायरस, टमाटर में सबसे प्रचलित बेगोमोवायरस, जो भारत में लीफ कर्ल रोग का कारण बनता है, उनके प्रतिरोध व्यवहार की पुष्टि की। सात बेगोमोविषाणु प्रजातियों के विशिष्ट प्राइमरों और एक जीनस विशिष्ट प्राइमर का उपयोग करके पीसीआर आधारित पहचान के माध्यम से इन सभी जीनोटाइप में बेगोमोविषाणु का अनुक्रमण, प्रतिनिधि अनुक्रमण के माध्यम से उनकी पुष्टि के बाद इन टमाटर जीनोटाइप में कई बेगोमोविषाणु प्रजातियों की उपस्थिति का पता चला है जो मिश्रित संक्रमण की संभावित घटना का संकेत देते हैं। क्षेत्र की स्थिति के तहत दो से छह अलग-अलग बेगोमोविषाणु, जबकि टोमैटो लीफ कर्ल नई दिल्ली विषाणु सात नमूनों में पाया गया था, अधिकांश नमूनों (तेरह) ने अकेले या अन्य बेगोमोविषाणु प्रजातियों के साथ संयोजन में क्रोटन येलो वेन मोज़ेक विषाणु (CYVMV) का जुड़ाव दिखाया। एक जीनोटाइप (ToLCD Tol-15), जिसमें कोई लक्षण नहीं दिखा, ने भी किसी भी प्राइमर के साथ प्रवर्धन नहीं दिया। एक अन्य जीनोटाइप (ToLCD Tol-1) ने केवल जीनस विशिष्ट प्राइमरों के साथ अन्य बेगोमोविषाणुओं की

उपस्थिति का संकेत दिया, जिसके लिए हमारे पास कोई प्राइमर नहीं था। इस नमूने से और दूसरे नमूने से, जहां केवल CYVMV संक्रमण का पता चला था, बेगोमोवायरस का पूरा जीनोम अलग, क्लोन और अनुक्रमित किया गया था। CYVMV संक्रमित नमूने के एक विस्तृत अनुक्रम विश्लेषण से पता चला है कि विषाणु वास्तव में किसी भी ज्ञात बेगोमोविषाणु के साथ 91% से कम अनुक्रम पहचान के साथ बेगोमोविषाणु की एक नई प्रजाति है, जबकि अन्य नमूने से बेगोमोविषाणु, जो विशिष्ट प्रजातियों के साथ कोई प्रवर्धन नहीं दिखाता है। प्राइमर, टमाटर लीफ कर्ल कर्नाटक विषाणु का एक स्ट्रेन था। नई प्रजातियों के लिए टोमैटो लीफ कर्ल *Ty*-पूसा विषाणु (ToLCTyPV) नाम प्रस्तावित किया गया था, जो संभवत: सीवाईवीएमवी के बीच प्रमुख पेरेंट्स और तंबाकू कर्ली शूट विषाणु (टीबीसीएसवी) के बीच माता-पिता के रूप में एक अंतर-विशिष्ट पुनर्संयोजन के कारण विकसित हुआ था। ToLCTyPV के खिलाफ एक आंशिक अग्रानुक्रम दोहराव निर्माण विकसित किया गया है। आगे के कृषि-इनोक्यूलेशन अध्ययन से हमें यह जानने में मदद मिलेगी कि क्या यह टमाटर में प्रतिरोध तोड़ने वाला विषाणु हो सकता है। इस प्रकार अध्ययन ने स्पष्ट रूप से प्रजनन कार्यक्रम में दाता लाइनों के रूप में उपयोग किए जाने वाले टमाटर जीनोटाइप की प्रतिरोध प्रतिक्रिया और उनमें बेगोमोविषाणु की घटना के परिदृश्य को स्पष्ट रूप से दिखाया।

मुख्य शब्द : टोमैटो लीफ कर्ल बीमारी , *Ty* रेसिस्टेंट जीन, बेगोमोविषाणु , क्रोटन येलो वेन मोज़ेक विषाणु , टोबैको कर्ली शूट विषाणु , रीकॉम्बिनेशन, टोमैटो लीफ कर्ल *Ty*-पूसा विषाणु , टोमैटो लीफ कर्ल कर्नाटक विषाणु ।

