Analysis of NBS-LRR mediated host response against Chilli leaf curl virus

Thesis submitted to

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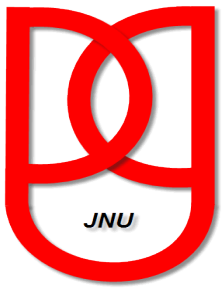
For the award of the degree of

DOCTOR OF PHILOSOPHY



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Date: 14, July, 2017

CERTIFICATE

This is to certify that the work embodied in this thesis entitled **"Analysis of NBS-LRR mediated host response against Chilli leaf curl virus"** has been carried out at School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. This work is original and has not been submitted so far in part or in full, for the award of any degree or diploma by any university.

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Dedicated to
My Parents and family

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- Ashish Kumar Singh

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ABBREVIATIONS

%	Percent
°C	Degree Celsius
A-rich	Adenine-rich region
BiFC	Bimolecular fluorescent complementation
cDNA	Complementary DNA
ChiLCA	*Chilli leaf curl alphasatellite*
ChiLCD	Chilli leaf curl disease
ChiLCV	*Chilli leaf curl virus*
CroYVMB	*Croton yellow vein mosaic betasatellite*
DAPI	4, 6-diamidino-2-phenylindole
DO	Drop out
DPI	Days-post inoculation
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxy nucleotide triphosphates
dpi	Days post inoculation
dsDNA	Double stranded DNA
EDTA	Ethylene diaminetetra acetic acid
EtBr	Ethidium bromide
g	Gram
GFP	Green fluorescent protein
GDarSLA	*Gossypium darwinii symptomless alphasatellite*
h	Hour
IR	Intergenic region
LiAc	Lithium acetate
kb	Kilo base pairs
LB	Luria-Bertani medium
M	Molar
min	Minutes
ml	Millilitre

mm	Millimeter
mM	Millimolar
ML	Maximum-likelihood
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nano gram
NJ	Neighbor-joining
OD	Optical density
ORF	Open reading frame
PaLCuV	*Papaya leaf curl virus*
PCR	Polymerase chain reaction
PepLCBV	*Pepper leaf curl Bangladesh virus*
PTR	Partial tandem repeat
RCA	Rolling circle amplification
RDP	Recombination detection program
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	Revolutions per minute
SCR	Satellite conserved region
SDT	Species demarcation tool
sec	Second
ssDNA	Single stranded DNA
ToLCBDB	*Tomato leaf curl Bangladesh betasatellite*
ToLCJoB	*Tomato leaf curl Joydebpur betasatellite*
ToLCGV	*Tomato leaf curl Gujarat virus*
ToLCNDV	*Tomato leaf curl New Delhi virus*
TRV	*Tobacco rattle virus*
var.	Variety
v/v	Volume by volume
w/v	Weight by volume
µg	Microgram
µl	Microliter
µM	Micromolar

Chilli (*Capsicum* spp.) is an important spice cum vegetable crop and a member of the family *Solanaceae*. Out of 20-27 species that are categorized within the Genus *Capsicum*, out of which only five are cultivated : *C. annuum, C. baccatum, C. chinense, C. frutescens*, and *C. pubescen*. It is believed that chilli was originated about 9500 years ago in the Latin American region of New Mexico and Guatemala (Milind and Sushila, 2012) and subsequently distributed to the rest of world. At present chilli is cultivated all around the world except few cold countries and India is the largest producer, consumer and exporter of this crop. Chilli is an important cash crop in India. Burning sensation and pungency of chilli is due to the capsaicin which is being used for the treatment of arthritis, disorders of sensory nerve fibers and psoriasis (Chhabra et al. 2012). Chillies are also known to have anti-cancerous compounds. Chilli is rich sources of vitamins, specifically vitamin A and C. Recently, vitamin-P has also been identified in green chilli (Milind and Sushila, 2012). Chillies are also packed with ions like potassium, magnesium and iron. In industries chilli is being used for flavor, colour, beverages and extraction of oleoresin and capsaicin. Farmers of the African and Asian countries use chilli plants to save the valuable crop from elephants by growing few rows of them around the field. The capsanthin pigment provides the red colour to the chilli.

According to the Food and Agriculture Organization of the United State (FAOSTAT; 2014), India has produced largest amount of chilli in the world, however, the yield is less than that of many other countries. The reduced yield of chilli in our country is primarily due to the biotic stress created by pathogens and insects. Chilli is known as host for different bacterial, fungal and viral pathogens. Recently viruses have emerged as a far-reaching threat for the cultivation of chilli (Varma and Malathi, 2003). Although large number of RNA and DNA viruses can infect chilli plants but ssDNA viruses causing leaf curl disease are the major constraints. Till now several viruses are known to cause leaf curl disease in chilli which may lead to the 100% crop loss. These viruses belong to the family *Geminiviridae*, characterized with the presence of single stranded circular DNA encapsidated in a twinned icosahedral protein capsid. On the basis of genome organization, insect-vector and host range of viruses, the *Geminiviridae* family is categorized into nine genera and named as *Becurtovirus*, *Capulavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Mastrevirus*, *Topocuvirus*, *Turncurtovirus* and *Begomovirus* (Zerbini et al. 2017). Among these genera, except *Begomovirus* other eight genera possess monopartite genome whereas *Begomovirus* may have either mono- or bipartite genomes. Among the geminiviruses,

Begomovirus is the largest genus (288 species) and are distributed throughout the world and cause the huge loss of wide range of crops. Begomoviruses are known to be one of the most devastating plant pathogens and are being transmitted by the insect vector, *Bemisia tabaci* (Varma and Malathi, 2003; Brown et al. 2012).

In response to the pathogen attack, host cell triggers a chain of responses such as hormone-mediated defense, activation of pattern triggered immunity and activation of effector triggered immunity (R-gene mediated resistance). Resistance or tolerance against viruses in plants is the result of the coordinated action of specific and non-specific defense machinery (Kushwaha et al. 2015a). Many mechanisms of defense against viruses have been studied such as hypersensitivity response (Hussain et al. 2005; Mubin et al. 2010), quantitative trait loci (Anbinder et al. 2009; Tomas et al. 2011), small interfering RNA (siRNA) (Ding 2010) and epigenetic control resulting to the RNA silencing (Raja et al. 2010).

Plant immune system has two interconnected lines of receptors - one commonly senses molecule outside the cell and other usually senses the molecule inside the cell. Receptors are linked with the specific preprogrammed defense response. Both the systems percept the invaders and trigger the anti pathogenic activities in infected as well as neighboring cells. Sometimes, defense activating signals are also spread to the other parts of plants or even neighboring plants. The first line of receptors is pattern recognition receptors (PRR) which recognize the evolutionary conserved pathogen-associated molecular patterns or microbial-associated molecular patterns (PAMPs or MAMPs). Recognition of PAMPs/MAAMPs triggers the basal resistance which includes the intracellular signaling, transcriptional reprogramming and biosynthesis of a complex output response that ultimately limits colonization of pathogens. Such type of defense is known as Pattern-Triggered Immunity (PTI) or PAMP-Triggered Immunity. PTI is non-specific in nature and defends the plants against all groups of pathogens. For the survival against PTI, pathogens inject small molecules in the plant cell to suppress PTI, known as effectors. In plant cell, effectors of pathogens are recognized by the product of R-gene which forms the second line of receptor. Recognition of specific effector by specific resistance protein activates strong anti-pathogenic response. Mechanism of resistance governed by the effector and resistance protein interaction is known as effector-triggered immunity (ETI). Result of ETI is hypersensitivity response (HR), a type of programmed cell death that leads to the restriction of pathogen at the site of infection and prevent its spread (Heath, 2000).

In plants resistance genes (R-genes) are important constituents of genetic resistance machinery (Flor, 1971, Dangl and Jones, 2001). Till now several R-genes are identified, cloned and studied. On the basic of either presence or absence of different domains, R-gene can be categorized into five classes (Baker et al. 1997). Out of these five classes of R-genes, the NBS-LRR (nucleotide-binding site and leucine-rich repeat domain containing protein) class is most abundant and well studied (Martin et al. 2003). The NBS-LRR class is subdivided into two subclasses, on the basic of presence of either coiled-coiled (CC) or Toll/interleukin-1 receptor-like (TIR) domain at the N-terminal they are named as either CC-NBS-LRR or TIR-NBS-LRR, respectively. In NBS-LRR, NBS domain is highly conserved, can bind and hydrolyze to the ATP and GTP (Tameling et al. 2002). The LRR domain is variable in between NBS-LRR proteins and involved in protein-protein interaction which is required for pathogen recognition (Kobe et al. 1995; Leister et al. 2000; Dangl et al. 2001).

Plants also have the adaptive immunity to fight against viruses, it includes pathogen-specific gene silencing activity through RNA interference mechanism. In plant cell, viruses trigger the RNA silencing machinery by producing double-stranded RNA (dsRNA). These dsRNA are recognized and cleaved into 21 to 24 nucleotide (nt) pieces by specific Dicer proteins (Hamilton et al. 2002). These small RNA, later on united into RNA induced silencing complex (RISC) which guide silencing of the target gene expression (Shimura and Pantaleo, 2011). Silencing of gene expression can be obtained either at transcriptional or at post transcriptional level. During post transcriptional gene silencing (PTGS) mRNA is either degraded or suppressed translationally. Transcriptional gene silencing (TGS) includes the modification of DNA or histone or both, which ultimately leads to the formation of heterochromatin or transcriptional repression. However, for the counter defense viruses are equipped with the suppressor proteins to suppress the host RNA silencing mechanism (Bivalkar-Mehla et al. 2011). For the survival viruses also apply effort to suppress the defense system of the plants (Singh et al. 2016). Begomoviruses encode different proteins which function as a suppressor of PTGS, TGS and other host defense machinery. For example, AC1, AC2, AC4, AV2 and βC1 of begomoviruses are known to suppress RNAi machinery (Vanithrani et al. 2004; Trinks et al. 2005; Rodriguez-Negrete et al. 2013). Thus the strategy of RNA silencing is utilized by both host and virus. Phenomenon of RNA silencing provides the innate immunity against viruses (Sharma et al.

2013). This strategy is being used by viruses to encounter the defense mechanism of plants by interrupting the molecular function and modulating the expression of gene (Ratcliff et al. 1997).

Plants are continuously exposed to the several pathogens in the field. Mixed infection of viruses is a common phenomenon in nature and mixed infection of geminiviruses may result in recombination, pseudorecombination or reassortment of viral genome leading to evolution of new viruses (Fondong et al. 2000; Pita et al. 2001; Rasheed et al. 2006; Chakraborty et al, 2008). However, genomic components retained gene arrangements typical of geminiviruses. Mixed viral infection to a plant might result in either synergistic or antagonistic interaction (Méndez-Lozano et al. 2003; Alves-Júnior et al. 2009). For plant viruses, synergistic interaction indicates the interaction of either two or more independent viruses in a single host, which is characterized by significant enhancement in symptom severity and accumulation of viral titre. Synergistic interaction of viruses has been reported between distinct viruses belonging to either same or different family/genera/species. Synergistic interaction between ssDNA viruses are known to occur, for example in between *Tomato leaf curl New Delhi virus* (ToLCNDV) and *Tomato leaf curl Gujarat virus* (ToLCGV) in tomato (Chakraborty et al. 2008); *African cassava mosaic virus* – [Cameroon] (ACMV[CM]) and *East African Cassava mosaic virus* (EACMV) in cassava and tobacco plants (Vanitharani et al. 2004); *Pepper huasteco yellow vein virus* (PHYVV) and *Pepper golden mosaic virus* (PepGMV) in chilli plants (Rentería-Canett et al. 2011); *Tomato rugose mosaic virus* (ToRMV) and *Tomato yellow spot virus* (ToYSV) in tomato (Alves-Júnior et al. 2009); *Beet curly top virus* (BCTV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in *Nicotiana benthamiana* (Caracuel et al. 2012)]. Synergistic interaction between RNA viruses like *Beet yellows virus* (BYV), *Beet western yellows virus* (BWYV), and *Beet chlorosis virus* (BChV) is also known (Wintermantel, 2005). Interactions between DNA and RNA viruses have also lead to synergism in plant (Mohamed, 2010).

Chilli plants have been extensively studied for identification of resistance against several pathogens. Numerous R-genes has been identified and studied from chilli plants (Wang et al. 2012). Although several R-genes have been identified against viruses but none of the R-gene has been characterized against the largest family of the plant viruses, *Geminiviridae*. Kushwaha et al. (2015a) have reported differential expression of a gene sharing homology with *NBS-LRR* gene in the resistant chilli cutivar Punjab Lal, expression of which increases in the presence of geminiviruses (Kushwaha et al. 2015a; b). For identification and functional characterization of

geminivirus responsive *NBS-LRR* gene further study is the need of the hour. Keeping in view of the information available about defense machinery of chilli plants against viruses and host-virus interaction, the following objectives were set up to study mechanism of natural resistance/tolerance in chilli plants:

- To identify ChiLCV- responsive NBS-LRR gene in chilli.
- To understand the mechanism of NBS-LRR mediated host response.
- To determine role of NBS-LRR during synergistic interaction between chilli infecting begomoviruses.

2.1 Chilli (*Capsicum* sp.)

Chilli is one of the most important spices cum vegetable crops of the family *Solanaceae* and belongs to genus *Capsicum*. Chillies are known from pre-historic times in Peru. They are believed to have originated in the tropical America. It is also said that chillies have originated in the Latin American regions of the New Mexico and Guatemala as a wild crop around 7500BC (Milind and Sushila, 2012), as per the remains of the pre-historic Peru. At present, chilli is cultivated worldwide except few temperate countries. India is the largest producer, consumer and exporter of chilli in the world and produces about 25% of word's total production fallowed by china. According to the Ministry of Agriculture & Farmers Welfare Government of India, during 2014-15, 170000 ha area was used for the chilli cultivation and 1983000 tonnes chilli was produced. The most important chilli growing states in India are Andhra Pradesh, Telangana, Karnataka, West Bengal and Madhya Pradesh which together constitute more than 75 per cent of the total production. Andhra Pradesh tops the list in dry chilli production followed by Telangana, Karnataka and West Bengal (Table 2.1).

Table 2.1 Total area harvested and the average production of 10 major chilli growing states in India

S N	State	Area (in '000 Ha)	Production (in '000 MT)
1	Andhra Pradesh	131.32	601.99
2	Telangana	78.94	279.78
3	Karnataka	89.56	111.55
4	West Bengal	63.60	100.00
5	Madhya Pradesh	54.41	93.57
6	Odisha	75.00	70.00
7	Gujarat	43.40	68.53
8	Maharashtra	99.50	45.60
9	Tamil Nadu	50.67	23.06
10	Punjab	10.60	17.70
	Total (all states)	**774.87**	**1492.14**

Chilli plant is an annual sub-herb and the fruits vary in shape, size, colour and degree of pungency (Fig. 2.1). The fruits are of diverse shapes and sizes depending upon the variety.

Fruits of chilli are known for sharp acidic flavor and are used for spices, vegetables, pickles, condiments and sauces. Chillies are rich sources of vitamins, especially vitamin A and C and are also packed with ions like potassium, magnesium and iron. Recently, vitamin-P has been identified in green chilli (Milind and Sushila 2012). In industries chilli is being used for flavor, colour, beverages and extraction of oleoresin and capsaicin. Chilli peppers are also used in medicine as a circulatory stimulant and analgesic (Fattori et al. 2016). Farmers of the African and Asian countries use chilli plants to save the valuable crop from elephants by growing few rows of them around the field. The pigment capsanthin provides red colour to chilli. *Capsicum* consists of approximately 20 – 27 species, five of which are domesticated: *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescen*. Phylogenetic relationship between species was investigated using biogeographical, morphological, chemosystematics, hybridization and genetic data. Fruits of *Capsicum* can vary tremendously in colour, shape, and size both between and within species. In India *C. annuum* is the most popular and cultivated variety. Chilli is a major spice crop of India and occupies about 26% of total spice cultivation (Fig. 2.2).

2.1.1 Chilli genome architect

Chilli genome has 12 pairs of chromosomes (2n = 24). Chilli genome of cultivars Zunla-1 and wild Chiltepin was sequenced recently using whole-genome shotgun approach (Qin et al. 2014). Chilli genome sequence has predicted 35,336 and 34,476 high-confidence protein-coding loci in the Zunla-1 and Chiltepin, respectively. Chilli genome also have 2,717,180 unique tag sequences. In the chilli genome 6,527 long noncoding RNAs and 5,581 siRNAs have been identified by Qin et al. (2014). Qin et al. (2014) have also discovered 176 miRNAs classified them into 64 families and also showed that compare to the other *Solanaceae* members and chilli plant have 141 miRNAs are conserved and 35 miRNAs are specific to it.

2.1.2 Taxonomic hierarchy of chilli

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Asteridae
Order	:	Solanales
Family	:	Solanaceae
Genus	:	Capsicum

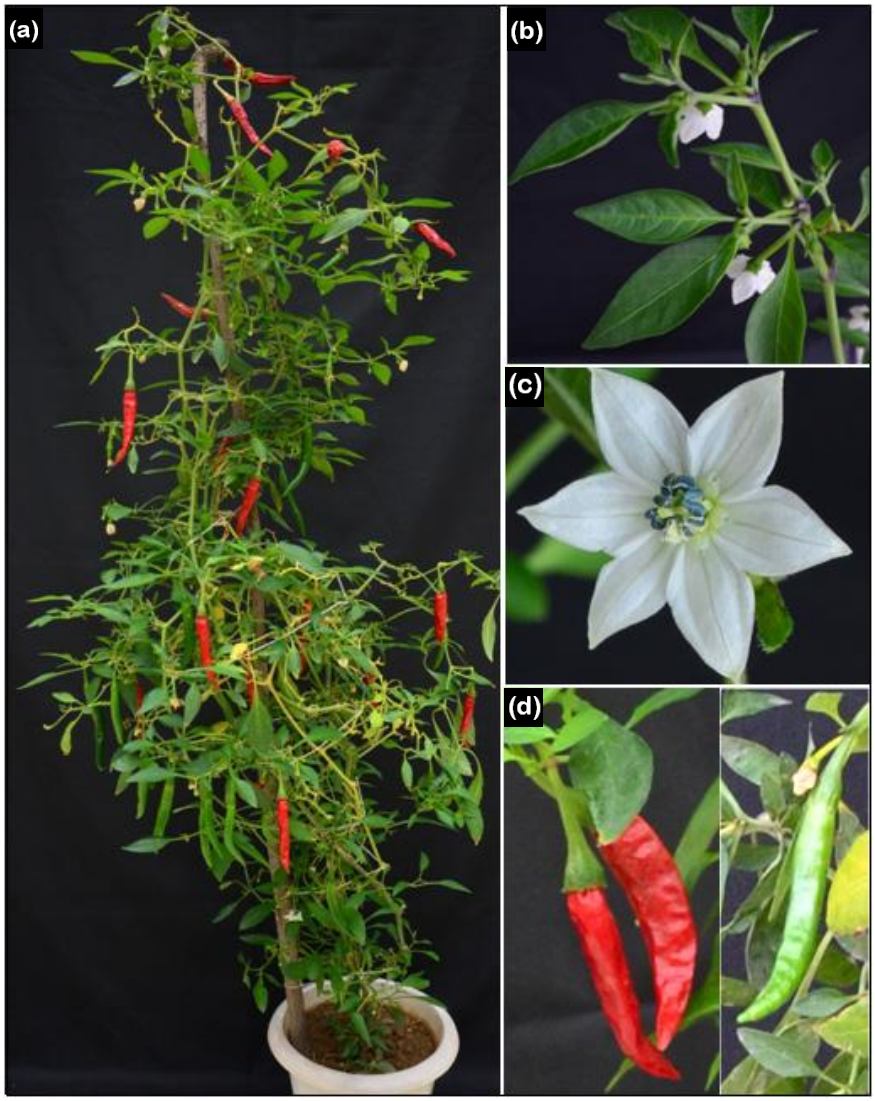


Fig. 2.1 Morphological description of chilli plant. **(a)** Phenotype of chilli plant bearing flowers and fruits. **(b)** Leaves. **(c)** Flower. **(d)** Fruits (ripened and unripened).

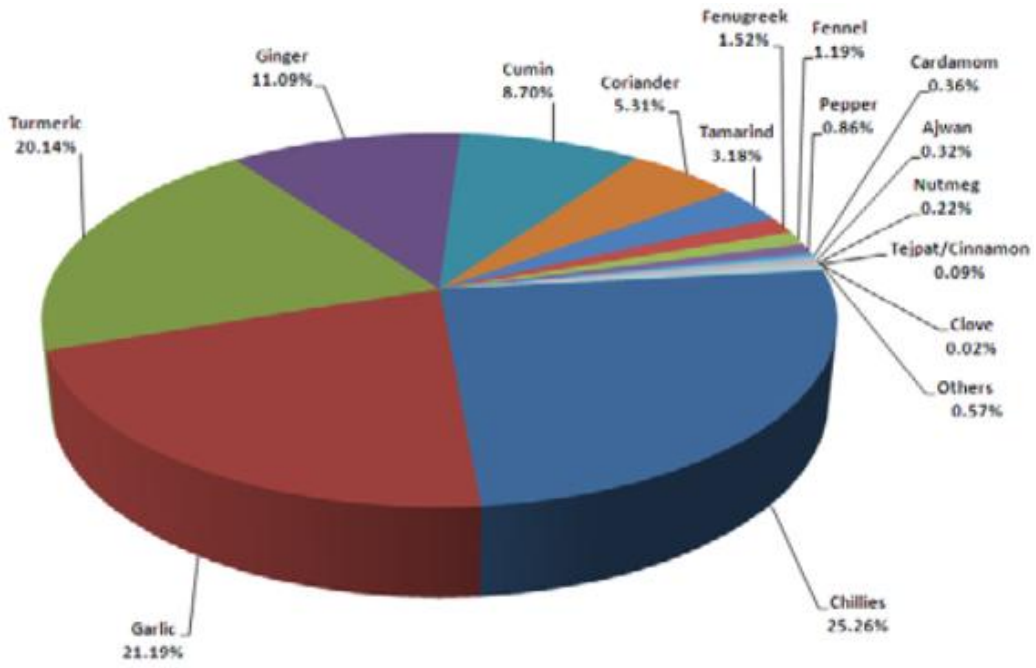


Fig. 2.2 Production share of different spice crops cultivated in India during 2013-14 season. Production of chilli was maximum followed by the garlic and turmeric. (http://nhb.gov.in/area-pro/NHB_Database_2015.pdf).

2.2 Chilli leaf curl disease (ChiLCD)

According to the Food and Agriculture Organization of the United State (FAOSTAT; 2014), India has produced the largest amount of chilli in the world still yield is less than many other countries. The reduced yield of chilli in our country is primarily due to the biotic stress created by pathogens and insects. Chilli is known as the suitable host for different bacterial, fungal and viral pathogens. Recently viruses have emerged as a far-reaching threat for cultivation of chilli (Varma and Malathi, 2003) which cause huge economic losses each year. Chilli leaf curl disease caused by chilli-begomoviruses is an emerging disease and has been reported from almost all chilli growing areas. *Chilli leaf curl virus* is one of the most devastating pathogens and the major constraint in chilli production. Various groups have reported the ChiLCD in Indian subcontinent (Gattani and Mathur 1951; Vasudeva, 1954; Mishra et al. 1963; Muniyappa and Veeresh, 1984). Association of begomovirus with ChiLCD has been confirmed recently (Khan et al. 2006; Senanayake et al. 2007; Chattopadhyay et al. 2008). The common symptoms of leaf curl disease are either upward or downward leaf curling, puckering, reduced pod size and in some cases additional symptoms like stunting, vein clearing and no fruit set also occur (Fig. 2.3).

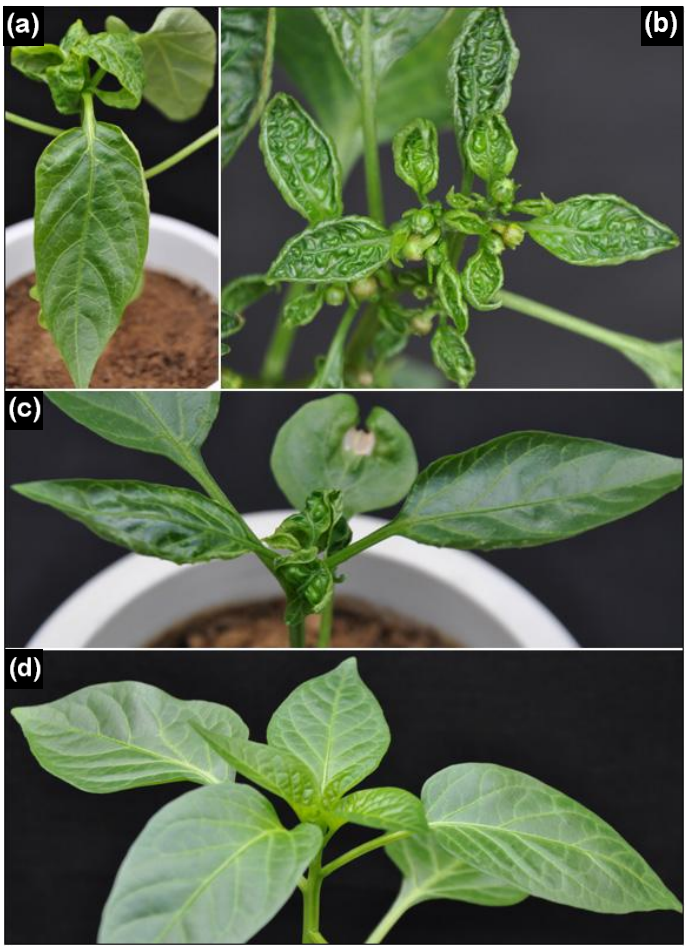


Fig. 2.3 Chilli plants showing typical leaf curl symptoms. (a), **(b)** and **(c)** Various type of leaf curl symptoms on chilli plants. **(d)** Healthy chilli plant.

2.3 The causal organism

Leaf curl disease of chilli is caused by *Chilli leaf curl virus* (ChiLCV) belongs to family *Geminiviridae* and transmitted by whiteflies (*Bemisia tabaci*). Till now a large number of chilli-infecting leaf curl viruses has been reported in India (Kumar et al. 2015).

2.4 Family *Geminiviridae*

Geminiviruses belong to the family *Geminiviridae*, characterized by twin icosahedral capsid and circular single-stranded DNA (ssDNA) genomes which replicate through double-stranded DNA (dsDNA) intermediates in the host cell (Hanley-Bowdoin et al. 2000). The term 'Geminivirus' was derived from the Latin word "geminus" meaning "twins" to describe the twinned particles and the name was proposed by Harrison et al. (1997). Size of

incomplete twined icosahedral particle is approximately 18 X 38 nm containing 22 pentameric capsomeres and each capsomere is about 8-nm in diameter (Francki et al. 1980; Zerbini et al. 2017). Geminiviruses are either monopartite or bipartite in nature i.e. they may contain one or two ssDNA genome of 2.5 to 3.0 kb. As twined capsid can capsidate only one ssDNA consequently, for a bipartite viruses, two twin particles are needed to obtain a full infection (Jeske, 2009).

2.4.1 Taxonomy

The members of family *Geminiviridae* show a lot of similarity, yet they have substantial diversity in terms of their genome structure, sequence, host range, vector transmission and tissue tropism. According to the genome structure, insect vector and host range, International Committee on Taxonomy of Viruses (ICTV) has subdivided family *Geminiviridae* is into nine genera: *Becurtovirus*, *Capulavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Mastrevirus*, *Topocuvirus*, *Turncurtovirus* and *Begomovirus* (Zerbini et al. 2017).

2.5 Genome organization of geminiviruses

2.5.1 *Becurtovirus*

Genus *Becurtovirus* has two recognized species, *Beet curly top Iran virus* and *Spinach curly top Arizona virus*. Contrast to other geminiviruses, members of the genus *Becurtovirus* possess unique nona-nucleotides (TAAGATTCC), genome organization with a spliced replication initiator protein and species demarcation threshold value for this genus has been fixed as 94% (Varsani et al. 2014). These viruses infect dicot plants such as *B. vulgaris*, *V. unguiculata*, *S. lycopersicum* and *P. vulgaris* in Iran (Yazdi et al. 2008; Soleimani et al. 2013; Gharouni Kardani et al. 2013) and *S. oleracea* in Arizona (Hernandez-Zepeda et al. 2013), Members of this genus infect dicot plants and are transmitted by leafhoppers (Zerbini et al. 2017).

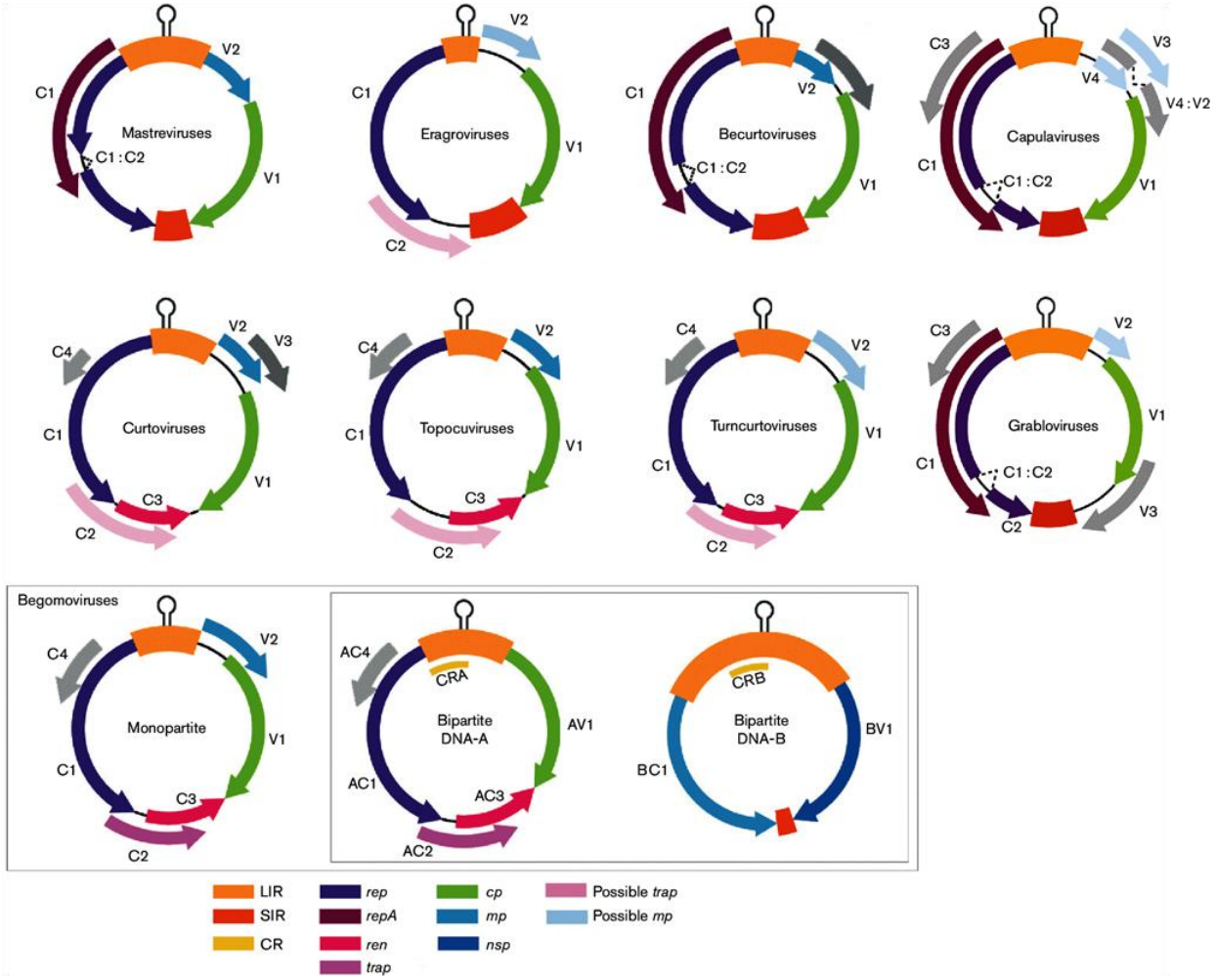


Fig. 2.4 Genome organization of different genera of the *Geminiviridae* family. The genome organization of different genera with type member; *Mastrevirus* (MSV-*Maize streak virus*), *Eragrovirus* (ECSV-*Eragrostis curvula streak virus*), *Becurtovirus* (BCTIV-*Beet curly top Iran virus*), *Curtovirus* (BCTV-*Beet curly top virus*), *Topocuvirus* (TPCTV-*Tomato pseudo curly top virus*), *Turncurtovirus* (TCTV-*Turnip curly top virus*), monopartite *Begomovirus* (TYLCV-*Tomato yellow leaf curl virus*) and bipartite BGMV-*Bean golden mosaic virus*. This figure is adapted from Zerbini et al. (2017).

2.5.2 *Capulavirus*

Capilavirus is a newly emerged genus with four species *Alfalfa leaf curl virus*, *Euphorbia caput-medusae latent virus*, *French bean severe leaf curl virus* and *Plantago lanceolata latent virus* (Varsani et al. 2014). *Capilavirus* have monopartite genome and being transmitted by aphid. The aphid species *Aphis craccivora* is the vector for *Alfalfa leaf curl virus*.

2.5.3 *Curtovirus*

Type species of this genus is *Beet curly top virus* which has genome of about 3 kb (Stanley et al. 1986). Curtoviruses infect dicotyledonous hosts (Varsani et al. 2014) and are transmitted by the beet leafhopper (*Circulifer tenellus*). Like *Mastrevirus*, they are also monopartite viruses but they share less sequence homology between them. *Curtovirus* have monopartite genome which includes 4 ORFs in the virion strand, 3 ORFs in the complementary strand with short intergenic region. Virion strand encodes a coat protein, ss/ds DNA regulator protein and a putative movement protein whereas complementary strand encodes a REP, Ren and pathogenicity associated protein and a protein involved in cell division (Hormuzdi and Bisaro, 1995).

2.5.4 *Eragrovirus*

Eragrovirus has one species *Eragrostis curvula streak virus* which infects monocot species (*E. curvula*) in South Africa (Varsani et al. 2009). These viruses encode a coat protein which resembles to the members of *Mastrevirus*, but the replication proteins are closely related to the viruses belonging to the genus, *Begomovirus*.

2.5.5 *Grablovirus*

Genus *Grablovirus*, has one species *Grapevine red blotch virus* and three-cornered alfalfa treehopper species *Spissistilus festinus* is the likely vector for this virus. *Grablovirus* genome has six ORFs and intergenic region. These viruses have the virion strand origin of replication nonanucleotide motif TAATATTAC and a unique genome arrangement (Varsani et al. 2017). Grabloviruses have the virion-strand origin of replication nonanucleotide motif TAATATTAC and a unique genome arrangement.

2.5.6 *Mastrevirus*

Mastreviruses can infect both monocot and dicot hosts its genome size 2.7-2.8 kb. Type species for this genus is *Maize streak virus*, a monopartite virus and is transmitted by leafhoppers. These viruses are mostly confined to the ‘old world’ infecting mainly monocots such as maize, sugarcane and wheat, whereas in Australia 5 distinct dicot-infecting mastreviruses are frequently found (Varsani et al. 2014). Recently in Middle East, India and

Africa, *Chickpea chlorotic dwarf virus*, a dicot-infecting mastrevirus has been reported (Kraberger et al. 2014). The genome of this viruses possess a nona-nucleotides (TAATATTAC) which is quite similar to the one noticed among other geminiviruses (Heyraud et al. 1993). Its genome encodes 2 ORFs each from virion and complementary strand which is separated by long intergenic region (LIR) and short intergenic region (SIR) (Kammann et al. 1991). LIR and SIR contain the origin of replication for the synthesis of virion and complementary strand, respectively. Virion strand encoded proteins are necessary for viral movement and encapsidation, whereas replication associated proteins are encoded in the complementary strand. Unique to this genus, these viruses are known to regulate their own gene expression through a post-transcriptional splicing event within monocots (Rojas et al. 2005). Recently, an unusual combination of mastrevirus-betasatellite-alphasatellite complex has been identified from wheat in India (Kumar et al. 2014).

2.5.7 *Topocuvirus*

Topocuviruses have monopartite genome of size ~ 3 kb in size. Type member of this genus is *Tomato pseudo curly top virus*. This virus encodes 2 ORFs in the virion strand and 4 ORFs in the complementary strand. It is found in the 'New World' and transmitted to dicot plants by the vector, treehoppers (*Micrutalis malleifera*). Based on the genome organization, this virus seems to a recombinant between *Mastrevirus* and *Begomovirus* (Briddon et al. 1996).

2.5.8 *Turncurtovirus*

Only member in this genus includes *Turnip curly top virus* reported either from *B. rapa* or *R. sativus* (Briddon et al. 2010). This phylogenetically distinct member is most closely resembles to the members of genus, *Curtovirus*. Its genome encodes 6 rather than 7 proteins. It contains nona-nucelotides (TAATATTAC) similar to *Mastrevirus, Begomovirus, Curtovirus* and *Topocuvirus*. For *Turncurtovirus,* the strain demarcation threshold of 95% has recently been assigned (Varsani et al. 2014).

2.5.9 *Begomovirus*

The largest genus of family *Geminiviridae* is *Begomovirus* with type species *Bean golden mosaic virus* (BGMV). Begomoviruses are further classified as monopartite (contain only

DNA-A like molecule) or bipartite (with DNA-A and DNA-B molecules). The insect vector, whiteflies (*Bemisia tabaci* Genn.) are reported to transmit these viruses and they also infect mostly dicot-hosts (Varma and Malathi, 2003; Brown et al. 2012). Based on genome organization and phylogenetic segregation, these begomoviruses are divided into two regions: 'Old world' that includes Africa, Asia, Australia and Europe; 'New world' that includes America, Brazil and Mexico (Rybicki, 1994; Nawaz-ul-Rehman and Fauquet, 2009). However, the probable centre of origin for these devastating pathogens is found to be around South East Asia (Nawaz-ul-Rehman and Fauquet, 2009). DNA-A component contains 6 ORFs, 2 ORFs in the virion strand and 4 in the complementary strand. Virion strand encodes coat protein (AV1) and pre-coat protein (AV2) which helps in virion encapsidation. Complementary strand encoded ORFs include replication associated protein (C1; Rep), transcription activator protein (C2; TrAP), replication enhancer protein (C3; REn) and a poorly characterized protein (C4) (Brown et al. 2012). DNA-B component found only in bipartite viruses encodes a movement protein (BC1; MP) and a nuclear shuttle protein (BV1; NSP) which helps in intra- as well as inter-cellular viral movement. DNA-A component of bipartite begomoviruses from the'New world' is found to lack AV2 ORF and hence, depends entirely on DNA-B encoded proteins for movement (Rybicki, 1994; Varsani et al. 2014). The virion- and complementary-strand transcription units in DNA-A and DNA-B are separated by a highly conserved non-coding region named common region (CR) (Lazarowitz and Shepherd, 1992). CR contains a nona-nucleotide region (TAATATTAC) forming stem loop structure which act as a cleavage site for Rep to initiate viral replication (Laufs et al. 1995). Virus-specific conserved region (iterons) called Rep-binding sites are located upstream to these stem loop structure (Fontes et al. 1994a, b). Monopartite begomoviruses are often found to be in association with satellite molecules namely, betasatellites and alphasatellites. Both these satellites encode a single ORF in the complementary and virion strand, respectively (Nawaz-ul-Rehman and Fauquet, 2009).

2.6 Replication in Geminiviruses

Geminivirus DNA replicates through rolling-circle mechanism, as observed in several prokaryotic viruses. Following entry into nucleus, the circular ssDNA is converted into circular dsDNA which is then packaged into nucleosomes. Viral minichromosomes remain extrachromosomal and serve as templates for transcription, replication and finally the production of progeny ssDNA that is encapsidated by coat protein again. The replication

processes have attracted most of the geminivirologists during the past two decades and have been extensively reviewed (Hanley-Bowdoin et al. 1999; Gutierrez, 2000; Gutierrez et al. 2004; Hanley-Bowdoin et al. 2004; Jeske, 2007). The strategy of replication is given in the Figure 2.5. The rolling circle replication (RCR) of geminiviruses is divided into two phases (Gutierrez, 2000): 1. Conversion of viral ssDNA into dsDNA forms on entering the nucleus of the initially infected cells. This step of synthesis of viral minus strand is carried out by cellular enzymes. 2. Rolling circle phase to replicate viral ssDNA on dsDNA templates. This step requires the participation of Rep protein. Rep is the only viral protein indispensible for RCR, as it is required for initiating DNA replication. Laufs et al. (1995) described in detail the role of Rep in initiation and termination of RCR of geminiviruses. Subsequently, an additional model of replication of geminiviruses and their satellites has been proposed (Jeske et al. 2001; Preiss and Jeske, 2003; Alberter et al. 2005). This model, recombination-dependent replication (RDR), was based on analyses of replication intermediates of AbMV (*Abutilon mosaic virus*), TYLCV (*Tomato yellow leaf curl virus*), BCTV (*Beet curly top virus*), TGMV (*Tomato golden mosaic virus*), ACMV (*African cassava mosaic virus*), ToLCV and one satellite molecule, DNA-β, using two-dimensional gel electrophoresis and electron microscopy. Apart from the previously identified RCR intermediates (Saunders et al. 1991), a range of intermediates suggested an additional RDR pathway. This is analogous to the pathway of T4 bacteriophage (Kreuzer, 2000) that has also been named the “join-copy” pathway (Mosig, 1998), “break-induced replication” (George and Kreuzer, 1996) and “bubble-migration synthesis” (Formosa and Alberts, 1986). The RDR model has three steps (Kreuzer, 2000; Mosig et al. 2001) as follows: 1. Processing of the broken double-stranded DNA to produce the 3′ end single stranded DNA required for DNA strand invasion. 2. Invasion of a homologous duplex by 3′ end single-stranded DNA to form a structure known as the `displacement loop' (D-loop or bubble loop). DNA strand invasion by the 3' end of ssDNA allows it to serve as a potential primer for DNA replication. 3. DNA heteroduplex extension (branch migration); at this step, the protein-directed branch migration occurs at the rear of the loop as DNA polymerase extends the leading-strand product at the front of the loop. Because both reactions occur at a similar rate, the size of the loop is roughly unchanged. This type of RDR does not need a topoisomerase, even when the circular DNA templates are supercoiled, and the two parental strands do not need to separate from each other. It also does not require participation of Rep in terms of its cognate virus recognition and nicking of ssDNA at the nonanucleotide sequence for initiation of replication. This

possibility is also supported by a recent study (Lin et al. 2003) in which mutants of ToLCV and its sat-DNA molecule that were impaired in their ability to bind Rep *in vitro*, were found infectious to tomato.

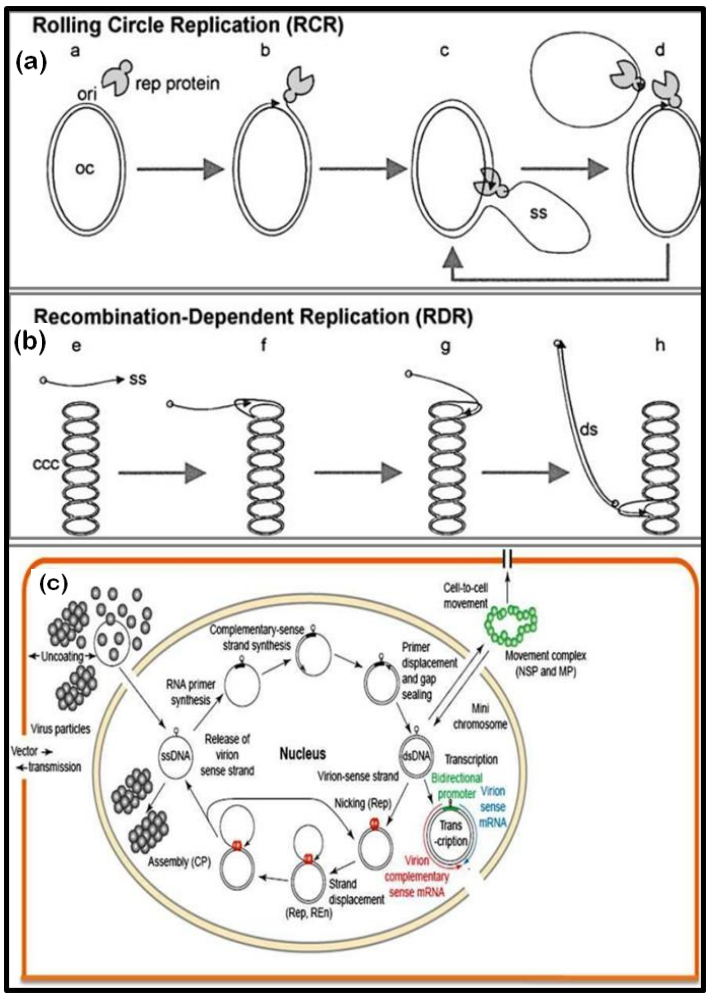


Fig. 2.5 Models for geminivirus DNA replication. (a) Model for rolling circle replication (RCR). AC1 binds to the circular DNA and makes a nick the in DNA, replace the DNA strand with newly synthesized strand and finally makes a new nick in ssDNA to come out with new strand. **(b)** Model for recombination-dependent replication (RDR) (Jeske et al. 2001). **(c)** Different stages of RCR used by geminiviruses for the replication (adapted from Vanitharani et al. 2005).

2.7 Geminivirus evolution and diversity

Viruses are small in size and no fossil structure is available. Therefore, it is very difficult to ascertain about the ancestors of geminiviruses. Geminiviruses replicate through rolling circle replication similar to the animal infecting pathogen circoviruses and anelloviruses, bacterial pathogen microviruses and to plasmids replicating in archaea, bacteria and algae, indicates that geminiviruses might have originated from these plasmid molecules or they share a

common ancestor of origin with these plasmids. However, it is presumed that geminiviruses were evolved as episomal DNA replicons of primeval prokaryotic organisms that have adapted to primitive eukaryotic progenitors of modern plants with the passage of time (Rojas et al. 2005). This hypothesis is supported by several evidences like, the conserved features of the replication initiator proteins of contemporary eukaryotic and prokaryotic DNA replicons (Olivas et al. 2002; Ilyina and Koonin, 1992), polycistronic mRNAs and ability of diverse geminivirus types to replicate in *Agrobacterium tumefaciens* (Frischmuth et al. 1990; Selth et al. 2002). Nawaz-ul-Rehman et al. (2009) reviewed the possibility of evolution of geminiviruses and their associated satellites DNA by taking all the information into the consideration. Rep protein is a very common throughout the evolution process such as the Rep protein of the *Wheat dwarf virus* (WDV a mastrevirus) and a Rep-like sequence encoded by a red algae (Porphyra pulchera) plasmid have very significant homology (>31% over 55 amino acids). They have assumed that plasmids of red algae and other primitive organisms, managed to acquire new genes, allowing this molecule to become free from its host and consequently capable of infecting plants, perhaps first monocot, as a pre-mastrevirus. These molecules have co-evolved with their hosts, at that duration these DNA replicons acquired new properties through recombination with host genome or other replicons (Rojas et al. 2005). Phylogenetic studies carried out by different researchers (Padidam et al. 1995; Rybicki, 1994) have also proposed that the primitive geminiviruses have single component, infects monocotyledonous hosts and was transmitted by leafhopper. Subsequently, continuous evolution facilitated the transmission through whiteflies and infection to more advanced dicotyledonous plants resulting in the ancestral old world begomoviruses. The acquisition of an additional second component seems to have occurred later on the evolutionary scale, although this might have happened before the separation of the continents. This has been assumed because bipartite begomoviruses are found in both old and new world (Rojas et al. 2005). Adaptation of the monopartite begomoviruses to acquire satellite DNA molecules like DNA-β may possibly have opened new possibilities for viruses to adapt and colonize on new hosts (Briddon et al. 2003; Rojas et al. 2005). Harrison and Robinson (1999) have proposed that genus *Curtovirus* might have originated from ancient recombination events between a *Mastrevirus* and a *Begomovirus.* Another recombination event possibly between ancient curtovirus and another virus which is not related with the modern geminiviruses resulted to generate genus *Topocuvirus* (Briddon et al. 1996). Recombination is the fundamental source of geminivirus evolution, which may be facilitated by the

recombination-dependent replication strategy (Preiss and Jeske, 2003) found in naturally occurring mixed infections (Harrison et al. 1997; Pita et al. 2001). Recently, role of compound microsatellites have also been established with recombination of geminiviruses (George et al. 2015).

2.8 Pseudo-Recombination and Synergism

Mixed infection of geminiviruses may result in either recombination or re-assortment of viral components leading to emergence of new virus species (Fondong et al. 2000; Pita et al. 2001; Rasheed et al. 2006; Chakraborty et al. 2003, 2008). However, genomic components retained gene arrangements typical of geminiviruses (Reddy et. al. 2012). Mixed viral infection might result in either synergistic or antagonistic interaction (Méndez-Lozano et al. 2003; Chakraborty et al. 2008; Alves-Júnior et al. 2009). These processes have biological and epidemiological implications which may cause more severe disease, sudden breakdown of host resistance and development of epidemics. Genome component exchange ability of begomoviruses was first shown experimentally by Stanley et al. (1985). Now progress has been reached to elucidate the factors controlling the potential for pseudorecombination between two viruses. Ramos et al. (2003) has reported that *Tomato mottle Taino virus* (ToMoTV) have the ability to transcomplement with *Potato yellow mosaic virus* (PYMV), but not with *Tomato mottle virus* (ToMoV), although the latter having a higher Rep and REn similarity with ToMoTV. Another example of compatibility indicates that a single DNA-A component of some geminiviruses can replicate with several different DNA-Bs and cause infection when co-inoculated with each of these (Karthikeyan et al. 2004). Such type of genetic re-assortments directly designates the potential for rapid evolutionary change. Two different virus species may or may not transcomplement the genomic components of each other which might depend on the IR and Rep compatibility. The previous assumption is well demonstrated in ToLCNDV and ToLCGV, where IR of DNA-A and DNA-B of ToLCNDV and ToLCGV respectively shares 83% sequence identity and they together produce more virulent severe disease symptoms than their naturally occurring partners. Co-infection of these two viruses also leads to asymmetric synergism where ToLCGV favours more replication of ToLCNDV but not *vice-versa* (Chakraborty et al. 2008). Other than compatibility between Rep and IR of two partners of a pseudorecombination set, some other factors are also found to be involved in determining pathogenesis and pseudo-recombinants. These factors may be either mutual efficacy of interaction in protein products of partners or

their interaction with host factors. These reasons may also be the cause of synergism between viruses. Synergism is the interaction between two or more unrelated viruses in a single host, which is characterized by significant enhancement in symptoms and accumulation of one or more viruses (Syller et al. 2012; Zhou et al. 2017; Xia et al. 2016). This in turn increases the disease severity in susceptible plants and may break natural resistance of a host plant. Many diseases of crops are the result of synergistic interaction of viruses (Untiveros et al. 2007; Scheets, 1998). Synergism of viruses can alter host range, tissue tropism (Ryabov et al. 2001), host range (García-Cano et al. 2006) and transmission rate (Li et al. 2014) along with viral DNA accumulaton (Scheets, 1998; Xia et al. 2016). Synergistic interaction of viruses has been reported between distinct viruses belonging to either same or different family/genera/species. Molecular determinants of synergistic interaction have also been studied. The ORF C2 of *Beet curly top virus* (*Curtovirus*) promotes a suitable cell environment to enhance the replication of geminiviruses in Rep assisted manner (Caracuel et al. 2012). Potyvirus encoded P1, helper component proteinase (HC-Pro), and a fraction of P3 enhances the pathogenicity and replication of cucumber mosaic virus and tobacco mosaic virus by suppression of post transcription gene silencing (PTGS) (Pruss et al 1997). Cucumber mosaic virus 2b protein blocks systemic signal of PTGS and supports for accumulation of Potato virus Y in the shoot apex (Ryang et al. 2004; Guo and Ding, 2002). Co-infection of polerovirus *Brassica yellows virus* (BrYV) and an umbravirus *Pea enation mosaic virus 2* (PEMV 2) enhances the titer of both the viruses by increasing the virus derived siRNA and breaking the phloem limitation of BrYV in *N. benthamiana* plant (Zhou et al. 2017). Infection of *Maize chlorotic mottle virus* (MCMV) and *Sugarcane mosaic virus* (SCMV) increases the accumulaton of MCMV by increasing the siRNA derived by both the viruses (Xia et al. 2016).

2.9 Plant immunity

Plants are continuously attacked by several pathogens, however, only a few among those pathogens are able to infect the plants whereas remaining are blocked by the plant immunity. Plant immunity is being considered as the inherent or induced potential of plants to fight or ward off biological attack by pathogens. Molecules released by attacking pathogens are recognised by plant cell surface receptors, and trigger specific signalling cascades that help to defend the plants against attack. Immunity provides the resistance/tolerant state to plants against pathogens. Disease resistance guards the plants by two methods, by pre-

formed structures and chemicals and by infection induced responses of plant immune system. Plant immune system has two interconnected line of defense; first line of defense is pattern triggered immunity and second line of defense is effector triggered immunity.

2.9.1 Pattern triggered immunity

First line of defense system perceives pathogen through recognition of the pathogen-associated molecular patterns (PAMPs) by the pattern-recognition receptors (PRRs). Often PAMPs are derived from non-pathogenic microbes and in such cases, are named as microbe-associated molecular patterns (MAMPs) (Ausubel, 2005). PAMPs/MAMPs recognition by PRRs induces the PAMP-triggered immunity or pathogen-triggered immunity or pattern-triggered immunity (PTI) or MAMP-triggered immunity. PTI is a complex set of responses intended for fighting against the attack of pathogen. The plant degradation products formed as a consequence of pathogen invasion are known as damage-associated molecular patterns (DAMPs). DAMPs include the endogenous peptides, constitutively present or newly synthesized compound which are released by the plants following pathogen attacks (Boller and Felix, 2009). DAMPs recognition also induces immune response to the PTI response (Yamaguchi and Huffaker, 2011).

2.9.1.1 Pattern-recognition receptors

Commonly PRRs are plasma membrane bound receptor like kineses (RLKs) or the proteins having extracellular domains allowing MAMP/DAMP recognition (Bohm et al. 2014; Tang et al. 2017). Example of some RPP/PAMP pairs are; Arabidopsis flagellin-sensitive 2 (FLS2) protein which recognizes the 22 amino acid long stretch of N-terminus of flagellin protein from *Pseudomonas aeruginosa* (Gomez-Gomez and Boller, 2000); EF-Tu receptor (EFR) of Arabidopsis, recognizes 18 amino acid long segment of elongation factor Tu (EF-Tu) called elf18 from *Escherichia coli* (Zipfel et al. 2006). XPS1 is a LRK recognizes xup25 in *Arabidopsis* (Mott et al. 2016). RLK protein, PEPR1 which is a DAMP identifies AtPep1 of *Arabidopsis* (Yamaguchi et al. 2006).

2.9.1.2 Downstream signaling of pattern-recognition receptors

Pathogen reorganization by PRRs follows a number of defense strategies which includes production of reactive oxygen species (ROS) which have toxic effects on pathogens (O'Brien et al. 2012); production and secretion of antimicrobial compounds such as phytoalexins (camalexin), defense related proteins/peptides (PR proteins) (Cowan, 1999; van Loon et al. 2006; Ahuja et al. 2012; Bednarek, 2012); closure of stomata to restrict the entry of bacteria (Melotto et al. 2008; Sawinski et al. 2013); limiting the transfer of nutrients from cytosol to the apoplast for bacterial growth check (Chen et al. 2010; Wang et al. 2012).

One of the earliest response after MAMP detection is the influx of extracellular Ca^{2+} into the cytosol (Ca^{2+} burst) (Jeworutzki et al. 2010; Ranf et al. 2011; Nomura et al. 2012). Ca^{2+} burst is positively regulated by BIK1 and BIK1 family proteins (Li et al. 2014). Ca^{2+} influx influences the opening of other transporters of membrane such as influx of H^{+}, efflux of K^{+}, Cl^{-} and $NO3^{-}$ which results into the extracellular alkalinization and ultimately depolarization of plasma membrane which effect the MAPK cascade (Jeworutzki et al. 2010).

PAMP recognition also triggers the extracellular ROS production, referred as ROS burst (Nuhse et al. 2007; Ranf et al. 2011; Kimura et al. 2017). ROS burst is mediated by respiratory burst oxidase homolog D (RBOHD) which is a plasma membrane-localized NADPH oxidase (Nuhse et al. 2007; Zhang et al. 2007; Ranf et al. 2011). RBOHD produces superoxide (O^{2-}) which is membrane–impermeable and converts to hydrogen peroxide (H_2O_2) by superoxide dismutases. H_2O_2 is permeable to the membrane, enters into cytosol and different membrane organelles and elevate the cytosolic Ca^{2+} level (Pei et al. 2000; Rentel and Knight, 2004). Increased Ca^{2+} level leads to the second peak or prolonged plateau of Ca^{2+} (Ranf et al. 2011).

Other than Ca^{2+} and ROS some small molecules such as Nitric oxide (NO) and its derivatives, collectively referred to as reactive nitrogen species were also involved at different steps of signal transduction. These steps includes the regulation of non-expresser of PR genes 1 (NPR1) (a master regulator of defense gene expression) oligomeric state by cysteine S-nitrosylation, or via the inhibition of RBOHD by cysteine S-nitrosylation (Tada et al. 2008; Yun et al. 2011). Pathogen-induced Ca2+ influx into the cytosol activates calmodulin (CaM) and/or CaM-like proteins, which then lead to downstream NO synthesis induction (Ma et al. 2008). The origin of NO biosynthesis remains, however, largely unclear (Gupta et al. 2011). Some lipids, such as phosphatidic acid (PA) and ceramides, were proposed to function as signaling molecules upon pathogen infection (Okazaki and Saito, 2014).

2.9.2 Effector triggered immunity

If a pathogen is able to evade the MTI, second line of defense starts working against it, known as ‘‘effector-triggered immunity’’ (ETI) (Jones and Dangl 2006). ETI employs specific immune receptors known as resistance (R) proteins which recognize pathogen encoded specific elicitor known as effectors. To develop pathogenesis, pathogens have to evade both PTI and ETI. Although ETI is totally dependent on the germ-line encoded molecules still it provides remarkable resistance against pathogen. ETI provide resistance through induction of hypersensitivity cell death response (HR). HR induction localizes the pathogen at the site of infection and ultimately prevents its spread.

2.9.2.1 Resistance protein (Effector-recognition receptor)

Over the past decade, a large number of R-genes/R-proteins are identified, cloned and studied. On the basic of presence of different domains, R-gene can be categorized into five classes (Baker et al. 1997; Liu et al. 2007) (Fig. 2.6). Out of the five classes of R-genes, NBS-LRR (nucleotide-binding site and leucine-rich repeat domain containing protein) class is the most numerous and most studied class (Martin et al. 2003). Plants have large number of NBS-LRR genes which are subdivided into two classes TIR-NBS-LRR and CC-NBS-LRR. TIR-NBS-LRR proteins have a motif homologous to the cytoplasmic domains of the Drosophila Toll protein and the mammalian interleukin-1 receptor (TIR) at the N-terminal. A coiled-coil (CC) domain is present at the N-terminal of CC-NBS-LRR.

Both the subfamilies can be identified (95%) by the presence of conserved residue, D (Aspartate) or W (Tryptophan), at the end of highly conserved kinase-2 motif in the NBS domain. Presence of D residue corresponds to the TIR-NBS-LRR subfamily whereas the W-residue corresponds to the non-TIR-NBS-LRR subfamily of R genes (Wan wt al. 2012).

Till now eight conserved motifs in NBS domains have been identified in both non-TIR- and TIR-NBS-LRR R-genes. Conserved motifs of NBSdomain include P-loop, kinase-2, kinase-3a, GLPL, RNBS-A-TIR, RNBS-D-TIR, RNBS-A-non-TIR and RNBS-D-non-TIR (Yue et al. 2012) (Fig 2.7). The first four conserved motifs are common in both, TIR and non-TIR-NBS-LRR subfamilies. RNBS-A-TIR and RNBS-D-TIR motifs are specific to the TIR-NBS-LRR subfamily. Remaining two motifs, RNBS-A-non-TIR and RNBS-D-non-TIR, belong to the non-TIR-NBS-LRR subfamily (Fig 2.7).