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APPENDIX

COMMON REAGENTS, BUFFERS AND MEDIA USED

i. ANTIBIOTICS

Ampicillin	Stock solution (100 mg/ml) of the antibiotic was made in double distilled water, filter sterilized (through 0.22 µ filter) and distributed into 200 µl aliquots and stored at - 20^0C. It was used at final concentration of 100 µg/ml.
Kanamycin	Stock solution (50 mg/ml) of the antibiotic was made in double distilled water, filter sterilized (through 0.22 µ filter) and distributed into 200 µl aliquots and stored at - 20^0C. It was used at final concentration of 50 µg/ml.

ii. ELECTROPHORESIS REAGENTS

50X TAE	Tris base	242.0 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100 ml
	Distilled water	to 1 L
Loading dye	1% Bromophenol blue	200 µl
	Glycerol	200 µl
	10% SDS	60 µl
	0.5 M EDTA	50 µl
	10X TAE	60 µl
	Distilled water	30 µl

iii. MEDIA

Luria Agar medium	Bacto-tryptone	10.0 g
	Bacto-yeast extract	5.0 g
	NaCl	10.0 g
	Agar	15.0
	Deionized water	950 ml

pH was adjusted to 7.0 with 5N NaOH and volume made upto 1 litre with deionized water. It was dispensed in 100 ml aliquots in 250 ml flasks and was sterilized by autoclaving for 20 min. at 15 p.s.i.

Luria Broth medium	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Deionized water	950 ml

pH was adjusted to 7.0 with 5N NaOH and volume made upto 1 litre with deionized water. The medium was aliquoted into 50 ml in 250 ml flasks and sterilized by autoclaving for 20 min at 15 p.s.i.

iv. PLASMID ISOLATION BUFFERS

Solution I (resuspension buffer)	25 mM Tris HCL (pH 8.0)
	50 mM Glucose
	10 mM EDTA
Solution II (lysis buffer)	0.2 N NaOH
	1% SDS
Solution III (neutralization buffer)	3 M Sodium acetate pH 4.8

v. DNA MOLECULAR WEIGHT MARKER

1 kilobase (1kb) and 100 bp plus DNA ladder of Thermo Scientific were used as marker. The 1 kb ladder is formed by fourteen DNA fragments of 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3.5 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 0.75 kb, 0.5 kb and 0.25 kb. The 100 bp ladder is formed by ten DNA fragments of 3000 bp, 2000 bp, 1500 bp, 1200 bp, 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 200 bp and 100 bp.

vi. PREPARATION OF COMMONLY USED STOCK SOLUTIONS

Solution	Method of preparation
1M $CaCl_2$	54.0 g of $CaCl_2.2H_2O$ was dissolved in 200 ml of pure water (Mill-Q or equivalent). The solution was sterilized by passing through a 0.22 micron filter and stored in 1 ml aliquots at 4^0C.
0.5 M EDTA (pH 8.0)	186.1 g of ethylenediamine tetra acetic acid disodium salt.$2H_2O$ was added to 800 ml of distilled water, stirred vigorously on a magnetic stirrer, pH was adjusted to 8.0 with NaOH (20.0 g of NaOH pellets). Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
Ethidium bromide (10 mg ml^{-1})	1.0 g of ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The solution was transferred to a dark bottle and stored at room temperature.
Phenol : Chloroform : isoamyl alcohol	Buffer saturated phenol, chloroform and isoamyl alcohol were mixed in the ratio of 25 : 24 : 1. The equilibrated mixture was stored under a layer of 0.01 M Tris-HCl (pH 7.6) at 4^0C in dark glass bottle.
Chloroform: isoamyl alcohol	Buffer saturated chloroform and isoamyl alcohol were mixed in the ratio of 24 : 1.
1 M $MgCl_2$	203.3 g of $MgCl_2.6H_2O$ was dissolved in 800 ml of distilled water. The volume was made upto 1L, dispensed into aliquots and sterilized by autoclaving.

3M Sodium acetate (pH 4.8) 408.1 g of NaOAc.3H2O was dissolved in 800 ml of distilled water. The pH was adjusted to 4.8 with glacial acetic acid. Volume made upto 1L with distilled water, dispensed into aliquots and sterilized by autoclaving.

5 M NaCl 233.8 g of NaCl was dissolved in 800 ml of distilled water, volume made upto 1L with distilled water, dispensed into aliquots and sterilized by autoclaving.