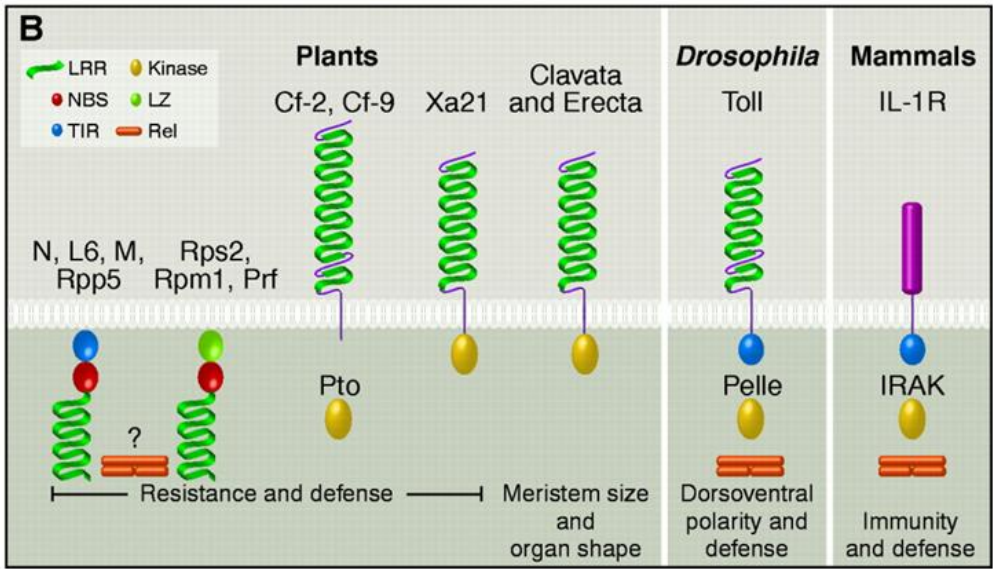


Fig.2.6 Genes belong to the defense and developmental pathway in plant, insect and mammals sharing structural domains. Structural features shared by the different resistance proteins are a leucine-rich repeat (LRR) motif or a serine-threonine kinase domain. Differnet domains of defense and developmental proteins are, kinase, serine-threonine kinase; LZ, leucine zipper; Rel, Rel-related transcription factors (Dorsal and Dif of *Drosophila* and NF-kB of mammals) and inhibitors (Cactus of *Drosophila* and IkB of mammals). This figure is adapted from (Baker et al. 1997).

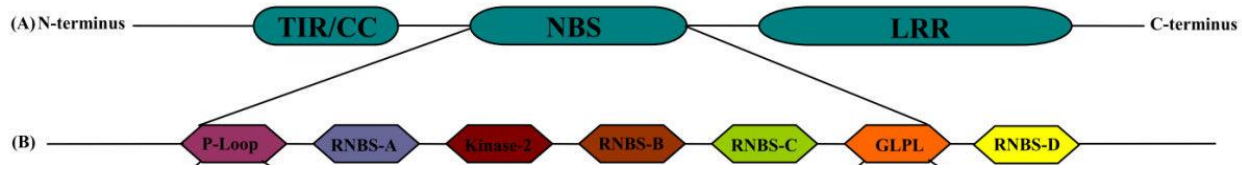


Fig. 2.7 Schematic representation of NBS-LRR class of R- gene. (a) Typical domains of NBS-LRR class of R-Proteins. **(b)** All conserved motifs of the NBS domain within NBS-LRR. This figure is adapted from (Wan et al. 2012).

The CC domain has loosely conserved structure with minimum three variants; CC, CC_{EDVID} or CC_R. The CC_{EDVID} variant is named because of highly conserved ‘‘EDVID’’ motif. This motif is absent in the other two variants. The CC_R class is named after their founding members the Arabidopsis RPW8 proteins.

2.10 Function of different domains of NBS-LRR protein

2.10.1 Leucine rich repeat (LRR) domain

LRR domains are located at the C-terminal end of NBS-LRR proteins and consist of tandem LRRs. LRR domain has barrel-like structures with a parallel β-sheet lining the inner concave surface and α-helical structures comprising much of the rest of the domain (Kobe and Deisenhofer, 1994). LRR domain is typically 10-40+ repeats of a motif of ≈24 amino acids which is highly variable. The core signature of LRR repeat motif is LxxLxxLxLxxLxx (N/C/T) x (x) LxxIPxx (L = leucine or other aliphatic residues, and x = any residue; reviewed in Jones and Jones, 1997). LRR has the β-sheet portion which works as the ligand-binding interface so LRR domain is involved in protein-protein interaction. LRR domain is involved in detection of avr protein of pathogen (Ellis et al. 1999; Luck et al. 2000). C-terminal of the LRR domain of Rx protein respond for the *Potato Virus X* coat protein (Rairdan and Moffett, 2006).

LRR domain is also involved in the regulation of activation of NBS-LRR protein by binding with CC and NBS domains (Rairdan and Moffett, 2006).

2.10.2 Nucleotide binding site (NBS) domain

The NBS domain (also called the NB, NB-ARC, Nod or NACHT domain) contains blocks of sequence that are conserved in both plant and animal proteins (van der Biezen et al. 1998; Takken et al. 2006). It includes the canonical nucleotide-binding kinase 1a or P-loop and kinase 2 motifs (also called Walker's A and B boxes) and the kinase 3a motif, as well as many blocks of conserved motifs (RNBS-A, RNBS-C, GLPL, RNBS-D and MHD) (van der Biezen et al. 1998; Meyers et al. 1999; Meyers et al. 2003). ATP binding to the NBS domain initiates a conformational change in plant NBS-LRR proteins, resulting in its activation. This phenomenon is similar to the mode of activation of small G proteins (Sprang, 1997).

2.10.3 N-terminal domain

NBS-LRR protein has CC or TIR domain at the N-terminal. Many reports have shown that CC domain is involved in dimerization along with NBS domain (Rairdan et al. 2008). TIR domain of NBS-LRR protein is also involved in the oligomerization (Xu et al. 2000). TIR domain is also involved in the pathogen recognition (Caplan et al. 2008). Dimerization/Oligomerization of NBS-LRR protein initiates downstream signaling for the activation of resistance response (Xu et al. 2000; Bernoux et al. 2011; Bonardi et al. 2012).

2.11 Localization of NBS-LRR

Different plant NBS-LRR proteins are localized inside different organelle present in the cell and this pattern of subc-ellular localization is important for their function (Qi and Innes, 2013). Several R proteins have a nucleo-cytoplasmic distribution and are relocated to the nucleus to regulate defense gene expression upon infection by pathogens (Wirthmueller et al. 2007; Caplan et al. 2008). In the presence of incompatible pathogens, barley (*Hordeum vulgare*) MLA protein triggers resistance response in the nucleus (Shen et al. 2007), while the cytoplasmic MLA induces production of cell death signals (Bai et al. 2012). However, the potato (*Solanum tuberosum*) Rx1 protein recognizes viral coat protein and elicits resistance in the cytoplasm, but the nuclear Rx1 balances this activity in different conditions (Slootweg et al. 2010; Tameling et al. 2010). By contrast, some R proteins are persistently localized at the endomembrane through their N-terminal motifs (Takemoto et al. 2012), although others are relocated from the cytoplasm to the endosomal compartments, for example, the potato R3a upon perception of the recognized effector AVR3aKI (Engelhardt et al. 2012). A subset of NBS-LRR proteins, such as Arabidopsis RPM1 and RPS5, are localized to the plasma membrane (PM). RPM1 is a CC-NBS-LRR which remains on plasma membrane before and after its activation (Boyes et al. 1998; Gao et al. 2011). The N-terminal acylation of the RPS5 CC domain and the RPS59s guardee PBS1 (which also contains an N-terminal S-acylation signal) are required for their PM localization (Ade et al., 2007; Qi et al., 2012, 2014). N-protein recognizes TMV coat protein in cytoplasm but nuclear function is crucial for the activation of downstream signaling (Slootweg et al. 2010). RRS1-R of *Arabidopsis* contains WRKY domain at the C-terminal, localizes in the nucleus (Deslandes et al. 2003).

2.12 Pathogen recognition by NBS-LRR

R-proteins are receptors for the recognition of pathogen effector protein. Considering many R-avr combinations, two distinct mechanisms of molecular recognition of pathogen effectors by NB-LRRs and a unique mechanism for non-NB-LRRs are known (Fig 2.8).

2.12.1 Direct recognition of the effector protein

The simplest way of pathogen recognition by plant NBS-LRR is, direct interaction with the pathogen avr protein. The relationship between corresponding host receptors and pathogen

effectors is defined by the "gene-for-gene" model (Zipfel, 2008) (Fig 2.8). It involves the direct effect of a specifically recognized effector on the receptor. Although it was possible to identify the receptors involved in such interactions but this phenomenon is very rare in the nature. Examples of direct interaction are; the interaction between Pita (CC-NBS-LRR of rice) and AVR-Pita from the fungus *Magnaporthe grisea* (Jia et al. 2000); RRS1-R from *Arabidopsis* with the bacterial wilt PopP2 effector (Deslandes et al. 2003) and interaction of L567 immune receptors (TIR-NBS-LRR) of flax with AvrL567 effectors flax rust fungus (Dodds et al. 2006).

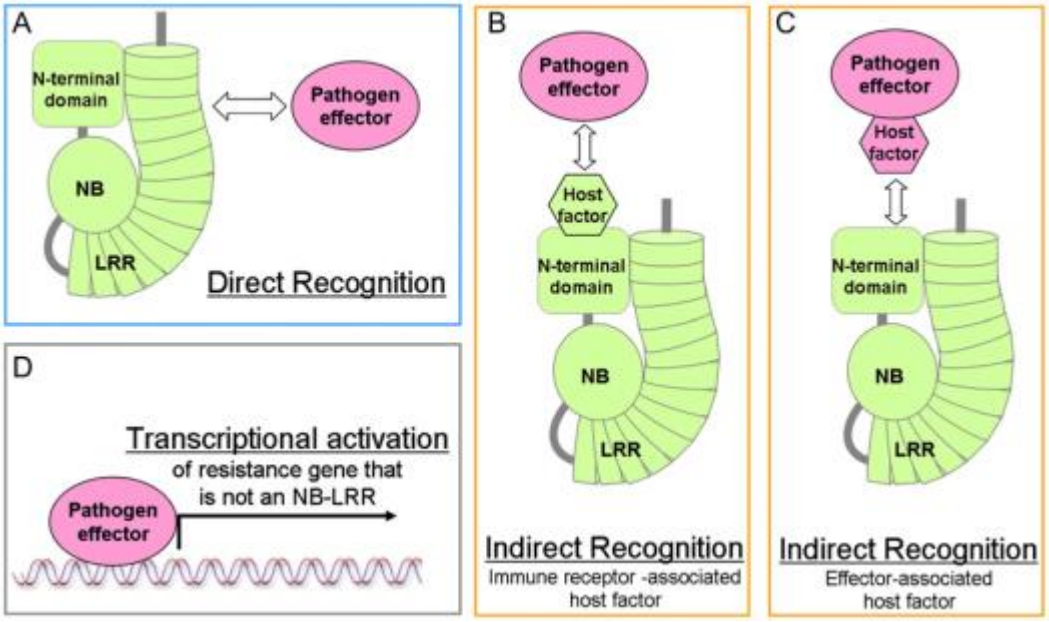


Fig.2.8 The Modes for recognition of Pathogens. (a) "gene-for-gene" hypothesis, pathogen recognition can occur if NB-LRR immune receptors (green) directly bind pathogen effectors (pink). **(b)** NB-LRRs can indirectly recognize pathogens through the N-terminal domain (CC or TIR) using an intermediary host factor. The host factor also referred to as guardee can be constitutively associated with the immune receptor. **(c)** It may first associate with the pathogen effector and then is subsequently recognized by the immune receptor. **(d)** The third type of recognition occurs when a pathogen effector mimics a transcription factor and directly induces the expression of a non-NB-LRR resistance protein. Figure adapted from Caplan et al. (2008).

2.12.2 Indirect recognition of the effector protein

Second method for avr recognition by R-protein is indirect recognition which is quite common but mechanically more complex (Fig 2.7). The cooperation of some of the host's additional proteins is necessary to initiate the resistance response. This phenomenon is explained by the so-called "guard model". Most of the effectors modify or alter the particular

host protein during pathogenesis. According to the "Guard Hypothesis", specific R-protein monitors the modification in the target protein of host and activates the defense response (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). The pathogen effector (guardee) is "guarded" by a suitable guard protein, namely an NBS-LRR receptor. Thus, direct detection of the pathogen effector molecules does not occur. Only the effect of effector is reflected by structural and/or functional changes in the host cell (Van der Biezen and Jones, 1998; Dangl and Jones, 2006). Example of indirect recognition is, RPS2 (CC-NBS-LRR of *Arabidopsis*) is activated when AvrRpt2, a cysteine protease from *P. syringae,* promotes cleavage of RIN4 (Axtell and Staskawicz, 2003; Mackey et al. 2003; Kim et al. 2005)

2.12.3 Recognition through effector-mediated transcriptional activation

Another method of avr recognition is relatively rare but is used by some non-NB-LRR immune receptors. It involves activation of R-protein by effector proteins that act as transcription factors. A pepper resistance protein Bs3 recognizes avrBs3 which localizes to the nucleus (Van den Ackerveken et al. 1996). AvrBs3 binds directly to the promoter of Bs3 gene resulting in the accumulation of Bs3 transcript, ultimately leading to HR (Romer et al. 2007). In similar manner AvrXa27, from *Xanthomonas oryzae* is recognized by rice Xa27, which encodes a novel resistance protein (Gu et al. 2005).

Available of genome sequences of chillies has attracted lot of attention for studying R-gene mediated resistance. Many R-genes have been identified from chilli plants through the PCR of conserved region of the NBS domain (Wan et al. 2012; Naresh et al. 2017). Some R-genes from chilli has been isolated and characterized to identify their functioning against specific pathogens. An well known CC-NBS-LRR gene of chilli, *Bs2* is characterized to provide resistance against *Xanthomonas campestris* pv. *vesicatoria*, contain bacterial avirulence gene *avrBs2*. Over expression of the hypersensitivity response-assisting (*Hrap*) gene of *C. annuum* in banana protects the banana plants from devastating bacterium *Xanthomonas campestris* pv. *musacearum* (Tripathi et al. 2010). R-gene against *Pepper mild mottle virus* (*Tobamovirus*) has also been identified in chilli plant (Tran et al. 2014). An R-gene analog of *Capsicum* (*CaRGA2*) is known to provide resistance against the oomycete pathogen, *Phytophthora capsici* Leonian which causes *Phytophthora* blight in chilli plants.

3.1 Materials

3.1.1 Viral clones

In this study the partial tandem repeat infectious clone of *Pepper leaf curl virus* isolate Varanasi (EF190217), *Pepper leaf curl virus betasatellite* (EF190215), *Tomato leaf curl New Delhi virus* DNA-A (ToNDA; KU196750), *Tomato leaf curl Gujarat virus* DNA-B (ToGB; KU196751), *Chilli leaf curl virus* (ChNDA; KR957353) and *Tomato leaf curl Bangladesh betasatellite* (Toβ; KR957354) were used.

3.1.2 Plant Material

In the present study, following plants were used for virus infection and/or transgenic preparation

Table 3.1: Different plants used in this study

S No	Plants	Source	Purpose
1	*Nicotiana benthamiana*	Central Tobacco Research Institute (CTRI), Rajamundary	Throughout the experiment as a model, Transgenic preparation
2	*Nicotiana tobacum* (cv. Xanthi)	Central Tobacco Research Institute (CTRI), Rajamundary	Transgenic preparation
3	*Capsicum annuum* (cv. Punjab Lal, Kashi Anmol, Kalyanpur Chanchal), *Capsicum chinense* (cv. Bhut Jolokia)	Indian Institute of Vegetable Research, Varanasi, Uttar Pradesh, India	Cloning of resistance gene, Infectivity of viruses, Transgenic preparation, detection of mixed infection

3.1.3 Microbial strains used

For cloning, agroinoculation, agroinfiltration, yeast two hybrid study and other experiments following microorganisms were used

Table 3.2: Different microbes and its strain used in this study

S No	Microbe	Strain	Genotype	Purpose
1	*Escherichia coli* (*E. coli*)	DH5α	*fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Cloning
2	*Agrobacterium tumefaciens*	EHA105	genotype C58 pTiBo542; T-region::aph, Km(S); A281 derivative harboring pEHA101, T-DNA replaced with nptII, elimination of T DNA boundaries unconfirmed, super-virulent	Transgenic preparation, Agroinfiltration, Agroinoculation
		GV2260	genotype C58 pTiB6S3DT-DNA, *atu3300* gene in its linear chromosome	Agroinfiltration, Agroinoculation
3	*Saccharomyces cerevisiae*	AH109	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : :GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3 : : MEL1UAS-MEL1 TATA-lacZ	Yeast two hybrid assay

3.1.4 Plasmid vectors used

Following vectors were used in this study

Table 3.3: List of plasmid vectors used in this study

S No	Vector	Source	Purpose
1	pJET1.2/blunt	Thermo Fisher Scientific, USA	Cloning of blunt end PCR product
2	pBINAR	MVL, SLS, JNU	Used as binary vector
3	pCAMBIA2300	MVL, SLS, JNU	Construction of viral infectious clone
4	pGBDC1	MVL, SLS, JNU	Clones used for Yeast Two-hybrid assay
5	pGADC1	MVL, SLS, JNU	Clones used for Yeast Two-hybrid assay
6	pSPYNE(R)173	MVL, SLS, JNU	Clones used for BiFC analysis
7	pSPYCE(M)	MVL, SLS, JNU	Clones used for BiFC analysis

3.1.5 Enzymes and kits used in this study

To complete the study following enzymes and kits were used

Table 3.4: List of enzymes and kits used in this study

S No	Enzymes and Kits	Source /Supplier company
1	Restriction enzymes	Thermo Fisher Scientific, USA and New England Biolabs, UK
2	T_4 DNA ligase	Thermo Fisher Scientific, USA
3	Klenow fragment	Thermo Fisher Scientific, USA
4	Antarctica phosphatase	New England Biolabs, UK
5	DNA ladder	Thermo Fisher Scientific, USA
6	Taq DNA polymerase	Thermo Fisher Scientific, USA
7	dNTPs	Thermo Fisher Scientific, USA
8	Plasmid purification kit (miniprep and mediprep)	Qiagen, Germany
9	Agarose gel extraction kit	Thermo Fisher Scientific, USA
10	Nucleotide removal kit	Qiagen, Germany
11	*pJET1.2/*Blunt Vector	Thermo Fisher Scientific, USA
12	*Reverse transcriptase*	Thermo Fisher Scientific, USA
13	*Oligo dT (18)*	Thermo Fisher Scientific, USA
14	*DNAase*I	Thermo Fisher Scientific, USA
15	RiboLock RNase Inhibitor	Thermo Fisher Scientific, USA
16	*Sybergreen*	Applied Biosystems, USA

3.1.6 Tissue culture media components

Tissue culture media was prepared using following components

Table 3.5: List of tissue culture media components

S No	Component	Source/Supplier company
1	Murashige-Skoog'S Salt	Merck, Darmstadt, Germany
2	Sucrose	Himedia, India
3	MES	Himedia, India
4	Myo-Inositol	Himedia, India
5	Nicotinic acid	Merck, Darmstadt, Germany
6	Pyridoxine hydrochloride	Merck, Darmstadt, Germany
7	Acetosyringone	Himedia, India
8	BAP	Merck, Darmstadt, Germany
9	Indole-3-acetic acid(IAA)	Merck, Darmstadt, Germany
10	Kanamycin	Duchefa Biochemie B.V, Netherlands
11	Cefotaxim	Duchefa Biochemie B.V, Netherlands
12	Clerigel™ (Gellan gum)	Himedia, India

3.1.7 Tissue culture plasticwares/glasswares

Table 3.6: List of plastic/glass wares used for tissue culture

S No	Plastic/glass wares	Source/Supplier company
1	Petri Plates	Tarsons, India
2	Jars	Tarsons, India
3	Magenta boxes	Tarsons, India
4	Filters	Mdi, India

3.1.8 Chemicals

All the chemicals used in this study were of high quality and purity. The chemicals were purchased from companies like M/s G.E. (GE Healthcare Life Sciences), Himedia, Qualigens, SIGMA, Fischer and Merck depending on the purpose of use.

3.1.9 Blotting membranes

Positively charged nylon membranes used for southern blotting were purchased from GE Healthcare Life Sciences, Chicago, USA and MDI Membrane Technologies, India.

3.2 Methods

3.2.1 Cloning of resistance genes

Different resistance genes were cloned from the resistant chilli cv. Punjab Lal plants. Genes were amplified from the cDNA prepared from *Pepper leaf curl virus* isolate Varanasi (GenBank accession no. EF190217) infected chilli plants at 21 days post inoculation (dpi).

3.2.1.1 Isolation of total RNA

RNA isolation was performed according to the Hamilton et al. (2002) using Trizol method. For small amount of RNA preparation, 100 mg leaf tissue (frozen in liquid nitrogen) was crushed in 1.5 ml microcentrifuge tube (MCT) with the help of micropestels. Crushed sample was homogenized with 500 µl Trizol (Merck, Darmstadt, Germany) and kept on ice until next step. Samples were mixed with 250 µl of chloroform by inverting several times and incubated at room temperature (RT) for 5 min without interruption followed by centrifugation at 12000g for 10 min at 4°C. The supernatant was collected in a new MCT and chloroform extraction procedure was repeated once again. Supernatant collected after two times chloroform extraction was gently mixed with 0.8 volume of chilled isopropanol. After mixing samples were incubated at 4°C for 20 min. For precipitation of RNA, tubes were centrifuged at 12000g for 10 min. After centrifugation supernatant was discarded and pellet was washed three times with 70% ethanol by centrifugation at 8000g for 10 min at 4°C. Pellet was air dried at room temperature till it became transparent. Finally, the pellet was dissolved in autoclaved DEPC water by heating at 65°C for 10 min and snap chilling. RNA was quantified with the help of nanodrop spectrophotometer (ND2000, Thermo Fisher Scientific, USA) and the quality of RNA preparations was analyzed on 1.2% agarose gel.

3.2.1.2 Preparation of cDNA

cDNA preparation was started with the degradation of DNA from total RNA. For the removal of DNA, 5.0 µg of total RNA was incubated with DNase for 30 min at 37°C. Subsequently, DNase was inactivated by the addition of 5 mM EDTA followed by heat inactivation at 65°C for 10 min. RNA (1.0 µg) treated with DNase was used for cDNA synthesis and final volume of reaction was made upto 20 µl. One µg of RNA was mixed with 0.5 µg oligo$(dT)_{18}$ and required amount of water and was incubated at 72°C for 10 min followed by chilling for 10 min. Further, 20 unit RNase inhibitor (Thermo Fisher Scientific, USA), 2.5 mM $MgCl_2$, 1.0 mM each dNTP, 200 unit reverse transcriptase (RT) (Thermo Fisher Scientific, USA) and 1X RT buffer were mixed with snap chilled RNA and incubated at 42°C for 60 min. Finally the reverse transcriptase was inactivated from cDNA by heating at 72°C for 10 min.

3.2.1.3 PCR amplification of R-genes

To amplify ChiLCV responsive R-gene two different set of primers were used for amplification using Pfu DNA polymerase.

Table 3.7: Primer sets used for the amplification of R-genes

Primer	Primer Sequence (5' – 3')	Primer Length (nt)
RPPFP	CCCGGGATGGGAGACCCAATCACAAGTG	28
RPPRP	GTCGACTCAGTGTACTTCCTCGACTTCACC	28
Bs2FP	CCCGGGATGGCTCATGCAAGTGTGGC	26
Bs2RP	CCCGGGCTAATGTTCTTCTGAATCAGAATCACTC	34

cDNA synthesized from ChiLCV- and mock-inoculated plants (21 dpi) samples were used as template for amplification of R-genes. For the amplification, 1U Pfu DNA polymerase (Agilent Technologies), 1X reaction buffer (provided with the enzyme), 1 mM individual dNTP and 0.5 µM each forward and reverse primers (Table 3.7) were used. Amplification reaction was carried out in thermal cycler (Applied Biosystem, Veriti^{TM} 96-Well Thermal Cycler) with the following program-

Table 3.8: PCR conditions used for amplification of the R-genes

S No	Step	Temperature	Duration	Cycles
1	Initial Denaturation	98°C	1.3 min	1
2	Denaturation	98°C	25 sec	28
3	Annealing	59°C	1 min	
4	Extension	72°C	3 min	
5	Final Extension	72°C	10 min	1
6	Hold	4°C		

3.2.1.4 Separation of amplified DNA and purification

PCR products were electrophoresed on 0.8% agarose gel at 60 volts for 2-3 hrs. DNA bands were excised and collected in a separate MCT and PCR products were purified with the help of gel extraction kit (Thermo scientific, Waltham, USA) according to the manufacturer's protocol.

3.2.1.5 Ligation of PCR product into cloning vector

PCR products (amplified R-genes) were incorporated into the cloning vector pJET1.2/blunt. Ligation reaction was carried out for about 12 hrs at 16°C. Ligation reaction was prepared with vector, insert, ATP, enzyme and buffer with following concentration-

Table 3.9: Composition of ligation mixture

S No	Reaction components	Stock	Working concentration/Amount
1	Vector	50 ng/ µl	25 ng
2	Insert	-	200 ng
3	ATP	100 mM	5 mM
4	T4 DNA Ligase	5U/ µl	5U
5	Reaction Buffer	10X	1X
6	Sterile Double Distilled Water	-	To adjust final volume 20 µL

3.2.2 Preparation of competent *E. coli* cells

Competent cells of *E. coli* DH5α strains were prepared by $CaCl_2$ method (Mandel and Higa, 1970). For competent cells preparation, a single colony of *E. coli* was inoculated into 3.0 ml of Luria-Bertani broth (LB broth) for primary culture. Next day (16 hrs after inoculation), 0.5 ml of the primary culture was inoculated into 50 ml of LB media and allowed to grow at 37°C, 220 rpm till the optical density of the culture (OD_{600}) reaches 0.50. Cells were chilled on ice for 15-20 min and centrifuged in 50 ml tubes at 6000 rpm for 5 min at 4°C. The supernatant was discarded and cells were resuspended in 30 ml of ice cold sterile solution of $CaCl_2$ and $MgCl_2$ (6.0 ml of 0.1 M $CaCl_2$ + 24 ml of 0.1 M $MgCl_2$), mixed thoroughly and again incubated on ice for 30 min. After incubation, cells were centrifuged at 6000 rpm for 5 min at 4°C. Supernatant was discarded and the cells were gently resuspended in solution containing 225 µl sterile glycerol (100%) and 1275 µl of 100 mM $CaCl_2$. The cells were then aliquoted into prechilled MCT and immediately stored at -80°C till further use.

3.2.3 Transformation of *E. coli*

E. coli competent cell's transformation was done according to Hanahan et al. (1991). Preserved *E. coli* competent cells were taken out and stored on ice and allowed to thaw completely. The ligation mixture/confirmed clone was added into the competent cells, mixed gently and again incubated on ice for 30 min. Competent cells mixed with ligation mixture/confirmed clone were incubated at 42°C, exactly for 2 min in a water bath to given heat shock and immediately transferred on ice for 5 min. Further, 1.0 ml of LB broth was added to each tube and incubated at 37°C for 1 hr with constant shaking at 220 rpm. Cells were centrifuged at 6000 rpm for 5 min at room temperature, except 100 µl remaining supernatant was discarded. The cells were gently resuspended in 100 µl LB broth and spread on LB agar plate containing appropriate antibiotics. Plates were allowed to dry in the laminar flow and incubated at 37°C for 16 h.

3.2.4 Plasmid DNA purification

Plasmid DNA was extracted by alkaline lysis method (minipreparation) according to Birnboim and Dolly (1979). *E. coli* cultures (1.5 ml) grown for 16 hrs were pelletized by centrifugation at 13000 rpm, for 1 min and resuspended in 100 µl of alkaline lysis solution I (resuspension buffer:

50 mM glucose, 25 mM Tris-Cl pH 8, 10 mM EDTA pH 8). Pellet was dissolved by vigorously vortexing and incubated at room temperature for 5 min. Freshly prepared alkaline lysis solution II (lysis buffer: 0.2 N NaOH, 1% SDS) 200 µl was added to the tubes and mixed by gently inverting the tubes and incubated on ice for 5 min. 150 µl of Pre-chilled alkaline lysis solution III (neutralization buffer: 5M potassium acetate, glacial acetic acid, water) was added, and tubes were mixed again by gentle inverting. Further tubes were incubated on ice for 10 min, in-between gently mixed 2-3 times. Tubes were centrifuged at 13000 rpm at 4°C for 15 min and supernatant was taken in fresh tubes. In these tubes equal volume of phenol: chloroform: isoamyl alcohol (24:24:1) solution was added and mixed thoroughly by inverting the tubes. The tubes were centrifuged at 13,000 rpm for 10 min at room temperature. The upper aqueous layer was collected in fresh tubes and mixed with 0.8 volume of chilled isopropanol by gentle inverted. Mixing was followed by the incubation of tubes at -20°C for 20 min and centrifugation at 13000 rpm at 4°C for 15 min for the precipitation and pelletization of DNA respectively. Supernatant was discarded and the pellet was washed with 0.5 ml of 70% ethanol by centrifugation at 13000 rpm at 4°C for 10 min. The supernatant was discarded and the pellet was dried completely to remove any traces of ethanol and resuspended into 30 µL of sterile double distilled water. The concentration of plasmid was analyzed by electrophorasis on 0.8% agarose gel and by spectrophotometer.

3.2.5 Restriction digestion of plasmid DNA

The reaction mixture for restriction digestion of plasmid DNA was prepared according to specific enzymes required for the digestion. On the basic of restriction site required for cloning plasmid DNA was treated with either single or double restriction enzymes. Restriction digestion with two enzymes was performed either sequentially or double digestion depended on the compatibility of the restriction enzymes in the reaction buffer (following manufacturer's recommendations). For sequential digestion, plasmid DNA was first digested with the restriction enzyme requiring low salt concentration, extracted with phenol:chloroform, followed by precipitation using isopropanol and then subjected to digestion with the second restriction enzyme. For providing the suitable conditions to the restriction enzyme tubes were incubated in a water bath at the 37°C for 3-4 h.

Table 3.10: Composition of restriction digestion reaction

S No	Component	Stock concentration	Working Concentration/Amount
1	Plasmid DNA	-	1.0 µg
2	Restriction Enzyme	10U/µl	1U
3	Buffer	10X	1X
4	Sterile Double Distilled Water	-	To adjust final volume 20 µL

3.2.6 Dephosphorylation of plasmid DNA

To prevent self ligation of vectors used for cloning at single restriction site, vectors were dephosphorylated at 5' end. Plasmid DNA digested at single restriction site was incubated for 20 min at 65°C to inactivate the restriction enzyme and subjected to dephosphorylation with antartica phosphatase (New England Biolabs, UK) (Table 3.11) for 10 min in specified reaction buffer in a water bath at 37°C. Antartica phosphatase was heat inactivated at 65°C for 20 min. Dephosphorylated plasmid DNA was resolved on 0.8% agarose gel with DNA marker and purified with DNA purification kit following manufacturer's instructions. The purified dephosphorylated plasmid was used as vector in ligation mixture set up.

Table 3.11: Dephosphorylation reaction mixture composition

S No	Components	Stock	Working Concentration/Amount
1	Vector DNA		1 µg
2	Antartica phosphatase	5U/µl	2.5U
3	Buffer	10X	1X
4	Sterile Double Distilled Water		To adjust final volume 20 µL

3.2.7 Preparation of competent *Agrobacterium* cells

Agrobacterium tumefaciens EHA105 strain was used to prepare competent *Agrobacterium* cells. A single colony of *Agrobacterium* from fresh LB agar plate was inoculated into 3 ml of LB broth supplemented with 1% glucose+rifampicin (30mg/l) and grown overnight at 28°C with shaking

at 220 rpm. Next day, 0.5 ml of this culture was used to inoculate 50 ml LB broth supplemented with 1% glucose+ rifampicin (30mg/l). The culture was incubated at 28°C with shaking at 220 rpm till OD_{600} reaches 0.50. The culture was taken out and chilled for 15 min on ice and cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C. The pellet was resuspended gently in 10 ml of ice cold 0.15 M NaCl and incubated on ice for 15-20 min. The cells were again centrifuged at 6000 rpm for 5 min at 4°C. The pellet was resuspended in 1.0 ml of ice cold 20 mM $CaCl_2$ and 100µl of competent cells were aliquoted into pre-chilled 1.5 ml MCT. The tubes were dipped in liquid N_2 for 5 min and immediately stored at -80°C till further use.

3.2.8 Transformation of *Agrobacterium* competent cells

Transformation of *Agrobacterium* competent cells with the plasmid construct was done according to Hofgen and Willmitzer (1988). Preserved *Agrobacterium* cells were taken out and incubated on ice to thaw. After complete thawing of cells, 1µg of construct DNA was added into competent cells and incubated on ice for 30 min. Further, cells were frozen into liquid N_2 for 2 min and again thawed in 37°C water bath for 2-3 min followed by the immediate transfer on ice for 5 min. One ml of LB broth supplemented with 1% glucose+ 30 mg/l rifampicin was added to the cells and incubated for the recovery of cells for 4-5 hrs at 28°C and 220 rpm conditions. After incubation cells were harvested by centrifugation at 6000 rpm for 5 min, excess of media was removed and the pellet was resuspended in remaining 100 µl media and cells were spread on selection plate (LB agar, 1% glucose, 30mg/l rifampicin and 50 mg/l kanamycin) and incubated at 28°C for 36-48 h.

3.2.9 Multiple sequence alignment and phylogenetic tree construction

Sequences of different R-genes present in chilli genome were extracted from the chilli genome database. Full length sequences of the R-genes in the chilli genome were identified by performing the BLAST search in chilli genome database (http://peppersequence.genomics.cn/page/species/blast.jsp) using the partial sequence of R-genes described by Wan et al. (2012) as a template. All the sequences, sharing homology with each known R-gene were collected for sequence alignment and phylogenetic analysis. Sequences were aligned using the MEGA program version 6.0 followed by generation of the phylogenetic

dendrogram with the help of neighbor-joining method (Tamura et al. 2013). The phylogenetic tree was verified by the bootstrap analysis with 1000 pseudo-replicates as implemented in MEGA 6.0. The bootstrap values were provided at the nodes of all the phylogenetic trees.

Phylogenetic analysis of different viruses used in this study was also performed. Vector sequence was trimmed from the raw sequence data by dint of VecScreen (http://www.ncbi.nlm.nih.gov/tools/ vecscreen/), and the remaining sequences were searched for homology at NCBI database (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). For identification of viral genomic components, Basic Local Alignment Search Tool (BLAST*n*) search was performed. Phylogenetic analysis of the sequences was carried out by generating dendrogram using neighbor-joining method with the help of MEGA 6 software using default setting (Tamura et al. 2013).

3.2.10 Agroinfiltration

The confirmed clones were mobilized into *A. tumefaciens* strain EHA105 by freeze-thaw method and selected on LB agar containing kanamycin (50 µg/ml) and rifampicin (30 µg/ml). Infiltration with *A. tumefaciens* harboring desired construct was performed according to the Hamilton et al. (2002). In brief, *Agrobacterium* cells were pelletized by centrifugation followed by resuspention of the cells in infiltration buffer keeping OD_{600} in-between 0.5-1.0. Suspension of cells was injected in the leaf from lower surface with the help of needleless syringe.

Table 3.12: Composition of infiltration buffer

S No	Components	Stock	Working Concentration/Amount
1	MES	0.5 M	10 mM
2	$MgCl_2$	100 mM	10 mM
3	Acetosyringone	100 mM	100 µM

3.2.11 Trypan blue staining

Staining of cell death was done according to the Koch and Slusarenko, (1990). Leaves were stained with lactophenol-trypan blue solution (10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, 10 mg of trypan blue, dissolved in 10 mL of distilled water). Whole leaf was dipped in

the stain and boiled for about 1 min. Destaining of excess trypan blue was performed with the help of chloral hydrate (2.5 gm chloral hydrate was dissolved in 1 ml double distilled water).

3.2.12 Isolation of total genomic DNA from plant

Total genomic DNA from the leaves of virus-infected and mock-inoculated plants was isolated according to the method described by Dellaporta et al. (1983) with some modifications. For small scale preparation, DNA isolation was carried out into 1.5 ml MCT with the help of micropestels.

About 200 mg leaf tissue was frozen in liquid nitrogen and ground with the help of micropestels till it became a fine powdery mass. 750 µl of extraction buffer (appendix I) was added to the ground sample and tissue was homogenized thoroughly after that stored on ice until proceeded for the next step. 100 µl of SDS (10%) was added to the homogenized slurry and properly mixed by frequent inverting of tube. Tubes, containing slurry were incubated at 65°C for 20 minutes, in-between mixed regularly after every 3 min interval. After that it was allowed to cool at room temperature for 5 – 10 min. Further 250 µl of 5M potassium acetate (pH 5.8) was added in the tubes and mixed properly by inverting the tubes several times and incubated on ice for 20 min. For separation of solid and liquid from slurry, tubes were centrifuged at 13000 rpm for 10 min at 4°C. After centrifugation, transparent supernatant was collected in new 2.0 ml tubes. In the next step, solution of Phenol: Chloroform: Isoamyl alcohal (25:24:1) was mixed thoroughly with supernatant in equal amount. Tubes were kept at room temperature for 5 min without any interruption to separate the aqueous and organic layer. Again MCTs were centrifuged at 13000 rpm for 10 min at room temperature which have created three layers, upper transparent aqueous layer, middle white colored protein layer and lower organic layer. Upper transparent aqueous layer was collected in 1.5 ml MCT. Further the aqueous layer was mixed thoroughly with the equal volume of Chloroform: Isoamyl alcohal (24:1) solution and incubated at room temperature without disturbing the layers, followed by the centrifugation at 13000 rpm for 10 min at room temperature. Again transparent aqueous layer was collected in new MCT and gently mixed with 0.8 volume of chilled isopropanol followed by the incubation at 4°C for 20 min. Further DNA was pelletized by centrifugation at 13000 rpm for 15 min at 4°C. Supernatant was discarded and the DNA pellet was washed three times with 500 µl 70% ethanol. Pellet was air dried at 37°C

and dissolved in ≈200 µl autoclaved double distilled water. DNA was quantified with the help of ND2000 nanodrop spectrophotometer and the quality of DNA preparations was analyzed on 0.8% agarose gel.

3.2.13 Southern hybridization

Viral DNA accumulation in plant samples was detected by Southern hybridization (Southern, 1975). Southern blot hybridization experiments were carried out according to the protocol described by Sambrook et al. (2001). For the southern blot hybridization experiments, 8.0 µg of total DNA isolated from the plant samples was electrophoresed in 0.8% agarose gel. Agarose gel having electrophoresed DNA was treated with 0.25M HCl for 15 min for the depurination of DNA. Depurination was followed by the denaturation of DNA by treating of gel with 0.5M NaOH and 1.5M NaCl for 45 - 60 min. Denaturation of DNA was followed by the neutralization with the solution containing 0.5M Tris-HCl and 1.5M NaCl. For neutralization, each gel was treated with neutralization solution twice, first for 30 min and second for 15 min. Ultimately DNA was transferred onto the positively charged nylon membrane using 10X SSC (3.0M NaCl, and 0.3M tri-Sodium citrate). Transfer was carried out through capillary method followed by the cross-linking of transferred DNA onto positively charged nylon membrane (MDI-India). DNA was crosslinked by exposure of membrane adhered with the DNA to the ultraviolet rays.

For hybridization, probe was prepared by random probe labeling method. Hybridization was carried out using radio labeled (αP^{32}-dCTP) probes specific for the viral genome. Hybridization was done in hybridization oven (GE Healthcare Life Sciences, Chicago, USA) at 65°C for 18 hrs. To remove the excess probe and non specific binding after hybridization, blots were washed twice with washing solution 1 (2X SSC and 0.2% SDS) at RT for 20 min and washing solution 2 (1X SSC and 0.5% SDS) at 65°C for 15.min to remove excess of probes to avoid nonspecific signals. Hybridized membrane was packed with the plastic wrap and kept in contact of phosphor imaging plate for 12–16 hrs followed by scanning of phosphor imaging plate using phosphorimager (GE Healthcare Life Sciences, Chicago, USA). The signals were quantified by using the image analysis software (Quantity One, Bio-Rad).

3.2.14 Quantitative real time PCR

Expression of R-gene was analyzed with the help of quantitative real time PCR (qRT PCR). Primers for the expression analysis of host genes were designed with the help of Primer Express v3.0 software (Applied Biosystem, California, USA). Amplification was performed with 1X SYBR Green master mix (Applied Biosystem, California, USA) containing DNA polymerase, dNTPs and a passive dye reference (ROX). qRT PCR was also supplemented with 2 µl of 1:5 dilution cDNA (used as template) and 50 pM of each primer. PCR program comprises of initial denaturation at 94°C for 5 min; 40 cycles of, denaturation at 94°C for 15 sec, annealing at 55°C for 35 sec and extension at 72°C for 35 sec. Three biological and three technical replicates were taken for each case. ct (ct-threshold cycle) value of host transcripts was normalized with ct value of Actin to get Δct value which was used to calculate $2^{-\Delta(\Delta CT)}$ values. $2^{-\Delta(\Delta CT)}$ values from mock- and virus-inoculated plants were used for graphical analysis of qRT PCR which was plotted using Sigma-plot 11.0 software (Post-hoc Holm-Sidak method) (Singh et al. 2013). To compare the relative expression level of host genes the Student's t-test was performed. Actin sequences derived from chilli var. Punjab lal (available in our laboratory) was used as the internal control. Actin was amplified using the primers (FP 5′ GAAGCTCAATCCAAACGTGGTATT 3′, RP 5′ CTCAAACATGATTTGTGTCATC 3′). To ensure that PCR product was derived from the mRNA only, parallel reactions lacking cDNA template (NTC) were also performed.

3.2.15 Protein-Protein interaction study

Interaction between virus-host and host-host proteins was studied with yeast two hybrid and BiFC assay.

3.2.15.1 Yeast two hybrid assay

To study the protein-protein interaction yeast two hybrid experiment was performed. Sterile technique was used throughout the yeast transformation protocol. Wide-bore pipette tips were used for pipetting yeast competent cells to reduce the shear forces associated with standard pipette tips. Competent cells were prepared for transformation.

3.2.15.1.1 Preparation of yeast competent cells

One ml of YPD broth was incubated in a 1.5-ml microcentrifuge tube with single yeast colony (*Saccharomyces cerevisiae* strain AH109) that was 2–3 mm in diameter in size and no more than one week old. The culture was vigorously vortexed until no cell clumps were visible. In a 250-ml flask filled with 50 ml of YPD broth, 1.0 ml of the yeast culture was added. The diluted culture was then incubated for 18–24 h at 30°C with constant shaking at 200 rpm. The OD_{600} was continuously checked and shaking was continued till OD_{600} reached ≥1.2. Then fully-grown yeast culture was added to 300 ml of YPD broth filled in a 1 liter flask till OD_{600} reached to 0.20. Next, the culture was incubated for 3-4 hrs at 30°C with constant shaking at 220 rpm until OD_{600} reached to the 0.60. The cells were harvested by centrifugation at 1000×g for 5 min at room temperature. The supernatant was discarded and the cells were resuspended in 50 ml of sterile deionized water for washing of pellet. Again, cells were centrifuged at 1000×g for 5 min at room temperature. The supernatant was discarded and the cells were resuspended in 1.5 ml of freshly prepared TE–LiAc solution. These yeast competent cells were used on the same day.

3.2.15.1.2 Transformation of yeast competent cells

The carrier DNA (salmon sperm DNA of 20 mg/ml concentration) was prepared by the boiling of salmon sperm DNA for 10 minutes followed by the snap chilling on ice. 100 µl of competent yeast cells were aliquoted in each MCT with the help of wide-bore pipette tips. 100 µg of carrier DNA was added to each tube. For transformations, 100 ng of the desired plasmids were also added to each tube. Further, 600 µl of TE–LiAc–PEG solution was added to each tube and the contents were mixed by vortexing. Mixing was followed by incubation at 30°C for 30 min with shaking at 200 rpm. After incubation, 70 µl of DMSO was added to each tube and the contents were mixed gently. Heat-shock treatment was given to the cells by incubating at 42°C water bath for 15 min. Then tubes were placed on ice for 10 minutes. The samples were finally centrifuged at 3000 rpm for 10 sec to pellet cells. Supernatant was carefully removed from the tubes. Residual supernatant was removed by spinning the tubes for a few seconds. 0.5 ml of 1× TE buffer was added to each tube and the tubes were vortexed to resuspend the cells. Using wide-bore pipette tips, the transformed cells were collected and spread on appropriate SD-selective plates. The plates were incubated at 30°C for 2–4 days until colonies appear.

3.2.15.1.3 Yeast two-hybrid interaction

Specific interactions between viral proteins with different host proteins and between different host proteins were determined by cloning them in Y2H vectors pGADC1 (AD) and pGBDC1 (BD). Purified plasmids of different combinations of both viral and host factors were cotransformed into yeast competent cells (AH109 strain of *S. cerevisiae*) and cotransformed cells were grown on SD/Leu^{-}/Trp^{-} (2 drop out) media containing petriplates. Specific interactions between two proteins were analyzed by further streaking of colonies from SD/Leu^{-}/Trp^{-} media petriplates to the SD/Leu^{-}/Trp^{-}/His^{-} (3 drop out) media and SD/Leu^{-}/Trp^{-}/His^{-} + 3AT media.

3.2.15.2 Bimolecular fluorescence complementation (BiFC) analysis

For BiFC analysis R-gene was inserted in N-terminal GFP and C-terminal GFP containing vectors, pSPYNE(R)173 and pSPYCE(M), respectively. Both types of vectors containing R-gene were transformed into *Agrobacterium* competent cells. *Agrobacterium* cells containing the desired construct were cultured in LB broth containing 50 mg/l kanamycin and 30 mg/l rifampicin and grown at 28°C at 220 rpm for 36 Hrs. Cell were harvested from fully grown culture by centrifugation at 6000 rpm for 5 min at RT. Harvested cells were resuspended in infiltration buffer by keeping OD_{600} = 0.50±0.01. These cells were incubated in dark at 28°C with 220 rpm shaking condition for 2 hrs. After incubation, leaves of 3-weeks old *Nicotiana benthamiana* plants were infiltrated with needle less syringe at the abaxial surface. Five days post infiltration, epidermis of the infiltrated region of *N. benthamiana* leaf was peeled off and mounted on the slide with 10% glycerol. Epidermis was kept on slide in such a way that the lower surface was facing upwards. 4, 6-diamidino-2- phenylindole (DAPI) and FM 4-64 stains were used for staining of nuclei and membranes, respectively. For visualization of DAPI fluorescence 340-380 nm excitation filter and a 435- 485 nm emission filter was used. Excitation filter of 465-495 nm and emission filter of 515–555 nm were used for visualizing the GFP fluorescence. Microscopic study was performed with the help of Nikon confocal microscope.

3.2.16 Preparation of transgenic chilli plants

Chilli (*Capsicum annuum* cv. Kashi Anmol) plants were used for over expression of the Capana07g001557 gene. Modified Murashige and Skoog media was used for the growth of plants during transgenic preparation.

Table 3.13: Murashige and Skoog media (MS media) composition

S No	Component	Stock	Working concentration/amount
1	M S basal salt	-	4.3 gm/L
2	Sucrose	-	3.0 %
3	MES	0.5 M	2 mM
4	Gamborg's Vitamin	50 X	0.1X
5	Myo-Inositol	50 mg/ml	0.1 mg/ml
6	Clerigel™ (Gellan gum)		0.25%
7	pH was adjusted to 5.8 by 1N NaOH		

3.2.16.1 Sterilization of chilli seeds

Chilli seed (*Capsicum annuum* cv. Kashi Anmol Wild Type) were washed three times with sterilized water. Further seeds were kept in 70% ethanol for 1 min with continuous shaking followed by three times washings with sterile water. Seeds were kept in 4% sodium hypochloride with continuous shaking for 8 min followed by six times washings with sterile water. Finally seeds were dried in sterile condition and spread on the half MS media containing petriplates. Petriplates were incubated at 25°C under 16/8 hrs light/dark conditions.

3.2.16.2 Preparation of explants

Hypocotyl of 7-10 days old seedling having only cotyledonary leaves (without true leaf) was used for the transgenic preparation. Seedling with 2-2.5 cm long stem was selected and cut from slight below than half so that the cotyledonary leaves containing part measured about 1.5 cm. Further, cotyledonary leaves containing part was taken and cotyledonary leaves were removed from it. Upper part of the stem without cotyledonary leaves, having apical meristem was used as

explants for the transformation and to develop transgenic. Finally explants were imbedded upright on pre-cultivation media for the adaptation in MS media. Explants were incubated for 48 hrs at 25°C under 16/8 hrs light/dark conditions.

Table 3.14: Pre-cultivation media composition

S No	Component	Stock	Working Concentration/amount
1	MS media		
2	BAP	10 mg/ml	10 mg/L
3	IAA	1 mg/ml	1 mg/L

3.2.16.3 Transformation of the explants

Agrobacterium harboring Capana07g001557 gene in binary vector (pBINAR) was grown in LB broth supplemented with 30 mg/l rifampicin and 50 mg/l kanamycin was for 36 hrs at 28°C. Cells were harvested by centrifugation at 6000 rpm for 5 min at RT in sterile condition. Cells were resuspended in MS liquid media and OD_{600} was adjusted to 0.30. Resuspended cells supplemented with 100 µM acetosyringone along with explants incubated on pre-cultivation media were filled in a sterile jar and incubated at 28°C for 10 min at 100 rpm in dark. Further explants were taken out from cell suspension, dried on sterile blotting sheets and embedded upright on co-cultivation media. Explants were incubated on co-cultivation media at 25°C for 48 hrs in dark and preceded for next step.

Table 3.15: Co-cultivation media composition

S No	Component	Stock	Working Concentration/amount
1	MS media		
2	BAP	10 mg/ml	10 mg/L
3	IAA	1 mg/ml	1 mg/L
4	Acetosyringone	100 mM	100 µM

3.2.16.4 Washing of the explants

To remove the *Agrobacterium*, explants were washed with 400 mg/L cefotaxime. For the washing, explants were collected in a sterile jar and filled with liquid MS media supplemented with 400 mg/L cefotaxime. Jar was incubated in dark at 28°C with shaking at 100 rpm. Cefotaxime treatment was followed by drying of the explants on sterile blotting sheet and transfer into the selection media. Again explants were embedded on selection media upright and incubated at 25°C in 16/8 hrs light/dark conditions.

Table 3.16: Selection media composition

S No	Component	Stock	Working Concentration/amount
1	MS media		
2	BAP	10 mg/ml	10 mg/L
3	IAA	1 mg/ml	1 mg/L
4	Kanamycin	50 mg/ml	50 mg/L
5	Cefotaxime	200 mg/ml	400 mg/L

3.2.16.5 Shoot regeneration from explants

After one week explants were subcultured in freshly prepared selection media. Few days after subculturing small buds appeared near to the apical meristem. Explants bearing small buds were subcultured three to four times in freshly prepared selection media after every two weeks, until 5-6 leaves were appeared.

3.2.16.6 Root regeneration from explants

Explants with shoot and leaves were subcultured in the rooting media. After one or two subculture white colored roots appeared from the explants embedded in the media. Subculture was done after the interval of two weeks and after three to four subcultures many, 5-6 cm roots appeared.

Table 3.17: Composition of rooting media

S No	Component	Stock	Working Concentration/amount	
1	M S salt	-	2.15 gm/L	
2	Sucrose	-	3.0 %	
3	MES	0.5 M	2 mM	
4	Gamborg's Vitamin	50X	0.1 X	Before Autoclave
5	Myo-Inositol	50 mg/ml	0.1 mg/ml	
6	Clerigel™ (Gellan gum)		0.25%	
7	pH was adjusted to 5.8 by 1N KOH			
8	Kanamycin	50 mg/ml	50 mg/L	After autoclave
9	Cefotaxime	200 mg/ml	400 mg/L	

3.2.16.7 Hardening of the plants

Plants grown on rooting media with developed vegetative organs (stem, leaf and root) were transferred for the hardening. During hardening plants were kept in high humidity condition at 25°C and 16/8 hrs light/dark for 14 days. High humidity condition was created by the covering of whole plant with transparent polythene bag.

3.2.17 Preparation of transgenic *N. benthamiana* / *N. tabacum*

Leaves of *Nicotiana benthamiana* and *Nicotiana tabacum* were used for the preparation of Capana07g001557 gene over expressing transgenic plants.

3.2.17.1 Sterilization of seeds

Seeds of *N. benthamiana* and *N. tabacum* were sterilized by washing the seeds with 4% sodium hypochloride for 5 min.

3.2.17.2 Preparation of explants

Leaves of *N. benthamiana* and *N. tabacum* plants grown in MS media under sterile conditions were used for the preparation of explants. Completely developed green leaves of *N. benthamiana* and *N. tabacum* were cut into 1 X 1 cm^2 part and used as explants for transgenic preparation. Explants was cut in sterile condition and transferred to the pre-cultivation media containing petriplates. Composition of pre-cultivation media was alike to the chilli pre-cultivation media. Petriplates containing explants were incubated at 25°C for 48 hrs at 16/8 hrs light/dark conditions.

3.2.17.3 Transformation of the explants

Capana07g001557 gene inserted into the binary vector (pBINAR) was used for the transformation of explants. For the transformation OD_{600} of *Agrobacterium* suspension in MSO (MS liquid media without solidifying agent) was 0.50 and the incubation time of explants in suspension was kept 15 min. All other steps of transformation were similar to chilli transformation. After infection explants were transferred to the co-cultivation media which was same as the chilli co-cultivation media. Explants were incubated in co-cultivation media for 48 hrs at 25°C in dark.

3.2.17.4 Washing of the explants

After incubation in dark explants was washed as the chilli explants. Washing was followed by the transfer of explants to the selection media. Composition of selection media was identical as chilli selection media except antibiotic concentration. Selection media for *N. benthamiana* and *N. tabacum* was supplemented with 75 mg/L kanamycin and 450 mg/L cefotaxime.

3.2.17.5 Shoot regeneration from explants

After one week explants were subcultured in freshly prepared selection media in magenta boxes. After one or two subcultures explants became white with some green pin points further buds appeared from these green points. Again few subcultures resulted to the development of buds into the shoots bearing leaves. Subculture was continued in fresh selection media after every 14 days till the shoots became 3- 4 cm long.

3.2.17.6 Root regeneration from the shoots

Under sterile condition shoots were cut (oblique cut) to detach from explants and transferred to the rooting media. In the rooting media shoots were embedded in upright status. After one or two subcultures white colored roots appeared from the lower tip of the shoot. Subculture was done after the interval of two weeks and after three to four subcultures many roots became 5-6 cm long. Again the rooting media was same as rooting media of chilli except the concentration of antibiotics. Rooting media for *N. benthamiana* and *N. tabacum* was supplemented with 75 mg/L kanamycin and 450 mg/L cefotaxime.

3.2.17.7 Hardening of the plants

Plants grown on rooting media developed all the vegetative organs (stem, leaf and root) were transferred for the hardening. During hardening plants were kept in high humidity condition at 25°C and 16/8 hrs light/dark for 14 days. High humidity condition was created by the covering the whole plant with transparent polythene bag.

3.2.17.8 Transfer of plants in soil and seed collection

After hardening plants were transferred in pots (6 inch) filled with soil and kept in green house. Green house was maintained at 25°C, 60% relative humidity and 16/8 hrs light/dark conditions. After two weeks of transfer, plants bearing floral buds were covered (upper part bearing flowers) with bag made of muslin cloth. Flowers were matured in the muslin cloth and developed into the matured fruits. Finally seeds were collected from mature red colored dried fruits.

3.2.18 Leaf disk assay

Leaf disk assay was performed with the leaf disk of *N. benthamiana*. For the experiment 2 cm^2 leaf disks were cut, incubated in pre-cultivation media, given infection with desired construct and washed the leaf disks in a similar manner as the *N. benthamiana* transgenic preparation. Unlike *N. benthamiana* transgenic preparation, during leaf disk assay samples were collected 7 days after the washing of leaf disks. From the leaf disks DNA was extracted and viral titer was

analyzed with southern blotting. For the infection of leaf disks equimolar mixture of *Agrobacterium* harboring the desired construct was used.

3.2.19 Isolation, cloning and sequencing of full-length viral genomes

Leaf samples from the resistant chilli cultivars showing severe leaf curling were collected from the experimental field of Jawaharlal Nehru University (JNU), New Delhi in 2009. To identify the causative virus of chilli leaf curl disease (ChiLCD), total genomic DNA was extracted from the diseased samples according to Dellaporta et al. (1983) and rolling circle amplification (RCA) was carried out using the TempliPhi 100 amplification kit (GE Amersham, Little Chalfont, United Kingdom) following the manufacturer's instruction. The RCA products were digested with *Kpn*I restriction enzyme and subsequently, linearized products were cloned into pUC18 vector at *Kpn*I site. In addition, PCR based amplification of full-length viral components were also carried out using degenerate primers (Briddon et al. 2002; George et al. 2014). Viral clones of 1.4 kb and 2.8 kb were sequenced commercially at the University of Delhi South Campus, New Delhi.

3.2.20 Infectivity analysis

3.2.20.1 Agro-inoculation

N. benthamiana plants of 5-6 leaf stage were used for agro-inoculation of viral infectious constructs. Agro-inoculation was performed on the *N. benthamiana* plants as described by Chattopadhyay et al. (2008). Primary culture of *A. tumefaciens* strain EHA105 harboring infectious viral construct was grown in 3 ml LB medium. Secondary culture was initiated into 50 ml LB media, primary culture was used as inoculum and allowed to grow at 28°C for 36 hrs at 220 rpm. The culture was pelletized at 6000 rpm for 5 min and re-suspended in autoclaved double distilled water added with 100 mM of acetosyringone. For each inoculum, the optical density (OD_{600}) was maintained as 1.0 and the equimolar concentration of each construct to be used for agro-inoculation was mixed. The plants to be inoculated were pricked at the petiole and

stem with a fine needle followed by the application of 50 µL of the inoculum at the injured site. The symptoms were measured following the symptom severity score given in the Table 3.18.

3.2.20.2 Particle bombardment (Biolistic inoculation)

C. annuum cv. Kalyanpur Chanchal plants were inoculated with the help of biolistic inoculation method following manufacturer's instructions (Bio Rad, Catalog numbers: 165-2431 and 165-2432). Gold particles of 0.6 µm diameter were coated with viral genome (infectious clone in binary vector). For the coating of viral genome 25 mg of gold particles (0.6 µm diameter) were mixed with 100 µl of 0.05 M spermidine by vortexing and sonicated to breakdown the clumps. 100 µl of viral genome (3 µg/µl, containing equal amount of ToNDA+ToGB or ChNDA+Toβ or mixture of ToNDA+ToGB+ChNDA+Toβ) was added to the gold particles mixed with spermidine. Further gold particles containing spermidine and viral genome were mixed with the 100 µl of 1M $CaCl_2$ while vortexing. Gold particles coated with viral genome were washed with 100% ethanol.

In the succeeding step, viral genome coated gold particles were coated in the inner surface of Gold-Coat tubing with the help of 0.1 mg/ml of PVP. Tubing was coated with gold particles with the help of Tubing Prep Station and dried with the help of nitrogen gas. Gold-Coat tubing containing gold particles was cut into the 0.5 inches small pieces with the help of tubing cutter. These small tubes known as cartridges were used for the gold particle bombardment. Ultimately gold particles were bombarded on two leaves of *C. annuum* cv. Kalyanpur Chanchal plants from by Helium gas at 300 psi with the help of Helios Gene Gun (Bio-Rad). The infected plants were maintained in green house at 25°C and 16/8 hrs light/dark conditions. The symptoms were measured following the symptom severity score given in the Table 3.18.

Table 3.18: Scales of symptom severity

S No	Symptoms	Symptom severity score
1	No symptoms	0
2	0-5% curling of top leaf	1
3	6-25% leaf curling, swelling of veins, yellowing of leaf	2
4	26-50% leaf curling, puckering, swelling of veins, yellowing of leaves	3
5	51-75% leaf curling, stunted plant growth, vein clearing or yellow patches (depends on virus)	4
6	>75% leaf curling, stunted plant growth, small leaves, deformed or no flowers	5

In an earlier study, suppression subtractive hybridization (SSH) cDNA library was made to understand the natural resistance of chillies against Chilli leaf curl virus (Kushwaha et al. 2015a). That study indicated that a gene (GenBank accession JK523032) identified by SSH experiment, bearing considerable homology with *NBS-LRR* gene was upregulated following ChiLCV infection in the resistant chilli variety Punjab Lal. Further, Reverse northern blotting experiment suggested that expression of that gene was >8 folds higher in virus infected chilli (*C. annuum* cv. Punjab Lal) plants than the mock-inoculated plants. Quantitative real time PCR (qRT-PCR) result also indicated that expression of this transcript did not have significant difference initially after infection (14 dpi) but at 21 dpi expression was >4 folds higher in the resistant cultivar (*C. annuum* cv. Punjab Lal) than susceptible cultivar (*C. annuum* cv. Kashi Anmol) of chilli.