10% Sodium dodecyl sulphate (SDS)

100.0 g of electrophoresis grade SDS was dissolved in 900 ml of distilled water, heated at 68^0C to assist dissolution and pH was adjusted to 7.2 by adding few drops of concentrated HCl. The volume was made up to 1L with distilled water, dispensed in aliquots.

10(N) NaOH Dissolve 400 g of NaOH in 800 ml of distilled water and make up the volume to 1L with distilled water.

1M Tris. HCl 121.1 g of Tris base was dissolved in 800 ml of distilled water. pH was adjusted to the desired value by adding concentrated HCl (for pH 7.4, HCl 70 ml; for pH 8.0, HCl 42 ml). The solution was allowed to cool down to room temperature before making final adjustment to the pH. The volume was made upto 1L with distilled water, dispensed into aliquots and sterilized by autoclaving

i. MATERIALS, SOURCE, OF CHEMICALS AND REAGENTS

Material	Source
Cells	
Escherichia coli DH5α	Stratagene, USA NEB, UK
Nucleic acid marker	

1 kb DNA ladder	Thermo Scientific, USA
100 bp plus DNA ladder	Thermo Scientific, USA
Enzymes	
Restriction endonucleases Alkaline phosphatase *GoTaq* DNA polymerase	Thermo Scientific, USA
Dream*Taq* DNA polymerase T4 DNA ligase	Thermo Scientific, USA
Primers Synthesized oligos	GCC Biotech, India
Antibiotics Kanamycin	Sigma Chemicals Co.
Ampicillin	Sigma Chemicals Co.
Gel purification kit	Promega corporation, USA

Sequences submitted to NCBI

LOCUS **MZ578457** 2765 bp DNA circular 18-JUL-2021
DEFINITION **Tomato leaf curl Ty Pusa virus complete genome.**
ACCESSION MZ578457
VERSION MZ578457.1
KEYWORDS .
SOURCE Tomato leaf curl Ty Pusa virus
ORGANISM Tomato leaf curl Ty Pusa virus
Unclassified.
REFERENCE 1 (bases 1 to 2765)
AUTHORS Mondal,F, Saxena,S., Hussain,Z., Mukherjee,S.K., Mandal,B. and Roy,A

TITLE Characterization of a new begomovirus species infecting Ty resistance gene containing tomato lines
JOURNAL unpublished
REFERENCE 2 (bases 1 to 2765)
AUTHORS Mondal,F., Saxena,S., Hussain,Z., Mukherjee,S.K., Mandal,B. and Roy,A.
TITLE Direct Submission
JOURNAL Submitted (15-JUL-2021) Advanced centre for Plant Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India, Pusa, New Delhi, Delhi 110012, India
COMMENT Bankit Comment: TOTAL # OF SEQS:1

##Assembly-Data-START##
Sequencing Technology: Sanger dideoxy sequencing
##Assembly-Data-END##
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LOCUS MZ578458 2760 bp DNA circular VRL 18-JUL-2021
DEFINITION **Tomato leaf curl Karnataka virus complete genome**.
ACCESSION MZ578458
VERSION MZ578458.1
KEYWORDS .
SOURCE Tomato leaf curl Karnataka virus (ToLCKV)
ORGANISM Tomato leaf curl Karnataka virus
Viruses; Monodnaviria; Shotokuvirae; Cressdnaviricota;
Repensiviricetes; Geplafuvirales; Geminiviridae; Begomovirus.
REFERENCE 1 (bases 1 to 2760)
AUTHORS Mondal,F., Saxena,S., Hussain,Z., Mukherjee,S.K., Mandal,B. and Roy,A

TITLE Characterization of Tomato leaf curl Karnataka virus infecting Ty
resistance gene containing tomato lines
JOURNAL unpublished
REFERENCE 2 (bases 1 to 2760)
AUTHORS Mondal,F., Saxena,S., Hussain,Z., Mukherjee,S.K., Mandal,B. and
Roy,A.
TITLE Direct Submission
JOURNAL Submitted (18-JUL-2021) Advanced centre for Plant Virology,
Division of Plant Pathology, Indian Agricultural Research
Institute, New Delhi, India, Pusa, New Delhi, Delhi 110012, India
COMMENT Bankit Comment: TOTAL # OF SEQS:1

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