For identification and functional characterization of this particular *NBS-LRR* gene, the present study was undertaken and the following results were obtained:

4.1 To identify ChiLCV- responsive *NBS-LRR* gene in chilli

4.1.1 Retrieval of full length *NBS-LRR* gene

Full length *NBS-LRR* gene retrieval was started after performing the blast (nucleotide BLAST) search using the sequence obtained from the SSH library indicated as above (GenBank accession JK523032). Blast analysis showed maximum homology with *RPP13like4* and *Bs2* genes of chilli. Primers (Table: 3.7) were designed to amplify both the genes. PCR amplification with Bs2 primers showed amplification from the cDNA template prepared from the virus-infected plants (21 dpi) but not with in the mock-inoculated plants (Fig. 4.1a). Electrophoresis of amplified product resolved the amplicons in two discrete sizes between 2500 bp to 3000 bp. Both the genes were cloned and sequenced for further study. Amplification with the RPP primers showed no amplification with cDNA prepared from the mock-inoculated plants, whereas very less amplification was obtained with 14 dpi virus-infected plant and substantial amplification with the cDNA isolated from the virus-infected plants at 21 dpi (Fig. 4.1d). Electrophoresis of amplified product revealed presence of only one type of DNA measuring of about 2.6 kb. Restriction digestion of the pJET1.2/blunt clone of amplified product elucidated the presence of two types of

DNA population in the amplicon. Further, both the clones were sequenced and studied further. Finally, four different *NBS-LRR* genes were amplified and cloned.

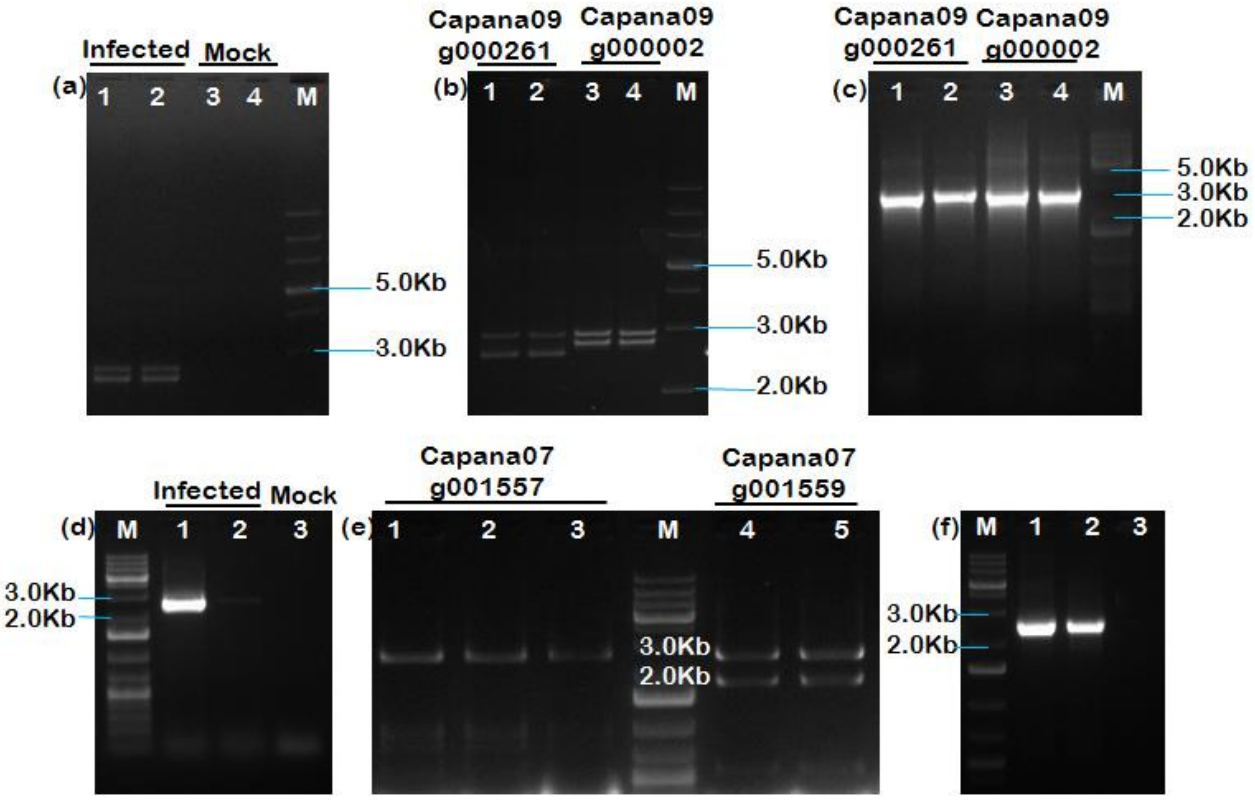


Fig. 4.1 Amplification and cloning of different *NBS-LRR* genes. (a) PCR amplification of *NBS-LRR* genes from chilli cDNA using Bs2 primers. Lane 1 and 2 cDNA was prepared from virus infected chilli plants, lane 3 and 4 cDNA prepared from mock infected chilli plants. M indicates for marker. **(b)** Confirmation of *NBS-LRR* gene in pJET1.2/blunt vector by digestion with *Nco*I. Lane 1 and 2 restriction digestion of *Capana09g000261* and lane 3 and 4 restriction digestion of *Capana09g000002*. **(c)** Confirmation of *NBS-LRR* gene in pJET1.2/blunt vector by PCR. Lane 1 and 2 PCR amplification of *Capana09g000002* and lane 3 and 4 PCR amplification of *Capana09g000261*. **(d)** PCR amplification of NBS-LRR gene from cDNA using RPP primers. Lane 1 and 2 cDNA was prepared from virus infected chilli plants and lane 3 cDNA prepared from mock infected chilli plants, M indicates for marker. **(e)** Confirmation of *NBS-LRR* gene in pJET1.2/blunt vector by digestion with *Bgl*II. Lane 1, 2 and 3 restriction digestion of *Capana07g001557* and lane 4 and 5 restriction digestion of *Capana07g001559*. **(f)** Confirmation of *NBS-LRR* gene in pJET1.2/blunt vector by PCR. Lane 1 PCR amplification of *Capana07g001557* and lane 2 PCR amplification of *Capana07g001559*.

4.1.2 Identification of position of the cloned *NBS-LRR* genes in the chilli genome

All four *NBS-LRR* genes were sequenced and analyzed for the presence of the open reading frame (ORF) with the help of ORF finder online tool (https://www.ncbi.nlm.nih.gov/orffinder/). Further, each ORF sequence was searched for homology in pepper genome database [Pepper Genome Database (release 2.0), http://peppersequence.genomics.cn/page/species/blast.jsp]. ORF finder results indicated that both the genes amplified by RPP primers contained ORF with length of 2649 bp. One of the genes amplified by RPP primers is *Capana07g001557* (length 2649) present in the chromosome 7 spanning between 190552994 to 190550346 nucleotide. The second gene amplified using RPP primers is *Capana07g001559* (length 2649) present in the chromosome 7 spanning between 190592039 to 190589391 nucleotide. One of the genes amplified by Bs2 primers has ORF of 2817 bp in length where as the another is of 2650 bp with multiple stop codons in it. The 2817 bp long gene amplified using Bs2 primers was *Capana09g000002* present in the chromosome 9 spanning between 524655 to 521910 nucleotide. The 2650 bp long transcript contains an ORF of 936 bp length located in the chromosome 9 spanning between 545068 to 545998 nucleotide and shows homology with *Capana09g000261*.

4.1.3 Phylogenetic analysis of the cloned *NBS-LRR* genes

Phylogenetic analysis of different *NBS-LRR* genes was preceded by collection of sequences of same kind of genes of chilli. Full-length sequences of different *NBS-LRR* genes were collected from the pepper genome database (http://peppersequence.genomics.cn/page/species/blast.jsp) by the blast search of known sequences. Finally, 56 different *NBS-LRR* genes were identified and translated to generate protein sequences for the preparation of phylogenetic tree. Phylogenetic tree was prepared by neighbor-joining method using MEGA 6.0 software. Analysis was coupled with well known TIR-NBS-LRR (N, L6 and M) and CC-NBS-LRR (RPM1, Gap2, and Prf) genes, served as positive control. Phylogenetic analysis revealed that all the cloned *NBS-LRR* genes belong to the CC-NBS-LRR category. Results also showed that out of 56 genes 43 belong to the CC-NBS-LRR family and 13 belong to the TIR-NBS-LRR family (Fig. 4.2a). All the NBS-LRR proteins were divided into 8 groups. Group I to VI belong to the CC-NBS-LRR family whereas VII and VIII belong to the TIR-NBS-LRR family (Fig. 4.2a). Capana09g000002

and Capana07g001559 are clustered in group I with Gap2 whereas Capana07g001557 and Capana07g001559 are grouped in group IV which does not have any well known NBS-LRR.

Multiple sequence alignment between P-loop and GLPL domains of different NBS-LRR proteins was done using MUSCLE algorithm. Alignment results reveled that Capana09g000002, Capana07g001557 and Capana07g001559 contain all the conserved domains (P-loop, RNBS-A-non TIR, Kinase-2, RNBS-B, RNBS-C and GLPL) whereas Capana09g000261 lacks the RNBS-C and GLPL domains (Fig. 4.2b).

4.1.4 Screening of ChiLCV responsive *NBS-LRR* gene

Hypersensitivity response (HR) is the characteristic feature of the resistance gene therefore all the cloned *NBS-LRR* genes were tested for the activation of HR. For the screening of ChiLCV responsive *NBS-LRR* gene, all the isolated *NBS-LRR* genes were cloned in binary vector (pBINAR) (Fig. 4.3). pBINAR clones were used for the transient expression of the *NBS-LRR* genes in model plant (*N. benthamiana*) and in the natural plant (*C. annuum*).

For the HR activation study all the *NBS-LRR* genes were transiently expressed in the 6^{th} and 7^{th} (from bottom) leaf of *N. benthamiana* plants. After 48 hrs of *NBS-LRR* infiltration, ChiLCV was also infiltrated in the same leaf. Both the infiltrations have provided the combination of R and avirulence (avr) factors in the same cell. Six days post infiltration of virus, HR lesions were observed at the site of infiltration on the leaf, infiltrated with *Capana07g001557* followed by the virus (Fig. 4.4a). Combinations of other *NBS-LRR* gene (*Capana09g000261, Capana09g000002* or *Capana07g001559*) and virus infiltration did not show significant HR lesion. Infiltration of *Agrobacterium* harboring pBINAR vector followed by infiltration with the virus served as the negative control of the experiment (Fig. 4.4a).

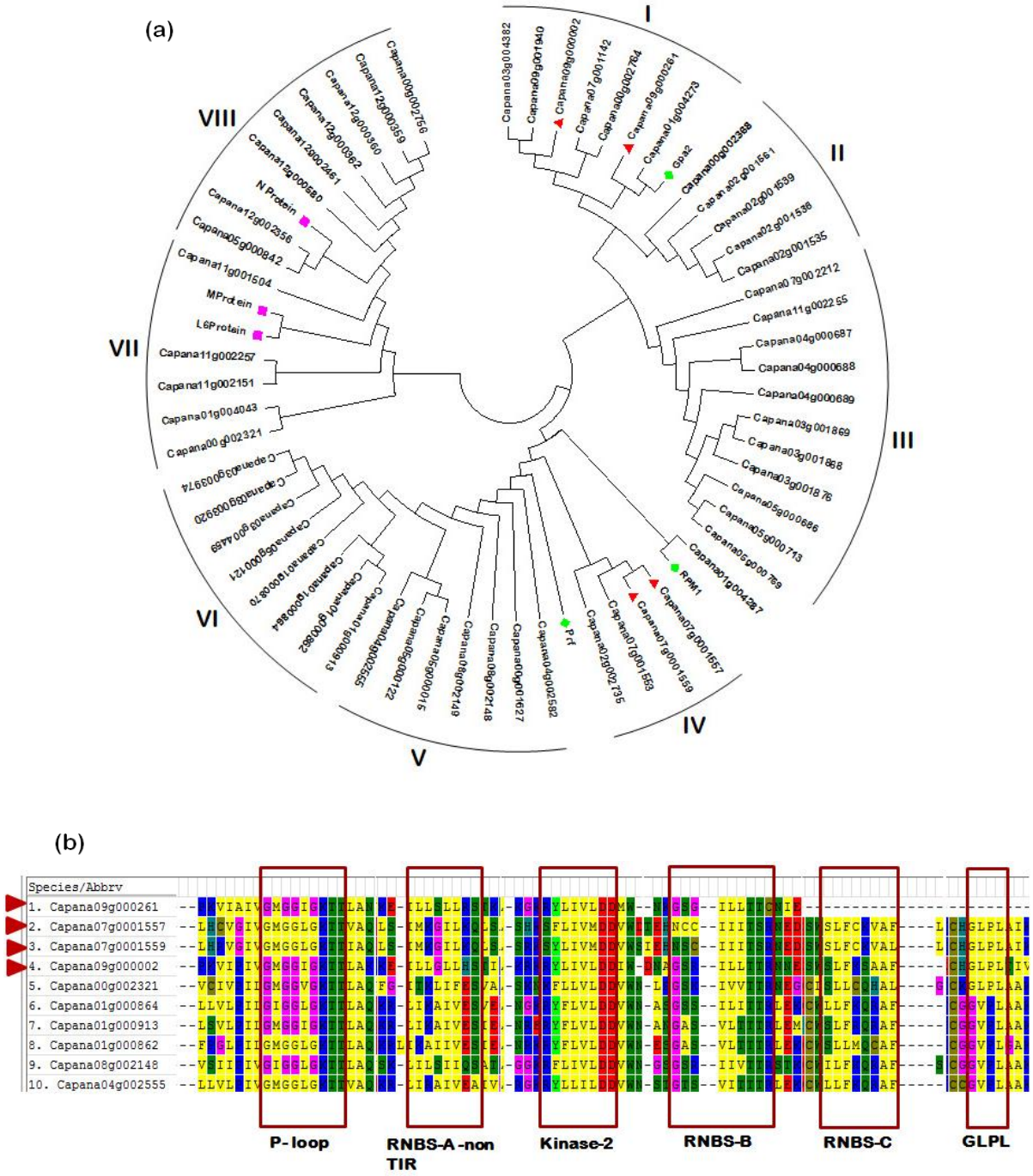


Fig. 4.2 Phylogenetic tree analysis and multiple sequence alignment of different NBS-LRR genes identified in chilli. (a) Phylogenetic tree was prepared by neighbor-joining method using MEGA 6.0 software. 56 full length amino acid sequences of different NBS-LRR proteins were grouped into 8 subgroups. Group I – VI are part of CC-NBS-LRR and VII – VIII belong to the TIR-NBS-LRR sub group. N, L6 and M are well known TIR-NBS-LRR genes indicated with pink square. RPM1, Gap2, and Prf are CC-NBS-LRR genes indicated by green square. **(b)** Multiple sequence alignment of amino acids between P-loop and GLPL domains of different NBS-LRR proteins, conserved domains are highlighted.

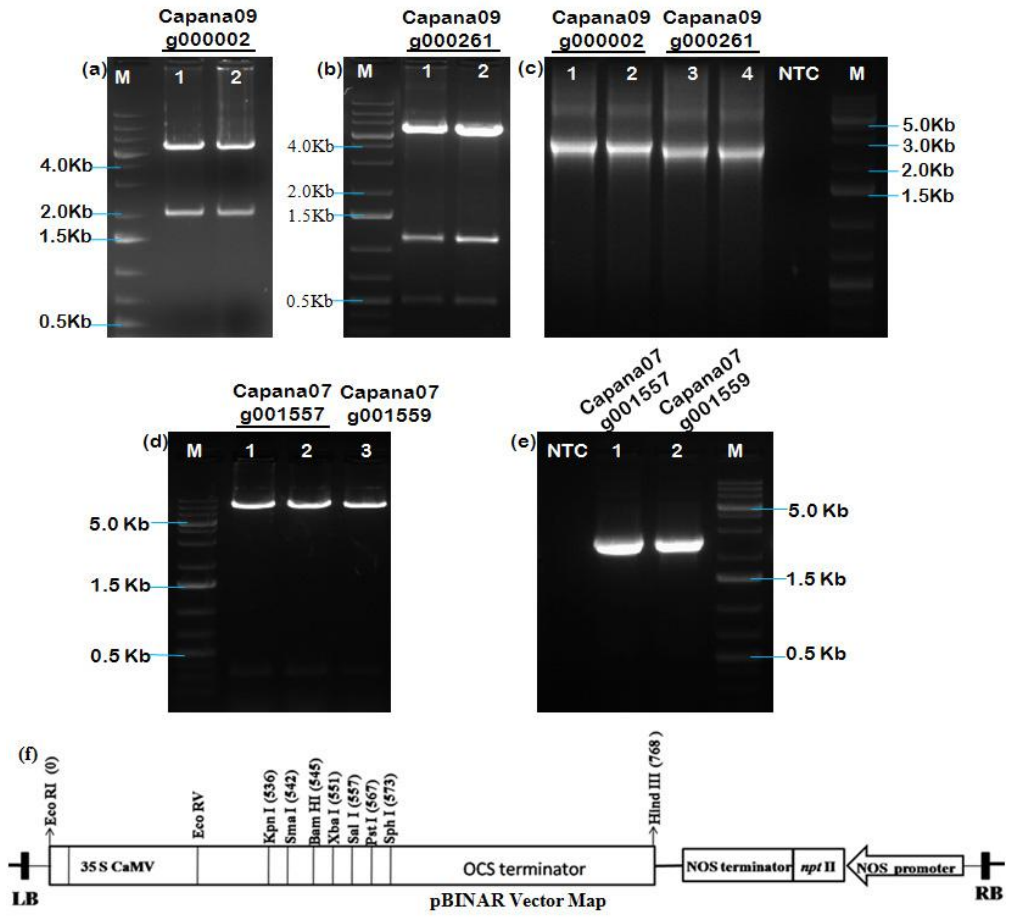


Fig. 4.3 Cloning and confirmation of different *NBS-LRR* genes in binary vector. (a) Confirmation of *Capana09g000002* in pBINAR by restriction digestion with *Kpn*I. **(b)** Confirmation of *Capana09g000261* in pBINAR by restriction digestion with *Pst*I. **(c)** Confirmation of NBS-LRR genes in pBINAR by PCR. Lane 1 and 2 are amplification of *Capana09g000002* and lane 3, 4 amplification of *Capana09g000261*. **(d)** Confirmation of *Capana07g001557* and *Capana07g001559* in pBINAR by restriction digestion with *Pst*I. **(e)** Confirmation of *NBS-LRR* genes in pBINAR by PCR. Lane 1 and 2 are the amplification of *Capana07g001557* and *Capana07g001559* respectively. Non template control (NTC) served as negative control for the amplification. **(f)** pBINAR vector map.

HR is a type of programmed cell death and dead cells can be visualized by trypan blue staining. Further trypan blue staining of all the infiltrated leaves showed cell death because of HR. Staining results also suggested maximum cell death in the leaves infiltrated with both *Capana07g001557* and virus combination. Leaves infiltrated with other combinations were also slightly stained with trypan blue but the intensity of staining was less than that of *Capana07g001557* and virus combination and similar to staining of negative control. Trypan

blue staining of leaves infiltrated with vector, *Capana09g000261, Capana09g000002* or *Capana07g001559* along with virus may be because of the cell death due to the compatible interaction between the virulence factors present in *Agrobacterium* and host factor.

Leaves of the chilli plants are sensitive for agroinfiltration and get detached from plant after 4 day post infiltration. Keeping it in mind agroinfiltration of different *NBS-LRR* genes was done together with virus. In chilli plants similar results were observed like that of *N. benthamiana*. HR was observed on the leaves infiltrated with *Capana07g001557* and virus at 4 days post infiltration (Fig. 4.4c). Leaves infiltrated with either *Capana09g000261, Capana09g000002, Capana07g001559* or vector together with virus did not show any HR lesion till 4 days post infiltration (Fig. 4.4c). Trypan blue staining of agroinfiltrated leaves of chilli showed blue patches only after the infiltration of *Capana07g001557* and ChiLCV (Fig. 4.4c). Combination of other *NBS-LRR* genes and ChiLCV infiltration does not show trypan blue staining of leaf (Fig. 4.4d).

4.1.5 *Capana07g001557* reduces ChiLCV DNA accumulation in *N. benthamiana* Leaf disk

Previous studies have suggested that the R-avr interaction induces hypersensitivity response and reduces the titer of pathogen. Therefore, leaf disk assay was performed to analyze the effect of different *NBS-LRR* genes on virus accumulation. *N. benthamiana* leaf disks were infected with *Agrobacterium* harboring different *NBS-LRR* genes cloned in pBINAR vector along with ChiLCV. Level of ChiLCV accumulation in leaf disks was analyzed with the help of Southern blot hybridization (Fig. 4.5). ChiLCV-AC1 specific probe labeled with αP^{32} was used for the detection of virus. Virus level was examined in three leaf disks of each combination to make it statistically correct. Results indicated reduced level of viral titer in the leaf disks infected with the virus and *Capana07g001557* containing *Agrobacterium* compare to the other R-genes and virus infected leaf disks (Fig. 4.5). Out of three leaf disks infected with virus and *Capana07g001557* combination, two were showing highly reduced virus level but one was showing viral titer similar to the other combinations R-genes and virus. Leaf disks infected with *Capana09g000261, Capana09g000002* or *Capana07g001559* containing *Agrobacterium* along with ChiLCV have showed virus level similar to the negative control (Fig. 4.5). Here level of

DNA accumulation in leaf disks infected with ChiLCV and pBINAR vector served as negative control. This result suggests that *Capana07g001557* reduces the viral DNA accumulation.

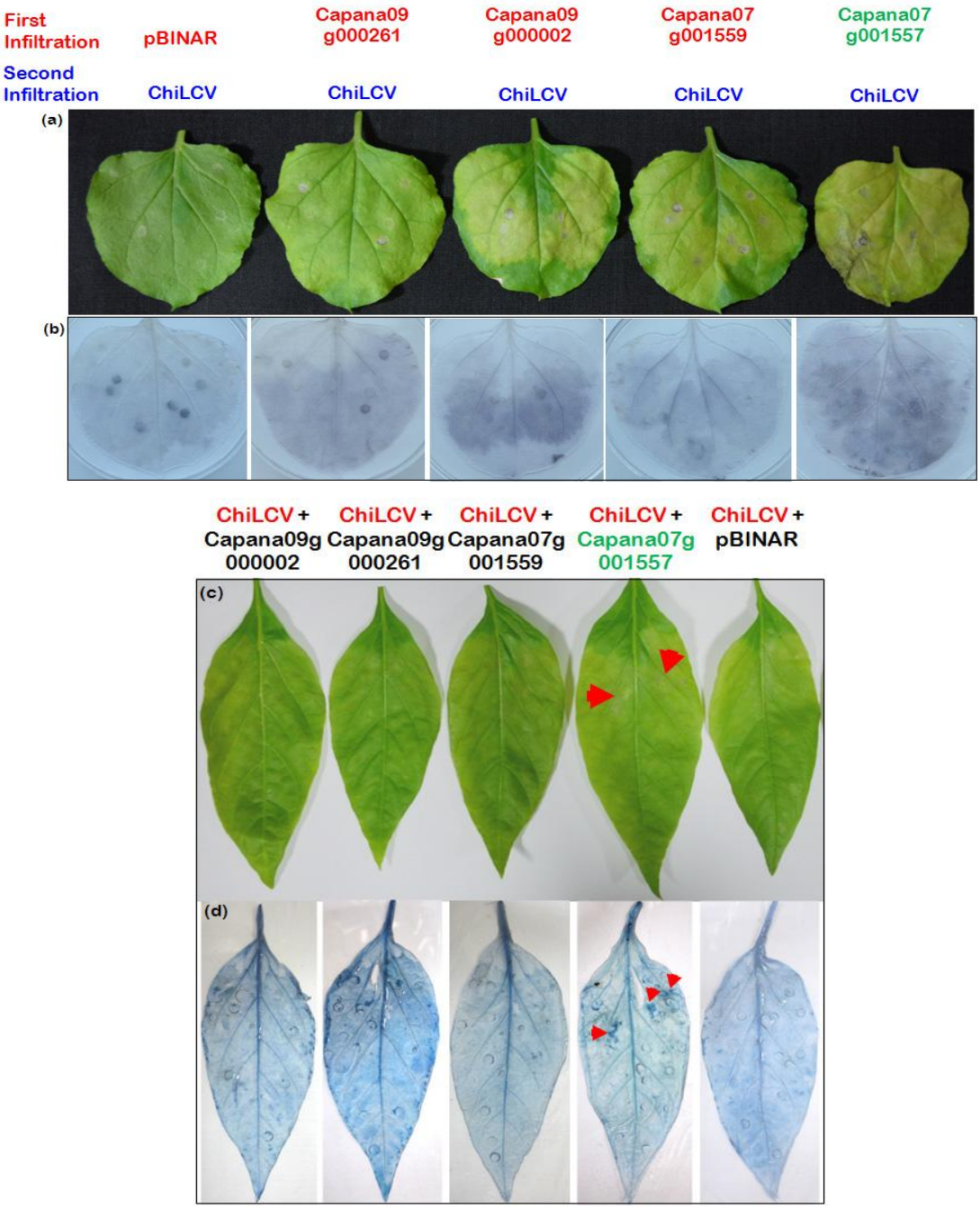


Fig. 4.4 HR activation by different *NBS-LRR* genes. (a) *N. benthamiana* leaves on 6 days after second infiltration, first agroinfiltration was done with different *NBS-LRR* genes followed by the second agroinfiltration of ChiLCV. **(b)** Trypan blue staining of agroinfiltrated *N. benthamiana* leaves. **(c)** Chilli leaves co-infiltrated with different *NBS-LRR* genes and ChiLCV in 4 days post infiltration. Red color arrow indicates the HR lesion on leaf. **(d)** Trypan blue staining of agroinfiltrated chilli leaves. Red color arrow indicates the trypan blue stained dead cells of chilli leaf.

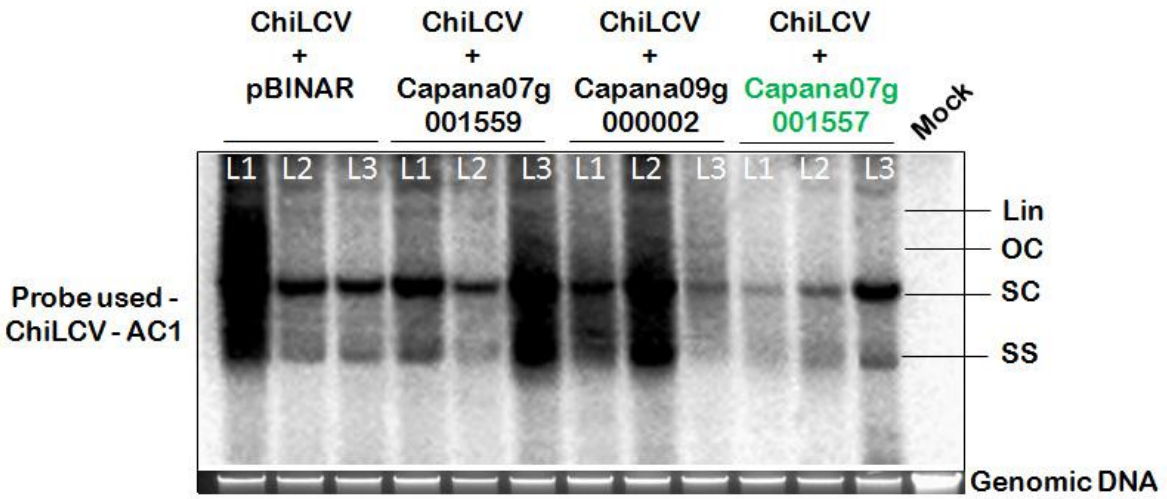


Fig. 4.5 Effect of *Capana07g001557* on viral DNA accumulation. A comparative analysis of the viral DNA accumulation in *N. benthamiana* leaf disks infected with ChiLCV along with different *NBS-LRR* genes. αP^{32} labeled ChiLCV-AC1 specific probe was used for southern blot hybridization. Different replicative forms of viral DNA are abbreviated as linear (Lin), open circular (OC), supercoiled (sc) and single-stranded (ss). Genomic DNA isolated from leaf disks stained with ethidium bromide (EtBr) served as the loading control.

4.2 To understand role of *NBS-LRR* during ChiLCV infection

Previous results have suggested that out of four cloned *NBS-LRR* genes, *Capana07g001557* is ChiLCV-responsive gene. Further experiments were performed to understand the role of *Capana07g001557* against ChiLCV infection.

4.2.1 ChiLCV-AC2 ORF enhances the expression of *Capana07g001557* in the resistant cultivar of chilli

Plants always have a basic level of expression of *NBS-LRR* gene which further gets enhanced in the presence of compatible pathogen. Kushwaha et al. (2015b) have shown that the expression of *Capana07g001557* increases after the infection of ChiLCV. To find out the viral protein responsible for the induction of *Capana07g001557* expression, gene expression was analyzed in the presence of each viral protein in chilli leaves. Each of the viral ORFs was transiently expressed in the 5th leaf (from bottom) of *C. annuum* cv. Punjab Lal. Expression of *Capana07g001557* was analyzed 4 days post infiltration by qRT-PCR. Mock (agroinfiltration of

empty vector) expression was considered as negative control. qRT-PCR results indicated that expression of the *Capana07g001557* was significantly increased ($p<0.001$) by the AC2 ORF of ChiLCV as compared to the mock (Fig. 4.6). *Capana07g001557* expression was unaffected in the leaves infiltrated with the other viral ORFs (AC1, AC3, AC4, AV1, AV2, βC1), difference in expression compared to the mock is statistically not significant (Fig. 4.6). Expression of *Capana07g001557* was also increased significantly ($p<0.001$) in the leaves infiltrated with the viral genome in comparison to the mock. Difference in *Capana07g001557* expression between AC2 ORF and ChiLCV is also statistically significant (Fig. 4.6).

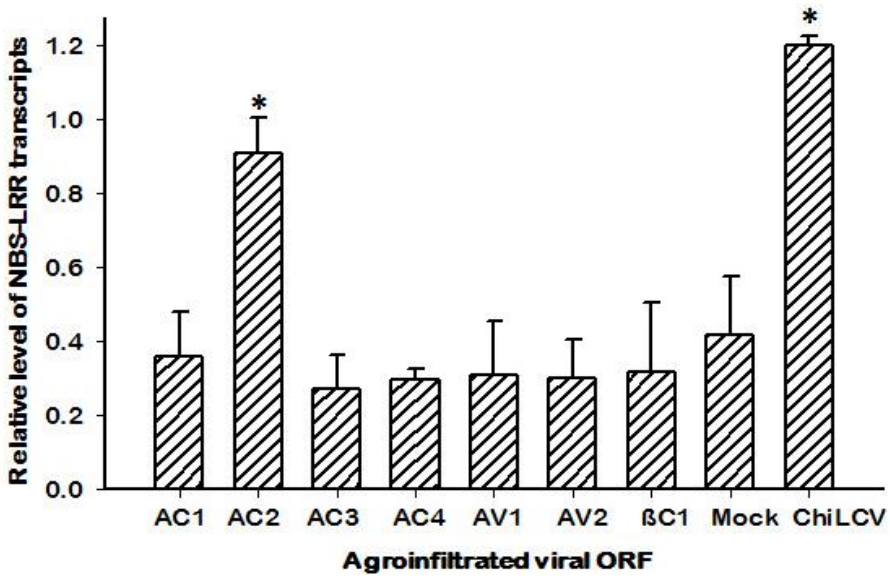


Fig. 4.6 Relative accumulation of Capana07g001557 transcripts. Expression of *Capana07g001557* gene in *C. annuum* cv. Punjab Lal leaves after agroinfiltration of different ChiLCV ORFs. Expression was analyzed in 5th leaf of chilli plants infiltrated with each viral ORF of ChiLCV with the help of qRT-PCR. * indicates the statistically significant upregulation of the gene expression compare to the mock.

4.2.2 Sub-cellular Localization of Capana07g001557

To understand the function of a protein it is necessary to know the site of function of protein in the cell. For sub-cellular localization study of the Capana07g001557 we have generated Capana07g001557 fused with green fluorescent protein (GFP) at the C-terminal end and driven by the CaMV35S promoter (Fig. 4.7i). Fusion construct was transformed into *Agrobacterium* cells and transiently expressed in *N. benthamiana* leaves. Microscopic study of the epidermal

cell revealed presence of Capana07g001557-GFP at the cell periphery (Fig. 4.7c), whereas the only GFP is spreaded through out the cell (Fig. 4.7h). DAPI actively binds to the AT rich DNA and produce blue color fluorescence so used as a marker of the nucleus (Fig. 4.7d). FM4-64 is red color dye and stains the cell membrane, vacuolar membrane and if stained for long time can also stain membrane of the big organelles (nucleus, ER) (Fig. 4.7b). GFP fluorescence of Capana07g001557-GFP does not overlap with DAPI fluorescence (Fig. 4.7a, f) but overlap with the FM4-64 fluorescence only at the cell periphery (Fig. 4.7a, f). Capana07g001557-GFP was also infiltrated with ChiLCV to show the localization of Capana07g001557 in the presence of avr factor. Again, Capana07g001557-GFP was found to be localized at the cell periphery (Fig. 4.7g). These results suggest that Capana07g001557 localizes at the cell membrane either presence or absence of virus and does not go into the nucleus.

4.2.3 ChiLCV-AC2 ORF induces HR in the presence of Capana07g001557

Resistance gene activates the hypersensitivity response in the presence of avirulence (avr) factor of pathogen. To identify the viral protein that acts as the avr protein and induces the HR activity by Capana07g001557, *Capana07g001557* gene was transiently over expressed followed by the over expression of each viral protein after 48 hrs, in same leaf (6^{th} and 7^{th} from bottom). Over expression of Capana07g001557 and ChiLCV AC2 proteins resulted in strong HR induction at 6 days post infiltration of the viral ORF (Fig. 4.8). Capana07g001557 and ChiLCV AC3 expression have also showed small HR lesion. Agroinfiltration of *Capana07g001557* with other viral ORF (AC1, AC4, AV1, AV2 or βC1) did not show any HR lesion (Fig. 4.8). None of the viral ORFs in the presence of empty vector also could induce HR lesion (Fig. 4.8). Over expression of *Capana07g001557* was also unable to activate HR till 8 days post infiltration (Fig. 4.8).

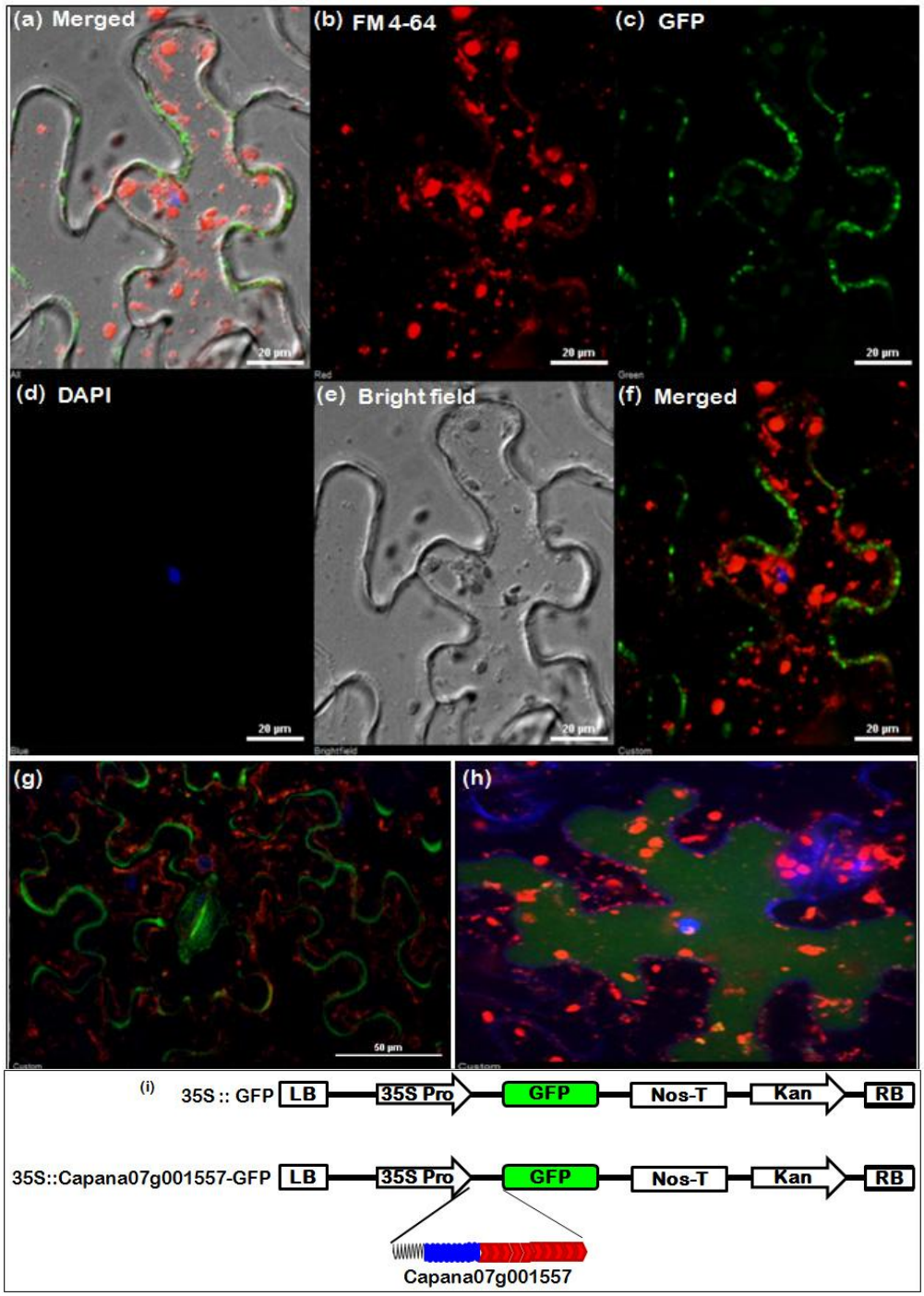


Fig. 4.7 Subcellular localization of Capana07g001557 protein in the epidermal cell of *N. benthamiana* leaf. (a) Merged picture of GFP fluorescence, DAPI stain and FM 4-64 stain cell against bright field background. **(b)** FM 4-64 stained cell. FM 4-64 is the marker of cell membrane and vacuolar membrane. **(c)** Fluorescence of GFP fused with Capana07g001557. **(d)** DAPI stained cell. DAPI is a marker of the nucleus. **(e)** Bright field image of epidermal cell. **(f)** Merged picture of GFP fluorescence, DAPI stain and FM 4-64 stain cell against dark background. **(g)** Localization of Capana07g001557 protein in presence of ChiLCV. **(h)** Localization of only GFP. **(i)** Constructs used for the localization study.

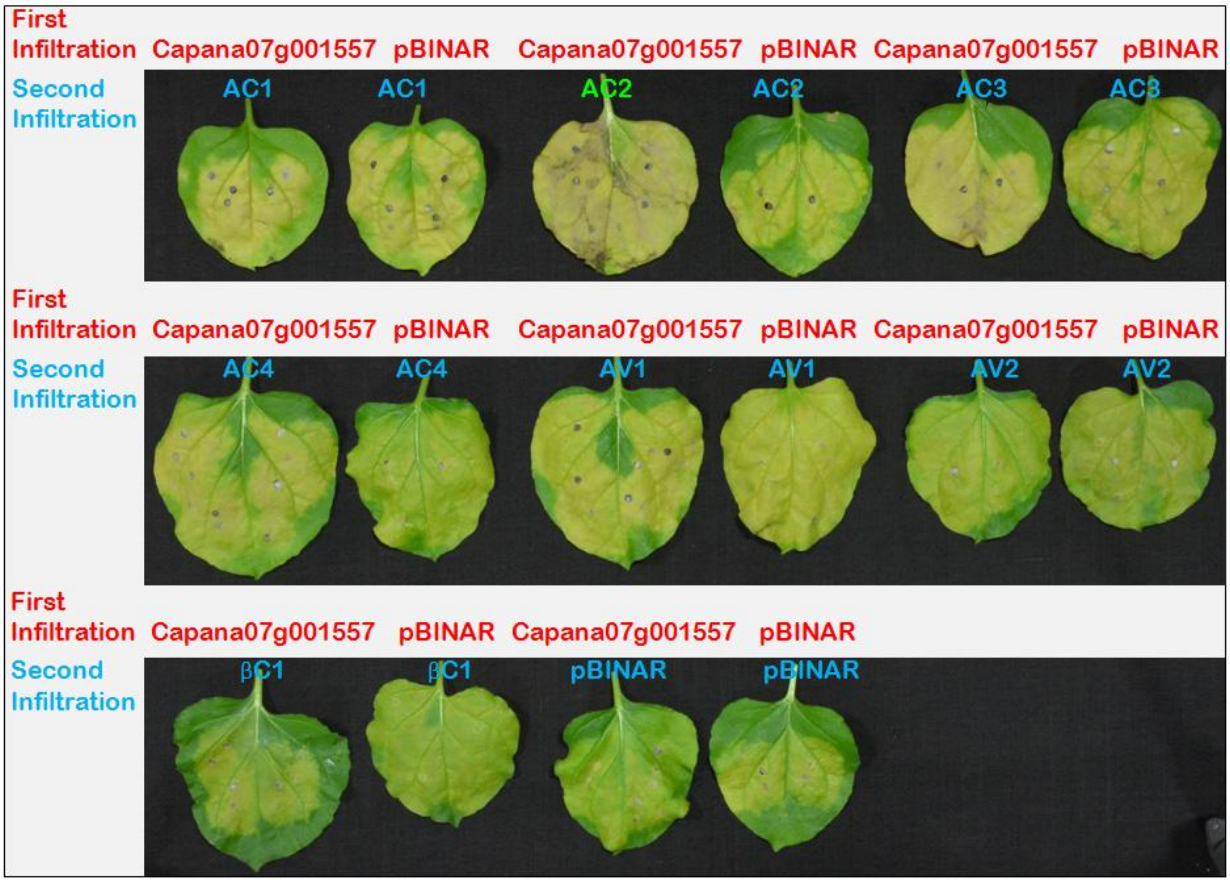


Fig. 4.8 Screening of HR inducing viral ORF. Agroinfiltration of *Capana07g001557* followed by the infiltration of different ChiLCV ORFs. Photograph was taken 6 days after second infiltration. Infiltration of *Capana07g001557* succeeded by the AC2 infiltration resulted in prominent HR induction, infiltration of AC3 also induced small HR lesion.

4.2.4 Capana07g001557does not interact physically with any viral protein

Direct interaction of NBS-LRR protein with the pathogen avr protein is very rare but few instances are known (Jia et al. 2000; Deslandes et al. 2003; Dodds et al. 2006). To identify, if any viral protein interact with Capana07g001557, yeast two hybrid (Y2H) experiment was performed. All the viral ORFs (AC1, AC2, AC3, AC4, AV1, AV2 and βC1) were cloned at the downstream of the activation domain to generate the fusion protein in yeast expression vector (pGADT7). *Capana07g001557* was cloned downstream of the binding domain in pGBKT7 vector. Yeast cells were co-transformed with *Capana07g001557* and any viral ORF containing constructs and grown on 2 drop out media (2-DO). Cells grown on 2-DO media were further transferred to the 3 drop out (3-DO) media and 3-DO to 3-DO+3AT media. It was observed that

yeast cells co-transformed with *Capana07g001557* and any of the viral ORF did not grow on 3-DO+3mM 3AT medium (Fig. 4.9). Results of Y2H experiment indicated that none of the viral protein directly interacts with Capana07g001557. Yeast cells co-transformed with the constructs served as positive control were grown on 3-DO+3mM 3AT medium after 72 hrs (Fig. 4.9). For the experiment, interaction of murine p53 protein (fused with GAL4 DNA binding domain) and SV40 large T-antigen protein (fused with GAL4 activation domain) was used as positive control. Yeast cells co-transformed with vectors (containing activation domain and binding domain) without any insert were used as negative controls.

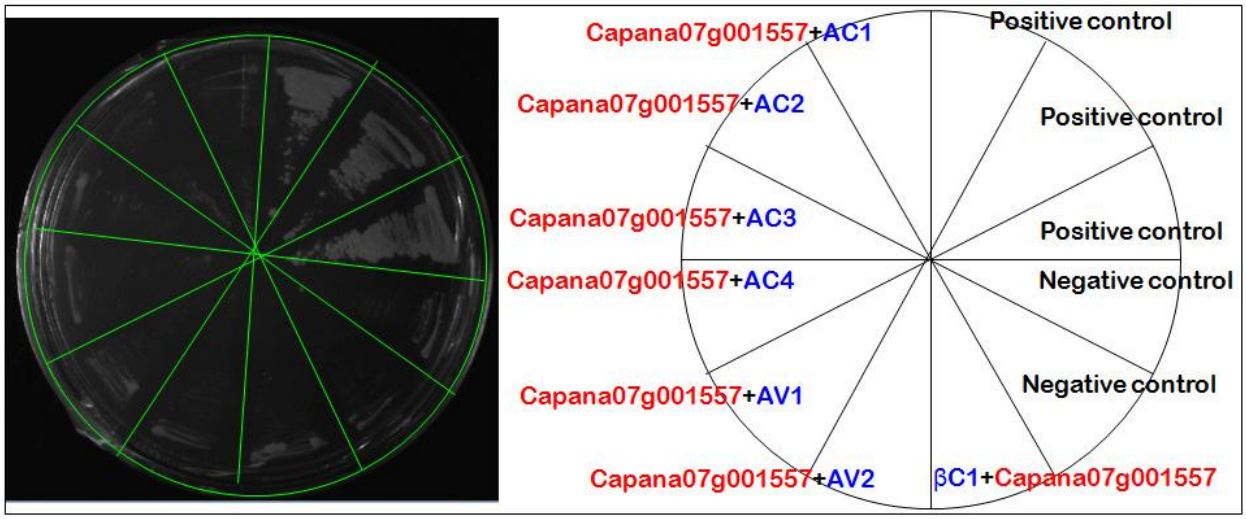


Fig. 4.9 Interaction between Capana07g001557and virus encoded proteins through Y2H assay. Yeast cells grown on 3-DO+3mM 3AT plate at the left side. Constructs used for the co-transformation of yeast cells are indicated at the right side, at same position where yeast cells were streaked at the left side.

4.2.5 Study of Capana07g001557 interacting proteins

Previous result has shown that none of the viral protein interacts with Capana07g001557. Earlier studies have shown that most of the NBS-LRR proteins do not interact directly with the pathogen effector but scan the status of host proteins targeted by pathogen effector (guard hypothesis). To identify the proteins interacting with Capana07g001557 which may be targeted by effector of ChiLCV, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) web resource was used. STRING is a biological database and web resource of known and predicted protein–protein interactions. STRING results showed that Capana07g001557 have maximum homology

with Solyc07g053010.1.1 RPP13-like protein 4-like protein of *Solanum lycopersicum*. STRING has also predicted that this protein may have interaction with Cyt-c1, HSP90 and SGT1. Interaction of Capana07g001557 with Cyt-c1, HSP90 and SGT1 was inspected with the help of Y2H experiment. Yeast cells co-transformed with the combination of *Capana07g001557* and other host gene (*Cyt-c1*, *HSP90* or *SGT1*) grown in 2-DO media and from 2-DO to 3-DO subsequently 3-DO 3mM+3AT media (Fig. 4.10). Other than the positive control, no yeast cells co-transformed with combination of *Capana07g001557* and other host gene could grow on 3-DO+3mM 3AT media (Fig. 4.10). To study the oligomerization property of Capana07g001557, *Capana07g001557* gene was cloned in both the vectors and co-transferred into the yeast cells. These co-transformed yeast cells were also not grown on 3-DO 3mM+3AT media (Fig. 4.10). These Y2H results indicated that Capana07g001557 neither forms oligomers nor interact with other host proteins (Cyt-c1, HSP90 or SGT1).

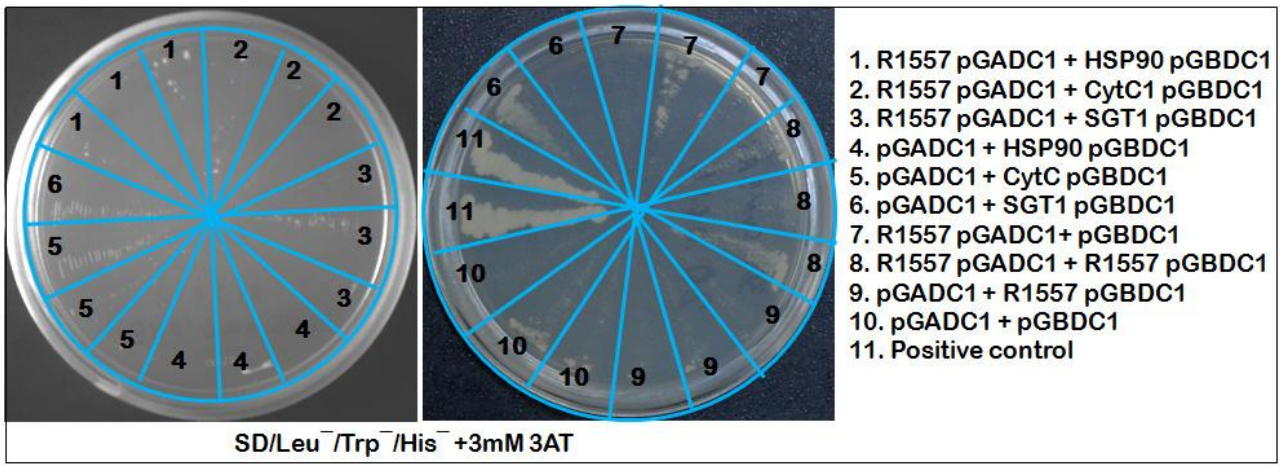


Fig. 4.10 Interaction study between Capana07g001557 and other host proteins through Y2H. Yeast cells co-transformed with different combinations of genes were grown on 3-DO+3mM 3AT plate. Constructs used for the co-transformation of yeast cells are provided at the right side. Three different colonies of yeast cells transformed with each combination were streaked on the 3-DO+3mM 3AT plates.

4.2.6 Over expression of *Capana07g001557* reduces spread of ChiLCV in infected cells

For the visualization of number of ChiLCV infected cells, ChiLCV based *GFP* expression vector (ChiLCV-*EGFP*) prepared by Kushwaha and Chakraborty, (2017) was used. In this vector ChiLCV-AV1 ORF was replaced with *EGFP* (enhanced green fluorescence protein) gene.

Agroinfiltration of ChiLCV-*EGFP* together with ChiLCV in *N. benthamiana* leaves followed by microscopic analysis showed green fluorescence in the virus infected cells. Infectivity of the virus can be measured by counting the number of green fluorescing cells. In this study, to find out the effect of *Capana07g001557* over expression on virus infectivity, different *NBS-LRR* genes (*Capana09g000261*, *Capana09g000002*, *Capana07g001557* and *Capana07g001559*) cloned in pBINAR vector were infiltrated in *N. benthamiana* leaves followed by the infiltration of ChiLCV-*EGFP*+ChiLCV. At 6 days post infiltration, microscopic study showed the number of cells expressing GFP in leaves infiltrated with *Capana09g000261*, *Capana09g000002*, or *Capana07g001559* was higher than the leaves infiltrated with *Capana07g001557* (Fig. 4.11a, 4.11b). In this experiment infiltration of pBINAR vector followed by the infiltration of ChiLCV-*EGFP*+ChiLCV used as control. Difference between the number of cells expressing GFP in the leaves infiltrated with *Capana09g000261*, *Capana09g000002*, or *Capana07g001559* and control was not significant (Fig. 4.11b). *Capana07g001557* expressing leaves showed significant reduction of number of GFP expressing cells as compared to the control and the signals were limited to a few cells only (Fig. 4.11b). Microscopic analysis suggests that Capana07g001557 reduces the infectivity of ChiLCV.

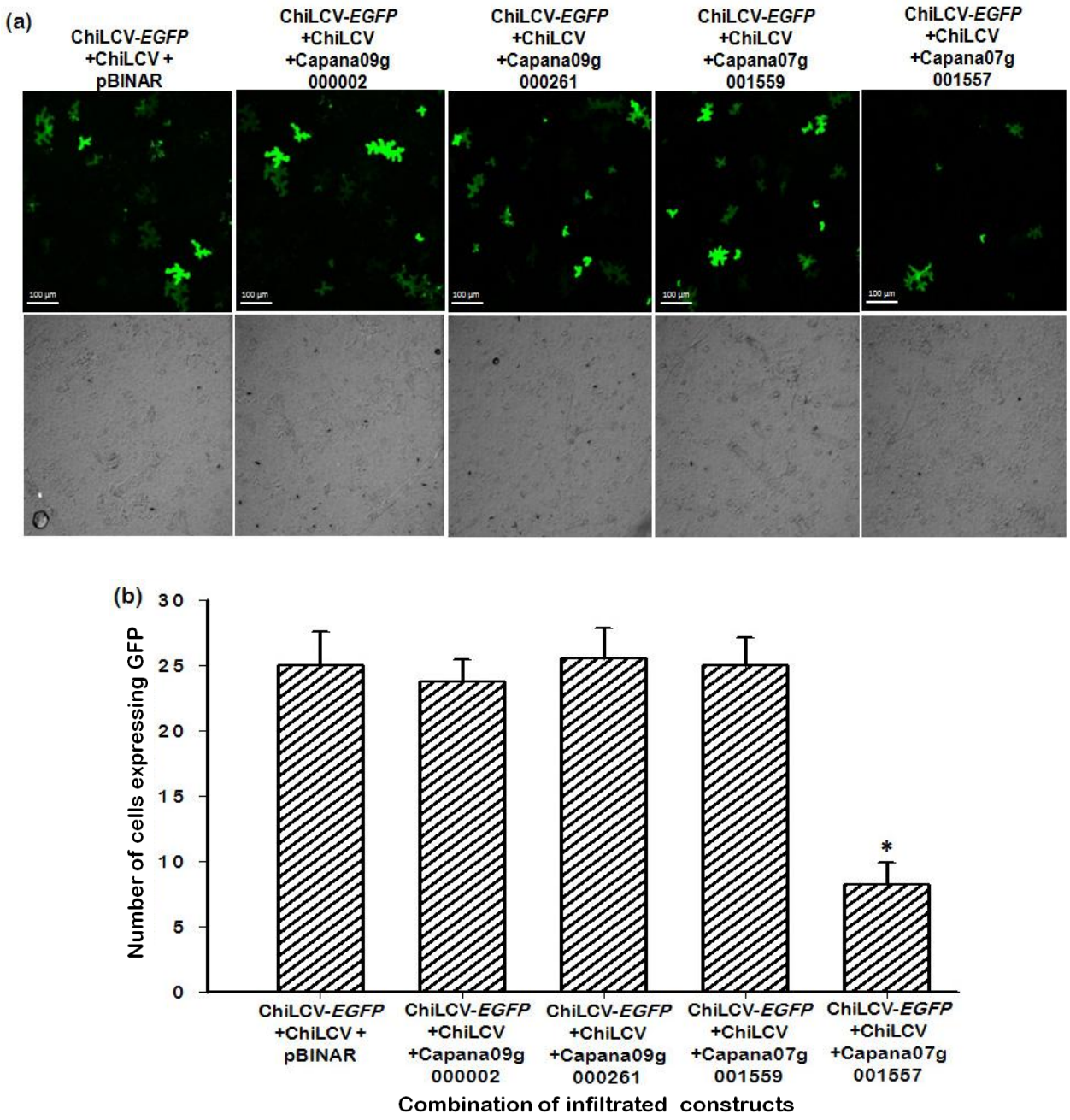


Fig. 4.11 Effect of different *NBS-LRR* genes on infectivity of ChiLCV. (a) Agroinfiltration of different *NBS-LRR* genes followed by the infiltration of ChiLCV-*EGFP*+ChiLCV. The scale bar measures 100 µM. Each green fluorescing cell presents the ChiLCV infected *N.* benthamiana cell. **(b)** Graphical representation of number of GFP expressing cells in the leaf of *N. benthamiana* infiltrated with different combinations of *NBS-LRR* genes and ChiLCV-*EGFP*+ChiLCV. * indicates the statistically significant difference in number of GFP expressing cells compare to the control.

4.2.7 Over expression of *Capana07g001557* also reduces the viral titer at the systemic part of plant

Previous results have shown that infection of ChiLCV along with *Capana07g001557* reduces the viral titer in leaf disks in comparison with the other *NBS-LRR* and negative control. *Capana07g001557* also reduces the infectivity of ChiLCV. We further tried to find the effect of *Capana07g001557* on viral DNA accumulation at the systemic leaf (located distally) of the plant. For the analysis of viral DNA accumulation at the systemic leaf of the plant, 6-7 leaf stage *N. benthamiana* plants were infiltrated with different cloned *NBS-LRR* genes in 3rd and 4th leaf. After 48 hrs of first infiltration same leaves were infiltrated with ChiLCV. Symptoms were observed on the plants at 28 days post virus infiltration (Fig. 4.12a). Plants infiltrated with pBINAR vector followed by the infiltration of ChiLCV showed severe symptoms and served as control (Fig. 4.12a). Plants infiltrated with *Capana09g000261*or *Capana09g000002* followed by the ChiLCV also showed the severe symptoms similar to the control plants (Fig. 4.12a). Infiltration of *Capana07g001559* followed by ChiLCV showed less severe symptoms than the negative control while *Capana07g001557* infiltration showed no symptoms (Fig. 4.12a).

Viral DNA accumulation in the systemic leaves of *N. benthamiana* plants was analyzed with the help of Southern blotting. Concurrent to the symptom severity, viral DNA accumulation was also found to be higher in the plants infiltrated with pBINAR vector, *Capana09g000261* and *Capana09g000002* as compared to the *Capana07g001559* infiltrated plants (Fig. 4.12b). Plants infiltrated with *Capana07g001557* followed by ChiLCV infiltration showed viral DNA accumulation below the detection level of Southern but was detected by PCR using specific primers (Fig. 4.12b).

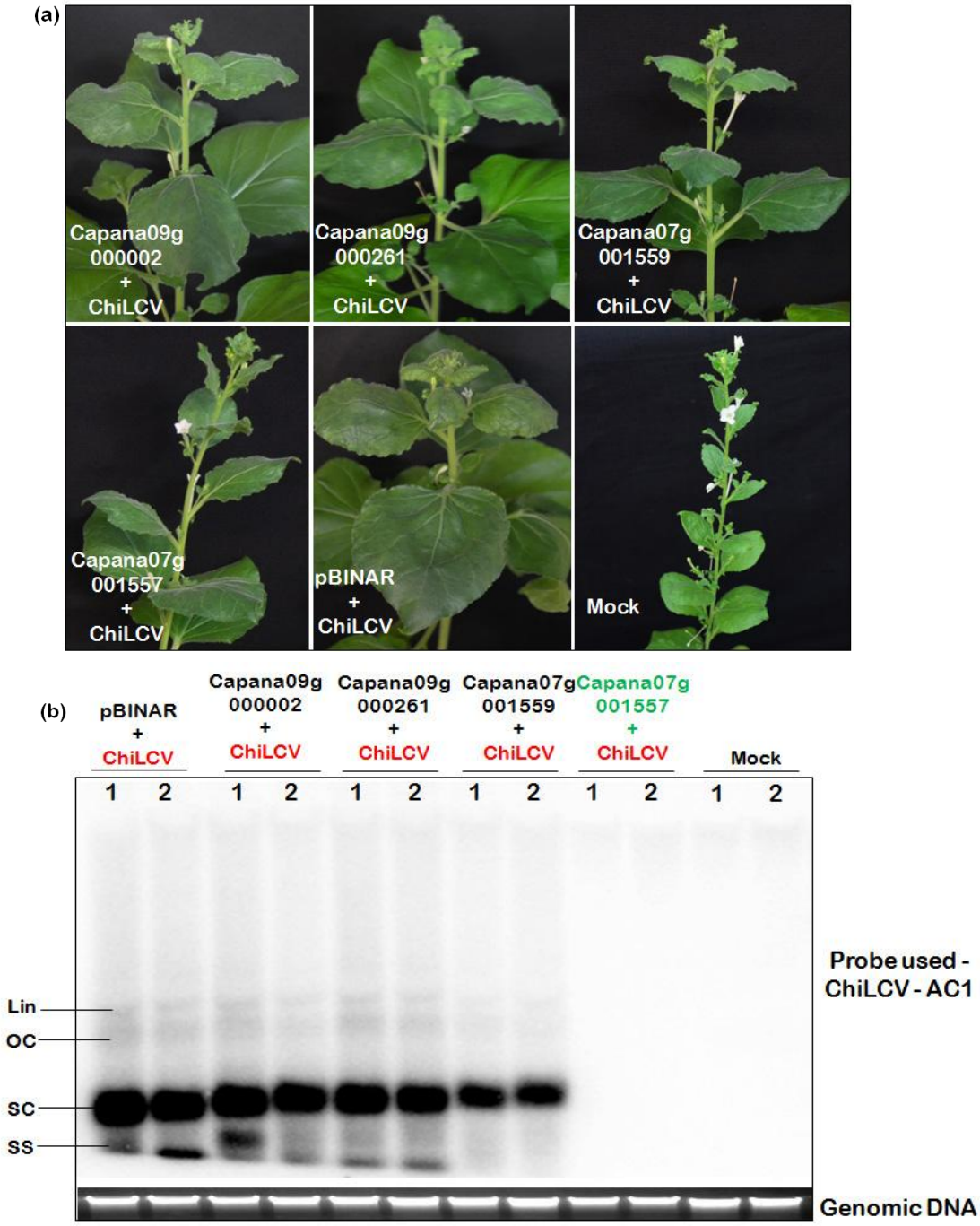


Fig. 4.12 Effect of different *NBS-LRR* genes on accumulation of viral DNA in systemic leaves. (a) Symptoms on the upper leaves of plants, infiltrated with different *NBS-LRR* genes followed by the ChiLCV infiltration in lower leaves of the plants. **(b)** Detection of ChiLCV DNA accumulation in the leaves of above plants with the help of southern blotting. Viral DNA titer was inspected in two different plants infiltrated with each combination. Different replicative forms of viral DNA are abbreviated as linear (Lin), open circular (OC), supercoiled (sc) and single-stranded (ss). Genomic DNA isolated from leaves stained with ethidium bromide (EtBr) served as the loading control.

4.2.8 Capana07g001557 dimerizes in planta in presence of ChiLCV

Previous reports have shown that R-protein can dimerize (Bernoux et al. 2011) but the Y2H results indicated that two Capana07g001557 protein molecules does not interact physically. To examine whether Capana07g001557 dimerizes in the presence of avirulence (avr) factor of virus, bimolecular fluorescence complementation (BiFC) experiment was performed. For the experiment *Capana07g001557* gene was cloned in both the BiFC vectors pSPYCE(M) and pSPYNE(R)173. pSPYCE(M) construct produces fusion protein contain C-terminal YFP (eYFP, C155) and Capana07g001557 at the N-terminal of YFP. pSPYNE(R)173 encodes fusion protein contain N-terminal YFP (eYFP, N 173) and full length Capana07g001557 at the C-terminal of YFP. Equimolar mixture of *Agrobacterium* harboring each type of construct was infiltrated in the *N. benthamiana* leaves, microscopic analysis did not show any fluorescence at 6 days post infiltration (Fig. 4.13). Agroinfiltratoin of both the constructs (*Capana07g001557* in pSPYCE(M) and *Capana07g001557* in pSPYNE(R) 173) along with ChiLCV showed fluorescence at the cell periphery on 6 days post infiltration (Fig. 4.13). Leaves infiltrated with *Capana07g001557* in pSPYCE(M)+pSPYNE(R)173+ChiLCV; either *Capana07g001557* in pSPYNE(R)173+ pSPYCE(M)+ ChiLCV or pSPYCE(M)+ pSPYNE(R)173+ ChiLCV was used as negative control. None of the negative control showed fluorescence under microscopy (Fig. 4.13). DAPI staining was carried out to stain the nucleus. FM4-64 staining was used as the marker for the cell membrane and vacuolar membrane. Microscopic results indicated that Capana07g001557 dimerizes in plant cell in the presence of ChiLCV. YFP fluorescence overlaps with the FM4-64 fluorescence at the cell periphery indicates that dimerization of Capana07g001557 occurs at the cell membrane.

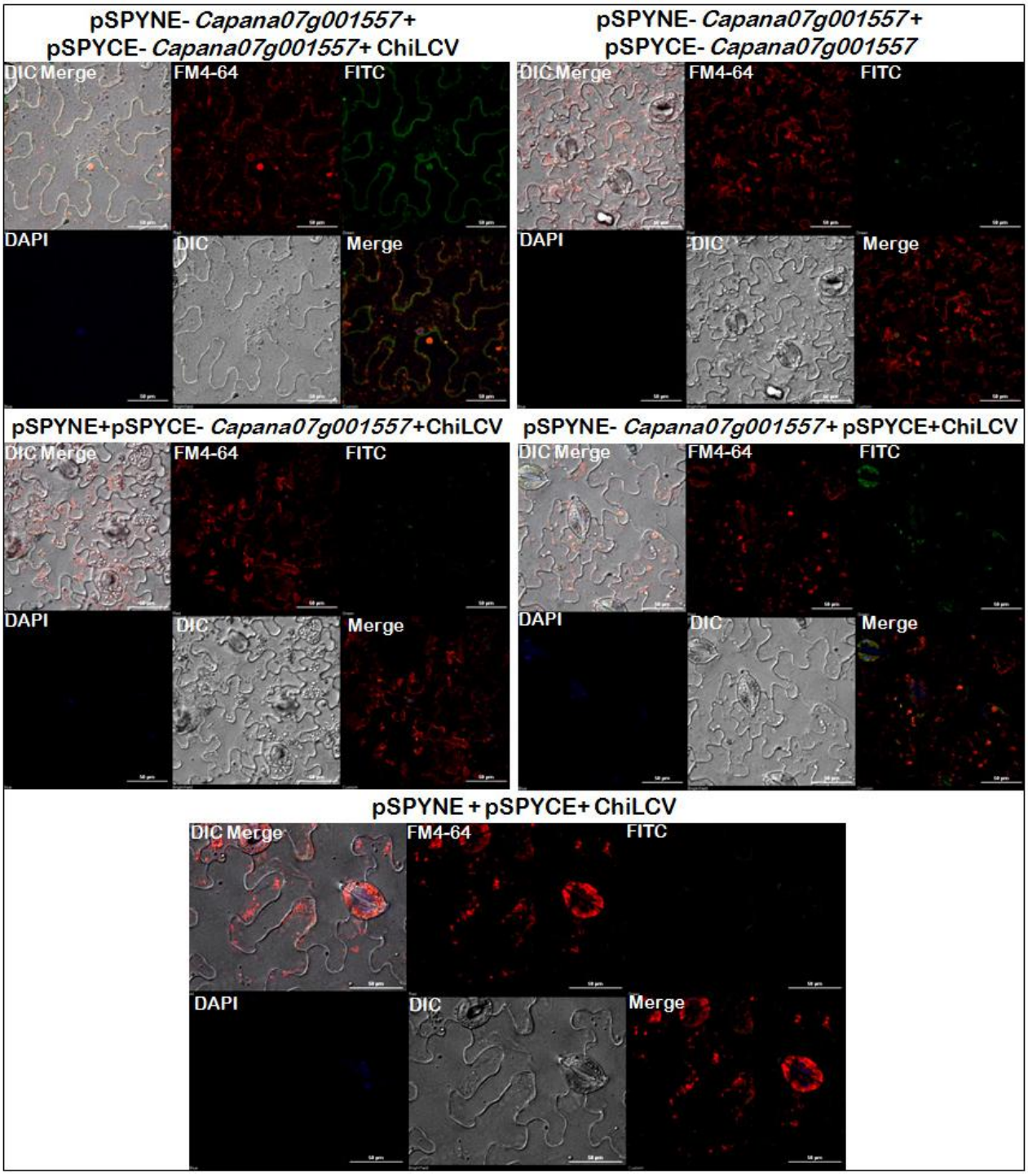


Fig. 4.13 Dimerization analysis of Capana07g001557 in planta through BiFC assay. Combination used for the infiltration of *N. benthamiana* leaf is mentioned at the top of figure. Nucleus was stained with DAPI, cell and vacuolar membrane was stained with FM4-64. Positive interaction of proteins resulted into the fluorescence on the membrane of whole cell uniformly. Each combination has the picture of DAPI, FM4-64 and FITC fluorescence; DIC image and merge picture against bright and dark background. Scale bar measures 50 µM.

4.2.9 Generation of chilli transgenic plant over expressing Capana07g001557

To study the role of *Capana07g001557* in chilli, *Capana07g00155* over expressing transgenic chilli (*C. annuum* cv. Kashi Anmol) plants were prepared. For the transgenic preparation the Kashi Anmol cultivar of chilli was selected because it is a ChiLCV-susceptible cultivar and the expression of *NBS-LRR* does not change significantly after ChiLCV infection (Kumar et al. 2006; Kushwaha et al. 2015a). Transgenic plants were regenerated from hypocotyl as mentioned in the materials and methods section. *Agrobacterium* EHA105 cells harboring *Capana07g001557* cloned in the pBINAR vector were used for the transgenic preparation. All the regenerated putative transgenic plants showed normal phenology (plant height, site of insertion of first pod, number of branches, internode length, foliar area, and total number of flowers and pods) compared to non-transgenic chilli plants (Fig. 4.14). Transgenic preparation of chilli was initiated with the 520 hypocotyls.

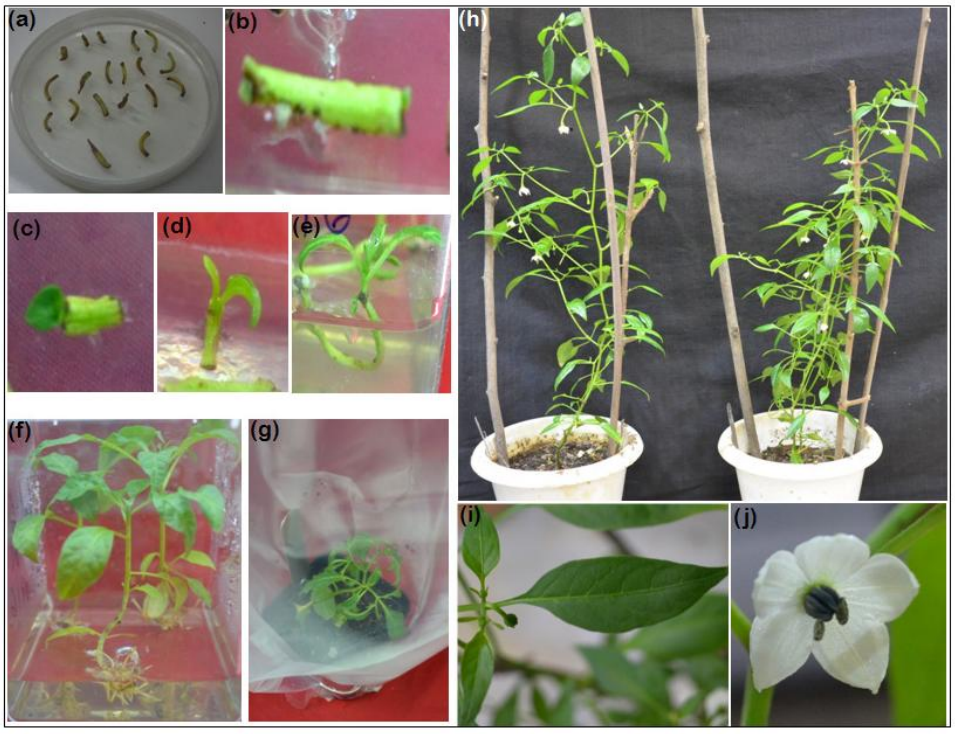


Fig. 4.14 Development of transgenic chilli (*C. annuum* cv. Kashi Anmol) plants overexpressing Capana07g001557. Transgenic plants at different stages of development. **(a)** Untransformed hypocotyls died in selection media. **(b)** Transformed hypocotyl showing green out growth. **(c)** and **(d)** Plantlet developed from green out growth. **(e)** Plantlets transferred onto shooting media. **(f)** Plantlet transferred in rooting media. **(g)** Completely developed plant kept for hardening. **(h)** Fully developed plants. **(i)** Leaves along with floral bud. **(j)** Flower of chilli plant.

Insertion of *Capana07g001557* in regenerated chilli plants was confirmed through the PCR with *Npt*II primers. To avoid the amplification of internal gene and non specific amplification, transgenic plants were confirmed through the amplification of *Npt*II gene instead of *Capana07g001557*. *Npt*II gene is present in between left border and right border of T-DNA present in the binary vector. In transgenic plant, *Npt*II gene also transfers along with transgene and provide resistance against kanamycin to the transformed plant. PCR results have indicated that out of 40 regenerated chilli plants 23 are PCR positive (Fig. 4.15a). Chilli transgenic lines 3, 5, 7, 8, 10, 11, 12, 13, 15, 16, 17, 18, 19, 51, 55, 57, 24, 25, 26, 28, 29, 30 and 31are PCR positive (Fig. 4.15a). PCR positive plants were further tested for *Agrobacterium* contamination through VirD2 PCR. VirD2 is an *Agrobacterium* specific gene and does not transfer to the plant during the process of transformation. PCR using VirD2 primers indicated that none of the transgenic plant bear *Agrobacterium* contamination as none of the plant showed VirD2 gene amplification (Fig. 4.15b).

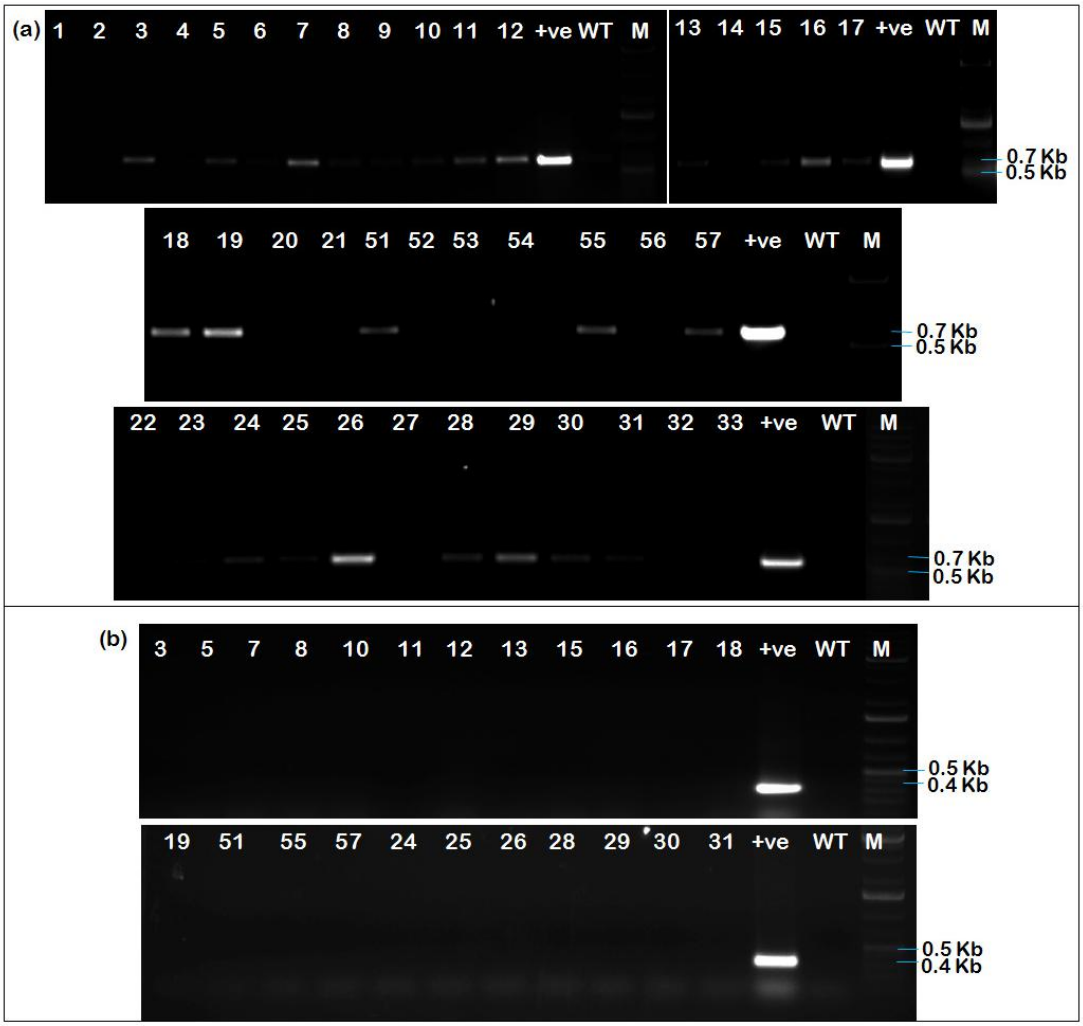


Fig. 4.15 Confirmation of the transgenic chilli plants through PCR. (a) PCR amplification of *Npt*II gene from regenerated chilli plants genomic DNA for the confirmation of transgene insertion.100 ng genomic DNA was used as template. pBINAR construct having *Capana07g001557* gene was used for the amplification of positive control. Genomic DNA of wild type plant was used as template for the negative control. **(b)** Amplification of VirD2 gene from the genomic DNA of transgenic plants (PCR positive plants) for the confirmation of *Agrobacterium* contamination.

4.2.10 Generation of Capana07g001557 over expressing *N. benthamiana* transgenic plants

Experimentation with chilli plants is difficult because of less infectivity through *Agrobacterium*, high incubation period and longer life cycle. To make the further experiments easier, Capana07g001557 over expressing *N. benthamiana* transgenic plants were developed. *N. benthamiana* plants were selected for the transgenic development because it is susceptible for all the geminiviruses, high infectivity and small life cycle. *N. benthamiana* transgenic development

was started with 240 explants. From these explants 73 shoots were generated and out of 73 shoots 18 were developed into complete plant (root, stem and leaf). All the 18 plants were kept for the hardening. All the plants were died due to the fungal infection during the hardening step.

Fig. 4.16 Develpomental stages of *N. benthamiana* transgenic plants. (a) Transformed leaf disk showing green patches, indicating transformed region started budding. **(b)** Plantlet developed from green budding region. **(c)** Plantlet transferred in rooting media. **(d)** Fully grown plantlets without roots transferred in rooting media. **(e)** Fully developed plant having leaves, stem and root in rooting media. **(f)** Completely developed plant kept for hardening. **(g)** Complete plant with leaves, stem, root and flowers. **(h)** Flower along with floral bud. **(i)** Individual leaf of the plant.

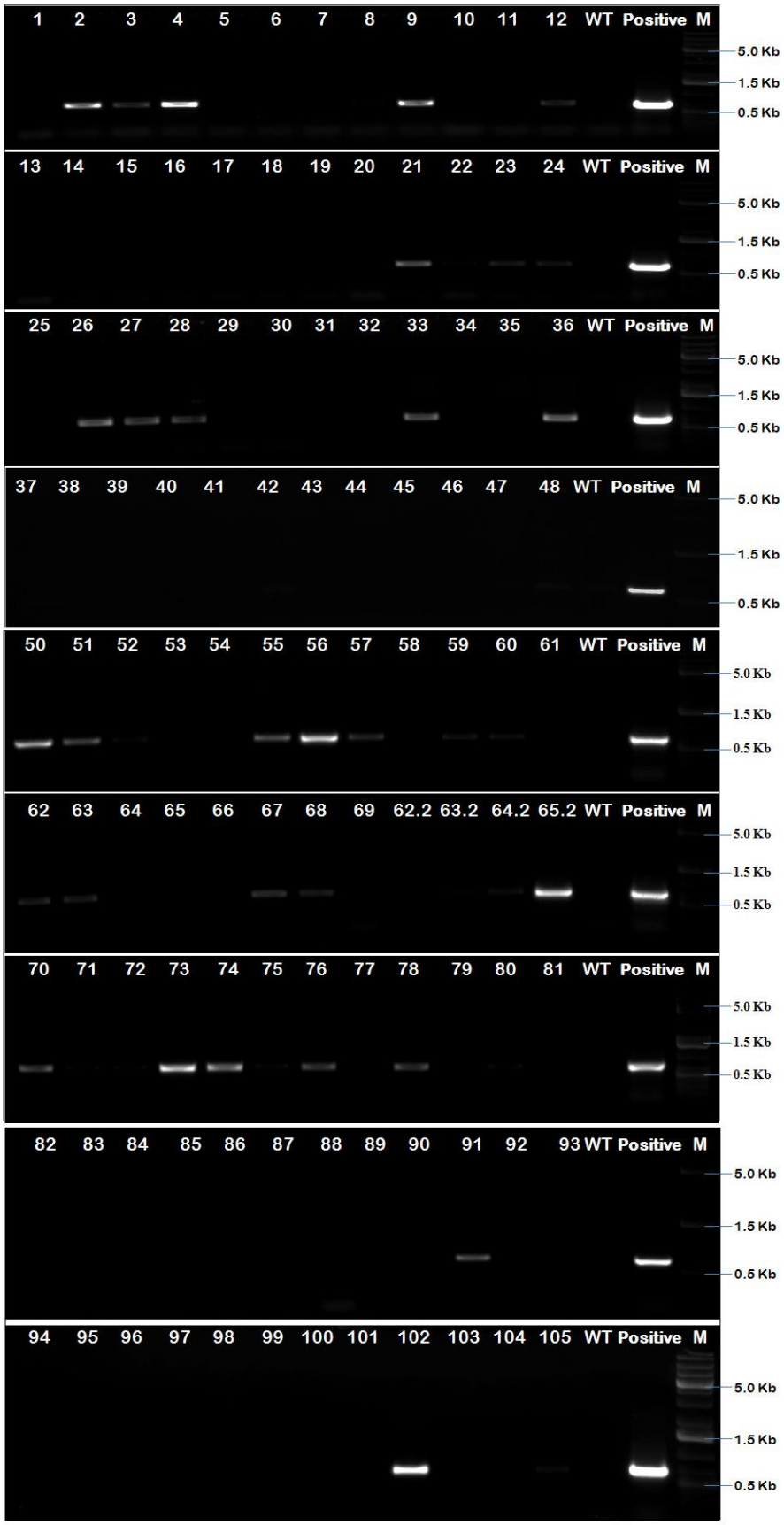
4.2.10 Generation of Capana07g001557 over expressing *N. tabacum* transgenic plants

All the *N. benthamiana* transgenic plants plants were died due to the fungal infection. Further, *Capana07g001557* over expressing *N. tabacum* cv. Xanthi plants were developed. For the development of transgenic plant *Capana07g001557* gene cloned in pBINAR vector was used. Transgenic preparation was started with the 490 explants. From these explants, 108 plants were developed. All the regenerated putative transgenic plants showed normal phenology (plant height, site of insertion of first pod, number of branches, internode length, foliar area, and total number of flowers and pods) compared to non-transgenic *N. tabacum* plants.

Insertion of *Capana07g001557* gene in transgenic plants (T0 generation) were confirmed by PCR. Through PCR, *Npt*II gene was amplified which is present in between the left border and right border of T-DNA and provide resistance to the transgenic plant against kanamycin. For the PCR confirmation 100 ng genomic DNA was used as template. For the confirmation of transgenic plants *Npt*II gene was amplified instead of the *Capana07g001557* gene because plants have large number of *NBS-LRR* genes and some of them have high nucleotide homology. High homology in between *NBS-LRR* genes may leads to the non specific PCR amplification. PCR results have suggested that 2,3,4,9,12, 21, 23, 24, 26, 27, 28, 33, 36, 48, 50, 51, 55, 56, 57, 59, 60, 62, 63, 67, 68, 64.2, 65.2, 70, 73, 74, 76, 78, 91, 102 and 105 are the PCR positive plants (Fig. 4.18a). Out of 108 regenerated tabacum plants 35 are PCR positive (Fig. 4.18a). To investigate the contamination of *Agrobacterium* in PCR positive plants, another PCR was performed with the genomic DNA of PCR positive plants and VirD2 primers. VirD2 is a gene of *Agrobacterium* genomic DNA which does not transfer into the transgenic plant. VirD2 PCR results have indicated that no any plant is infected with the *Agrobacterium* (Fig. 4.18b).

Fig. 4.17 Different stages of *N. tabacum* transgenic plant development. (a) Transformed leaf disk showing initial stage shoot development. **(b)** Plantlet developed from green budding region of leaf disk. **(c)** Plantlet transferred in shooting media. **(d)** Fully grown plantlets without roots in shooting media. **(e)** Fully developed plant in rooting media. **(f)** Completely developed plant kept for hardening. **(g)** Complete plant with leaves, stem, root and flowers. **(h)** Flower along with floral bud. **(i)** Individual flower. **(j)** leaves of the fully developed transgenic *N. tabacum* plant.

(a)


1 2 3 4 5 6 7 8 9 10 11 12 WT Positive M
13 14 15 16 17 18 19 20 21 22 23 24 WT Positive M
25 26 27 28 29 30 31 32 33 34 35 36 WT Positive M
37 38 39 40 41 42 43 44 45 46 47 48 WT Positive M
50 51 52 53 54 55 56 57 58 59 60 61 WT Positive M
62 63 64 65 66 67 68 69 62.2 63.2 64.2 65.2 WT Positive M
70 71 72 73 74 75 76 77 78 79 80 81 WT Positive M
82 83 84 85 86 87 88 89 90 91 92 93 WT Positive M
94 95 96 97 98 99 100 101 102 103 104 105 WT Positive M
5.0 Kb
1.5 Kb
0.5 Kb

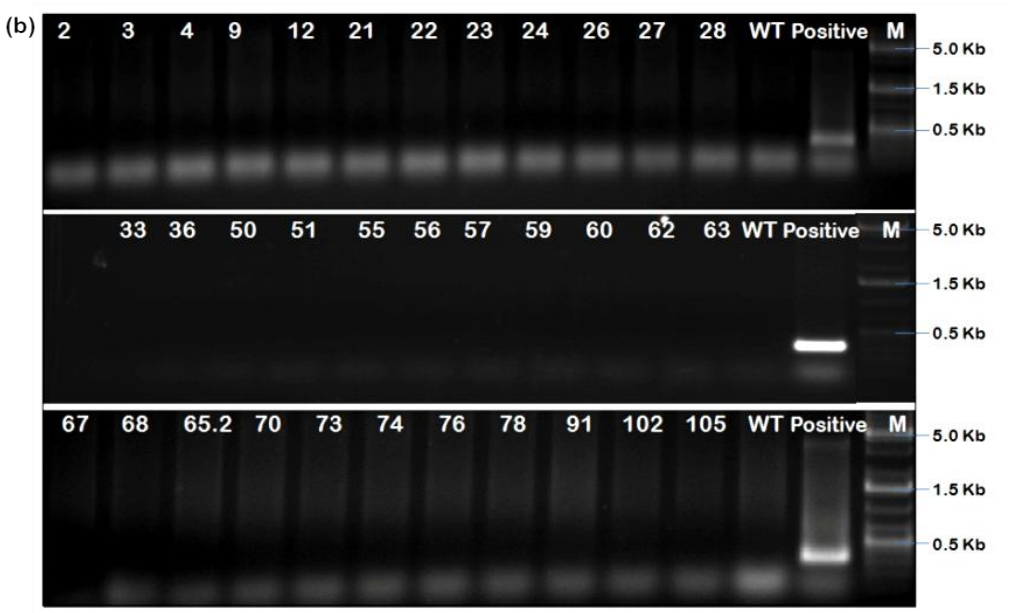


Fig. 4.18 Confirmation of the transgenic *N. tabacum* cv. Xanthi plants through PCR. (a) PCR amplification of *Npt*II gene from regenerated tabacum plants genomic DNA for the confirmation of insertion of transgene.100 ng genomic DNA was used as template. Clone of *Capana07g001557* gene in pBINAR vector was used as template for the amplification of positive control. Genomic DNA of wild type plant was used as template for the negative control. M indicates for DNA marker. **(b)** Amplification of VirD2 gene from the genomic DNA of transgenic plants (PCR positive plants) for the confirmation of *Agrobacterium* contamination.

4.2.11 Viral DNA accumulation in *Capana07g001557* gene over expressing *N. tabacum* plants

Previous results have shown that transient expression of *Capana07g001557* gene reduces the accumulation of ChiLCV. Further to confirm the role of *Capana07g001557* gene against ChiLCV, two different leaves of PCR positive transgenic *N. tabacum* plants (*Capana07g001557* gene over expressing plants) were infiltrated with ChiLCV and ChiLCV isolate Nagpur (NgA+Ngβ). At 6 days post infiltration viral DNA accumulation was analyzed through Southern blot hybridization. Hybridization was carried out using the AC1 specific probe of both the viruses. Accumulation of ChiLCV in the leaves of transgenic as well as wild type (WT) *N. tabacum* plants was below detection level so could not be analyzed with the help of Southern blotting. NgA+Ngβ were able to infect the *N. tabacum* plants efficiently and can be detected through Southern blotting (Fig. 4.19). Southern blotting results showed that the accumulation of

NgA+Ngβ in wild type plants was almost similar to the many transgenic plants (transgenic line 24, 26, 27, 59, 60, 70, 74, 78 and 91) (Fig. 4.19). Some of the transgenic lines (2, 3, 4, 9, 21, 28, 33 and 102) have shown small reduction in viral DNA accumulation compare to the wild type plants (Fig. 4.19). Remaining transgenic lines (12, 51, 55, 56, 57 and 73) showed drastic reduction in viral DNA accumulation compare to the wild type (WT) plants (Fig. 4.19). Interestingly, from the Southern blot analyses, it was evident that in some of the transgenic lines (24, 27, 59, 70 and 78) amount of super coiled form of viral DNA was similar to the WT plants but the single stranded form of viral genome was very less as compared to the non-transgenic (WT) plants (Fig. 4.19). WT plants infiltrated with the empty vector (mock) were used as the negative control.

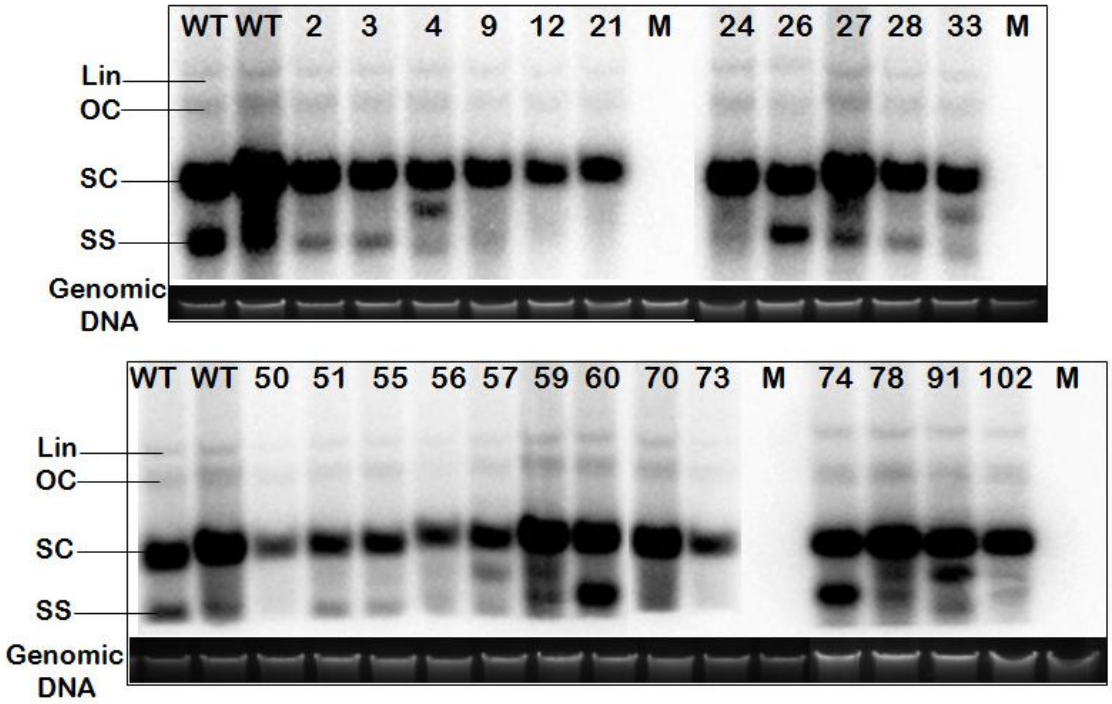


Fig. 4.19 Viral DNA accumulation in *Capana07g001557* over expressing transgenic *N. tabacum* plants. Comparative accumulation of ChiLCV isolate Nagpur in different transgenic lines and WT *N. tabacum* plants. NgA-AC1 specific probe labeled with αP^{32} was used for the hybridization. Different replicative forms of viral DNA are abbreviated as linear (Lin), open circular (OC), supercoiled (sc) and single-stranded (ss). Genomic DNA isolated from infiltrated leaves stained with ethidium bromide (EtBr) served as the loading control.

4.3 Determination of role of *NBS-LRR* during synergistic interaction between chilli infecting begomoviruses

4.3.1 Breakdown of natural resistance in chilli

Severe leaf curl disease was observed on the hitherto known resistant chilli cultivar Kalyanpur Chanchal during 2009 in the experimental field of Jawaharlal Nehru University, New Delhi. All the experimental plants of this cultivar showed typical symptoms of begomovirus infection such as severe leaf curling and stunted plant growth. This study reports for the first time breakdown of natural resistance in chilli. We further extended the survey to other chilli cultivars grown in the same field. It was noticed that cultivars like 'Bhut Jolokia' and 'Kashi Anmol' also displayed severe leaf curl disease symptoms. The characteristic disease symptoms were upward leaf curling, vein thickening, chlorosis, small leaves, leaf crinkling, yellow patches, and boat shaped leaf structure on chilli cultivars Kalyanpur Chanchal and Kashi Anmol (Fig. 4.20a, Table 4.1). However, plants belonging to the chilli cultivar Bhut Jolokia showed symptoms of severe leaf curling with vein thickening, leaf rolling, chlorotic spots and stunted plant growth (Fig. 4.20a, Table 4.1). Notably, in all three cases 100% disease incidence was observed (Table 4.1).

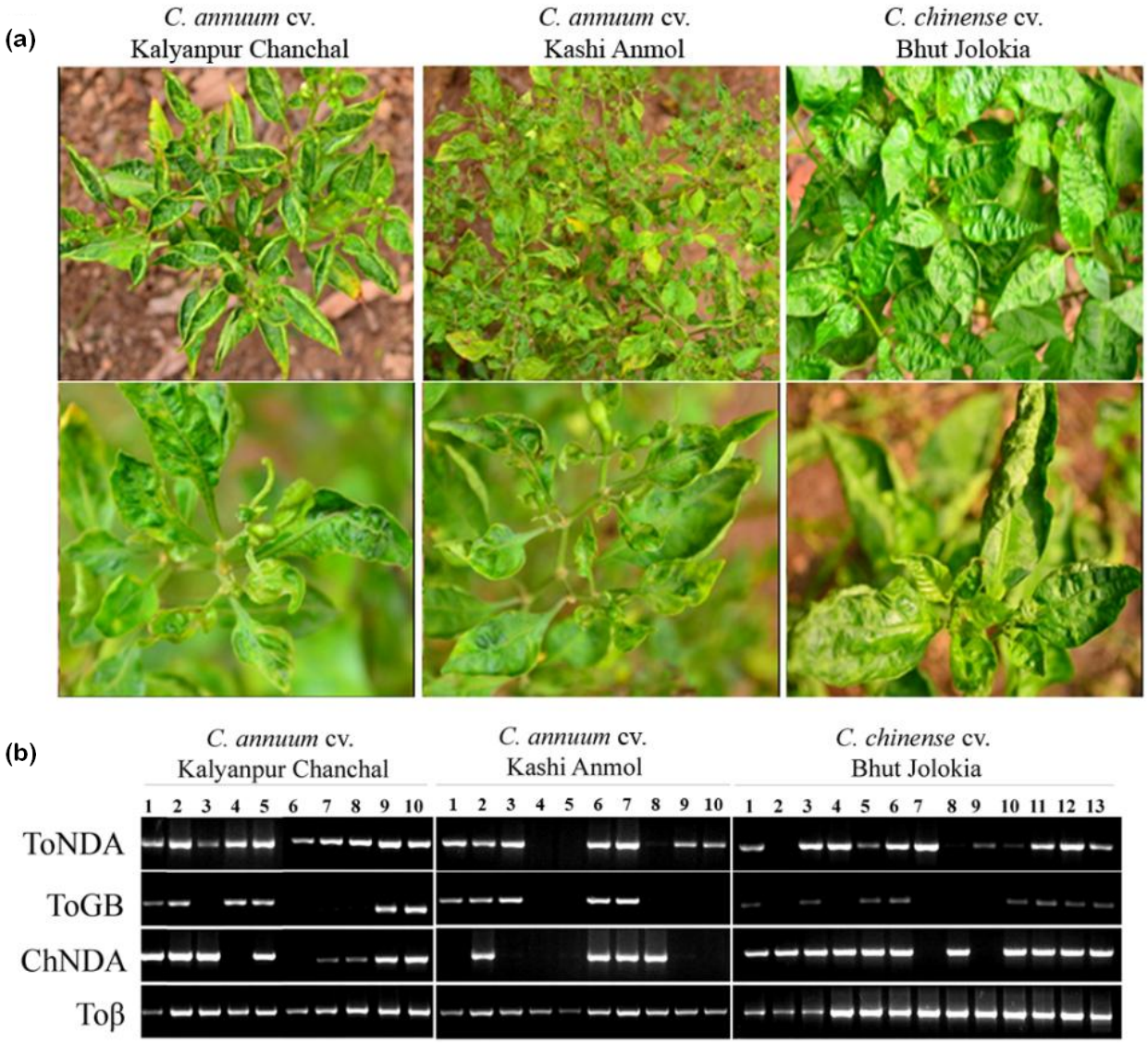


Fig. 4.20 Typical symptoms of leaf curl disease on plants belonging to *Capsicum* spp and detection of begomoviruses and associated betasatellite. Mixed infection of diverse begomoviruses and betasatellite resulted in breakdown of resistance in chilli cultivars. **(a)** A survey was carried out and symptomatic chilli plants belonging to three cultivars (as indicated) showing severe leaf curl symptoms were observed in natural condition. **(b)** Total plant DNA was used to isolate the resistant breaking strains of viruses. **(d)** Detection of begomovirus was carried out by polymerase chain reaction using ToNDA, ToGB, ChNDA and Toβ specific primers.

Table 4.1: Field occurrence and detection of begomoviruses on symptomatic chilli plants

Chilli cultivars	Symptoms	Total number of plants surveyed / symptomatic	ToNDA infected plants# / surveyed	ToGB infected plants# / surveyed	ChNDA infected plants# / surveyed	Toβ infected plants# / surveyed	ToNDA+ ToGB+ ChNDA+ Toβ infected plants# / surveyed
C. annuum cv. Kalyanpur Chanchal	ULC, VT, YN, LM, LC, YP, Bsl	10/10	10 / 10	8 / 10	8 / 10	10 / 10	7 / 10
C.annuum cv. Kashi anmol	ULC, VT, Ch, SL, LC, YP, Bsl	10/10	8 / 10	7 / 10	8 / 10	10 / 10	6 / 10
C. chinense cv. Bhut Jolokia	ULC, VT, CS, LR, St	13/13	12 / 13	8 / 13	11 / 13	13 / 13	8 / 13

ULC, upward leaf curling; VT, Vein thickening; YP, Yellow Patches on leaves; SL, small leaves; Bsl, Boat shaped leaf; LR, Leaf rolling; LM, Leaf mottling; LC, leaf crinkling; YN, Leaf yellowing and necrotic spots; Ch, Chlorosis; CS, Chlorotic spots (weak); St, stunting of plants

PCR positive plants

4.3.2 Cloning and detection of begomovirus genomic components associated with breakdown of resistance in chilli

To identify the begomoviruses associated with the leaf curl disease, rolling circle amplification (RCA) was performed using DNA isolated from the symptomatic plants of the resistant chilli cultivar Kalyanpur Chanchal (Fig. 4.21a). Restriction digestion of the RCA product with *Kpn*I resulted in two populations of DNA fragments, 2.7 kb and 1.3 kb (Fig. 4.21a). In addition, PCR using ChiLCV specific primers have resulted in the amplification of ~2.7 kb viral genome from the infected plants. These viral molecules were cloned into pUC18 vector and then sequenced subsequently. Sequencing of the viral genomic components revealed the presence of four different types of viral genomic components. A sequence of viral DNA (2763 nt; accession no KR957353) showed maximum homology (97.45%) with New Delhi isolate of *Chilli leaf curl virus* (accession no JN663866) and therefore, was considered as an isolate of this virus (hereafter mentioned as ChNDA). Similarly, two other viral genomic components of 2.7 kb in size shared 98% and 92.1% nucleotide per cent identities with the *Tomato leaf curl New Delhi* virus DNA A genome (accession no HM007113; referred hereafter as ToNDA) and Tomato leaf curl Gujarat

virus DNA-B component (accession no KP235538; referred hereafter as ToGB), respectively. In addition, a viral nucleotide sequence (1370 nt; accession no KR957354) possessed 97.45% sequence identity with *Tomato leaf curl Bangladesh betasatellite* (accession no JN663869; mentioned as Toβ) (Fig. 4.21a). According to the revised species demarcation threshold for begomoviruses (Brown et al. 2015) and betasatellites (Briddon et al. 2008), the cloned viral molecules were considered to be the isolates of *Chilli leaf curl virus* (DNA-A), *Tomato leaf curl New Delhi virus* (DNA-A), *Tomato leaf curl Gujarat virus* (DNA-B) and *Tomato leaf curl Bangladesh betasatellite*. Based on the nucleotide sequence identity, the cloned viral genomic components were positioned along with their respective begomovirus species and the betasatellite group in the respective phylogenetic trees (Fig. 4.21b, 4.21c, 4.21d, 4.21e).

In order to test the presence of these genomic components among all the symptomatic plants of the resistant cultivar Kalyanpur Chanchal grown in the experimental field, PCR using specific primers was carried out. PCR based amplification studies revealed that almost each chilli plant contained ToNDA and Toβ molecules, while 80% of the infected plants showed the infection of ToGB and ChNDA (Fig. 4.20, Table 4.1). Overall 70% of the infected plants of this cultivar showed the presence of all the four viral genomic components. We further studied the presence of these four components in other two chilli cultivars i.e., *C. chinense* cv. Bhut Jolokia and *C. annuum* cv. Kashi Anmol. Results indicated that in the case of *C. chinense* cv. Bhut Jolokia, all plants were found to be infected with Toβ, while 92.3%, 61.5% and 84.6% of the symptomatic plants contained ToNDA, ToGB and ChNDA, respectively. All these four viral components were present in 61.5% of the test plants (Fig. 4.20, Table 4.1). Similarly, *C. annuum* cv. Kashi Anmol showed ToNDA in 80%, ToGB in 70%, ChNDA 80% and Toβ in 100% plants (Fig. 4.20, Table 4.1). Presence of all the four genomic components was ascertained in 60% of the symptomatic plants of cultivar Kashi Anmol (Fig. 4.20, Table 4.1).

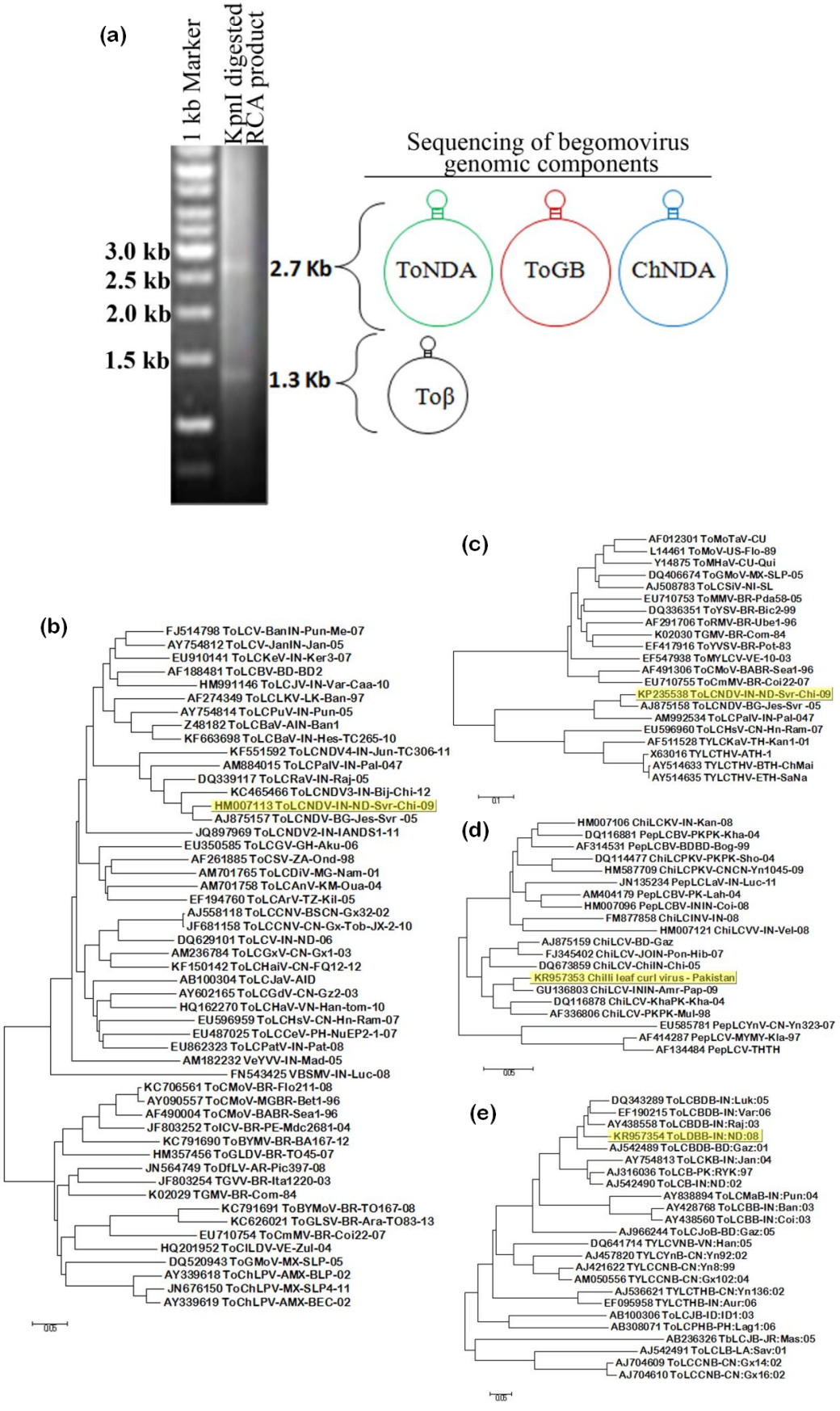

(a)
1 kb Marker
KpnI digested RCA product
3.0 kb
2.5 kb
2.0 kb
1.5 kb
Sequencing of begomovirus genomic components
2.7 Kb
1.3 Kb
ToNDA
ToGB
ChNDA
Toβ
(b)
FJ514798 ToLCV-BanIN-Pun-Me-07
AY754812 ToLCV-JanIN-Jan-05
EU910141 ToLCKeV-IN-Ker3-07
AF188481 ToLCBV-BD-BD2
HM991146 ToLCJV-IN-Var-Caa-10
AF274349 ToLCLKV-LK-Ban-97
AY754814 ToLCPuV-IN-Pun-05
Z48182 ToLCBaV-AIN-Ban1
KF663698 ToLCBaV-IN-Hes-TC265-10
KF551592 ToLCNDV4-IN-Jun-TC306-11
AM884015 ToLCPalV-IN-Pal-047
DQ339117 ToLCRaV-IN-Raj-05
KC465466 ToLCNDV3-IN-Bij-Chi-12
HM007113 ToLCNDV-IN-ND-Svr-Chi-09
AJ875157 ToLCNDV-BG-Jes-Svr -05
JQ897969 ToLCNDV2-IN-IANDS1-11
EU350585 ToLCGV-GH-Aku-06
AF261885 ToCSV-ZA-Ond-98
AM701765 ToLCDiV-MG-Nam-01
AM701758 ToLCAnV-KM-Oua-04
EF194760 ToLCArV-TZ-Kil-05
AJ558118 ToLCCNV-BSCN-Gx32-02
JF681158 ToLCCNV-CN-Gx-Tob-JX-2-10
DQ629101 ToLCV-IN-ND-06
AM236784 ToLCGxV-CN-Gx1-03
KF150142 ToLCHaiV-CN-FQ12-12
AB100304 ToLCJaV-AID
AY602165 ToLCGdV-CN-Gz2-03
HQ162270 ToLCHaV-VN-Han-tom-10
EU596959 ToLCHsV-CN-Hn-Ram-07
EU487025 ToLCCeV-PH-NuEP2-1-07
EU862323 ToLCPatV-IN-Pat-08
AM182232 VeYVV-IN-Mad-05
FN543425 VBSMV-IN-Luc-08
KC706561 ToCMoV-BR-Flo211-08
AY090557 ToCMoV-MGBR-Bet1-96
AF490004 ToCMoV-BABR-Sea1-96
JF803252 ToICV-BR-PE-Mdc2681-04
KC791690 ToBYMV-BR-BA167-12
HM357456 ToGLDV-BR-TO45-07
JN564749 ToDfLV-AR-Pic397-08
JF803254 TGVV-BR-Ita1220-03
K02029 TGMV-BR-Com-84
KC791691 ToBYMoV-BR-TO167-08
KC626021 ToGLSV-BR-Ara-TO83-13
EU710754 ToCmMV-BR-Coi22-07
HQ201952 ToCILDV-VE-Zul-04
DQ520943 ToGMoV-MX-SLP-05
AY339618 ToChLPV-AMX-BLP-02
JN676150 ToChLPV-MX-SLP4-11
AY339619 ToChLPV-AMX-BEC-02
0.05
(c)
AF012301 ToMoTaV-CU
L14461 ToMoV-US-Flo-89
Y14875 ToMHaV-CU-Qui
DQ406674 ToGMoV-MX-SLP-05
AJ508783 ToLCSiV-NI-SL
EU710753 ToMMV-BR-Pda58-05
DQ336351 ToYSV-BR-Bic2-99
AF291706 ToRMV-BR-Ube1-96
K02030 TGMV-BR-Com-84
EF417916 ToYVSV-BR-Pot-83
EF547938 ToMYLCV-VE-10-03
AF491306 ToCMoV-BABR-Sea1-96
EU710755 ToCmMV-BR-Coi22-07
KP235538 ToLCNDV-IN-ND-Svr-Chi-09
AJ875158 ToLCNDV-BG-Jes-Svr -05
AM992534 ToLCPalV-IN-Pal-047
EU596960 ToLCHsV-CN-Hn-Ram-07
AF511528 TYLCKaV-TH-Kan1-01
X63016 TYLCTHV-ATH-1
AY514633 TYLCTHV-BTH-ChMai
AY514635 TYLCTHV-ETH-SaNa
0.1
(d)
HM007106 ChiLCKV-IN-Kan-08
DQ116881 PepLCBV-PKPK-Kha-04
AF314531 PepLCBV-BDBD-Bog-99
DQ114477 ChiLCPKV-PKPK-Sho-04
HM587709 ChiLCPKV-CNCN-Yn1045-09
JN135234 PepLCLaV-IN-Luc-11
AM404179 PepLCBV-PK-Lah-04
HM007096 PepLCBV-ININ-Coi-08
FM877858 ChiLCINV-IN-08
HM007121 ChiLCVV-IN-Vel-08
AJ875159 ChiLCV-BD-Gaz
FJ345402 ChiLCV-JOIN-Pon-Hib-07
DQ673859 ChiLCV-ChiIN-Chi-05
KR957353 Chilli leaf curl virus - Pakistan
GU136803 ChiLCV-ININ-Amr-Pap-09
DQ116878 ChiLCV-KhaPK-Kha-04
AF336806 ChiLCV-PKPK-Mul-98
EU585781 PepLCYnV-CN-Yn323-07
AF414287 PepLCV-MYMY-Kla-97
AF134484 PepLCV-THTH
0.05
(e)
DQ343289 ToLCBDB-IN:Luk:05
EF190215 ToLCBDB-IN:Var:06
AY438558 ToLCBDB-IN:Raj:03
KR957354 ToLDBB-IN:ND:08
AJ542489 ToLCBDB-BD:Gaz:01
AY754813 ToLCKB-IN:Jan:04
AJ316036 ToLCB-PK:RYK:97
AJ542490 ToLCB-IN:ND:02
AY838894 ToLCMaB-IN:Pun:04
AY428768 ToLCBB-IN:Ban:03
AY438560 ToLCBB-IN:Coi:03
AJ966244 ToLCJoB-BD:Gaz:05
DQ641714 TYLCVNB-VN:Han:05
AJ457820 TYLCYnB-CN:Yn92:02
AJ421622 TYLCCNB-CN:Yn8:99
AM050556 TYLCCNB-CN:Gx102:04
AJ536621 TYLCTHB-CN:Yn136:02
EF095958 TYLCTHB-IN:Aur:06
AB100306 ToLCJB-ID:ID1:03
AB308071 ToLCPHB-PH:Lag1:06
AB236326 TbLCJB-JR:Mas:05
AJ542491 ToLCLB-LA:Sav:01
AJ704609 ToLCCNB-CN:Gx14:02
AJ704610 ToLCCNB-CN:Gx16:02
0.05

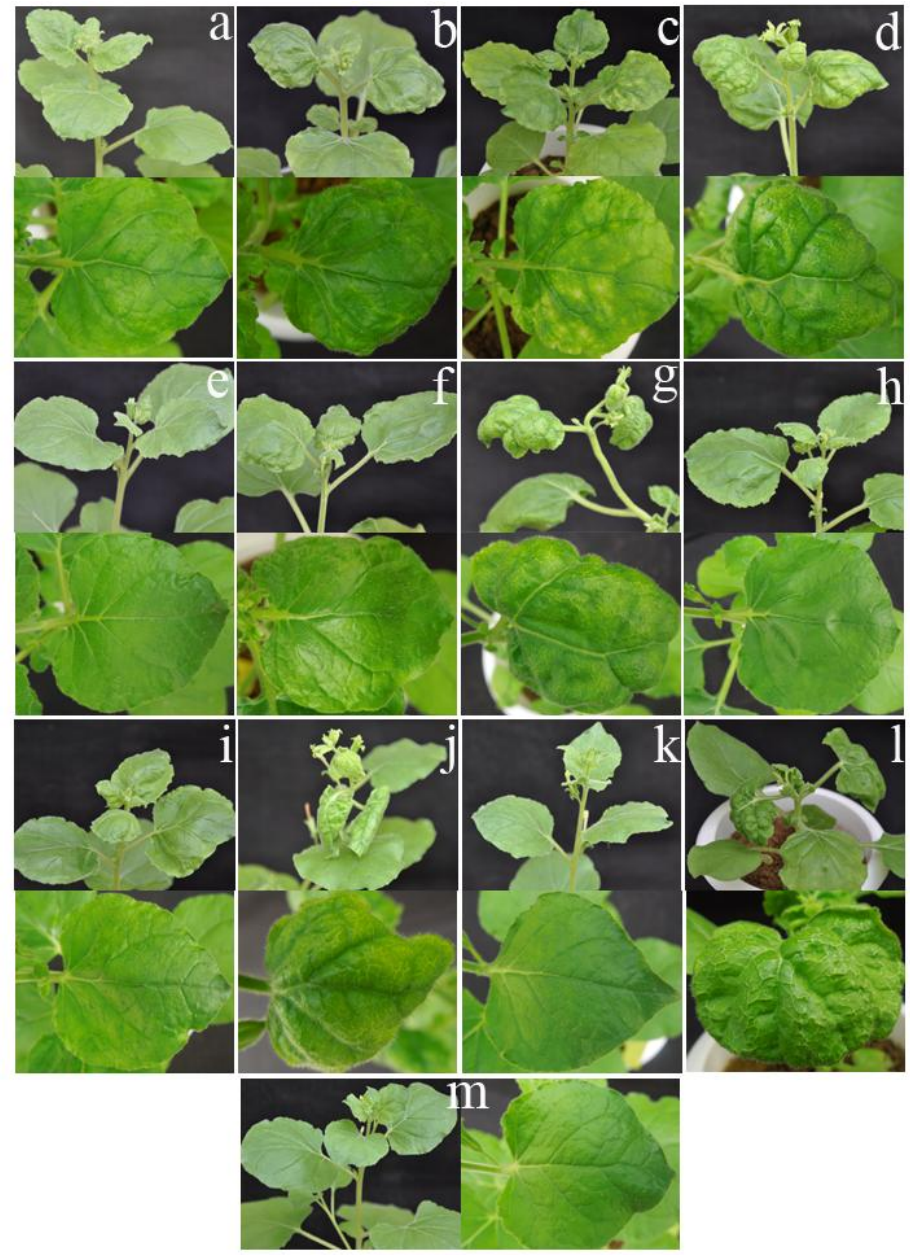
Fig. 4.21 Products of RCA and their phylogenetic analysis. (a) Two population of DNA obtained after restriction digestion of RCA product with *Kpn* I enzyme. Neighbour-joining phylogenetic dendrogram of viral genomic components isolated from resistant chilli cultivar Kalyanpur Chanchal. **(b)** ToNDA, **(c)** ToGB, **(d)** ChNDA and **(e)** Toβ.

4.3.3 Interaction among chilli-infecting begomoviruses in *N. benthamiana*

Infectious tandem repeats of all the isolated viral genomes and the betasatellite were constructed and were initially used to study the interaction in *N. benthamiana*. *N. benthamiana* plants infected with ToNDA alone showed initial symptom appearance at 10 dpi and the symptoms gradually increased upto level 3 at 20 dpi (Fig. 4.22o). ToNDA inoculated plants showed characteristics symptoms such as mild downward leaf curling, thickening of veins and small leaves (Fig. 4.22a, Table 4.2). However, plants co-inoculated with ToNDA and ToGB showed the first symptom at 6 dpi and exhibited maximum symptom severity (level 5) at 15 dpi (Fig. 4.22n, Table 4.2). ToNDA and ToGB inoculated plants developed severe symptoms such as leaf curling, vein thickening, stunting, leaf puckering, yellow patches on leaf lamina and twisting of petiole (Fig. 4.22b, Table 4.2). Notably, plants inoculated with ChNDA alone produced very mild symptoms on *N. benthamiana* (Fig. 4.22e), however, severe symptoms were observed on plants inoculated with ChNDA and Toβ (Fig. 4.22f). ChNDA and Toβ co-inoculated plants developed initial symptoms as downward leaf curling, vein thickening with leaf puckering at 10 dpi while symptom severity enhanced persistently and reached up to level 3 at 19 dpi (Fig. 4.22f, 4.22n, Table 4.2). On the other hand, plants co-inoculated with ToNDA and ChNDA displayed symptoms similar to ToNDA inoculated plants (Fig. 4.22i). ChNDA and ToGB inoculated plants exhibited very mild symptoms similar to ChNDA inoculated plants (Fig. 4.22k). Evaluation of the ToNDA and Toβ co-inoculated plants revealed that the earliest symptoms appeared on 6 dpi and the severity gradually increased to the highest level (symptom severity 5) on 16 dpi (Fig. 4.22j, 4.22o). ToNDA and Toβ inoculated plants exhibited leaf curling, vein thickening, leaf puckering, vein clearing, twisting of petiole and internodes elongation (Fig. 4.22j, Table 4.2).

Initial symptom was recorded at 6 dpi on the plants co-inoculated with ToNDA, ToGB and ChNDA and the severity progressed to the maximum level (symptom score 5) at 14 dpi (Fig. 4.22n, Table 4.2). Symptoms appeared were similar to the ToNDA and ToGB co-inoculated

plants, although with increased severity (Fig. 4.22c). Plants co-inoculated with ToNDA, ToGB and Toβ also showed severe leaf curl disease which appeared initially at 6 dpi and acquired maximum severity (symptom level 5) at 15 dpi (Fig. 4.22d, 4.22n, Table 4.2). Co-inoculation of ChNDA, Toβ and ToNDA displayed symptoms comparable to the ToNDA and Toβ co-inoculated plants (Fig. 4.22g, Table 4.2). Initial symptoms on these plants were observed at 6 dpi which attained maximum severity (level 5) at 15 dpi (Fig. 4.22n). Co-inoculation of ChNDA, Toβ and ToGB induced similar symptoms as ChNDA and Toβ co-inoculated plants and first symptoms appeared at 12 dpi which reached maximum severity (level 3) at 20 dpi (Fig. 4.22h, 4.22o). *N. benthamiana* plants co-inoculated with all the four infectious molecules exhibited the most severe symptom (level-5) such as downward leaf curling, vein thickening, stunting, leaf puckering, veinal chlorosis and twisting of petiole. On these plants, initial symptom appeared at 5 dpi, which subsequently reached maximum severity (symptom level 5) at 14 dpi (Fig. 4.22l, 4.22n, Table 4.2).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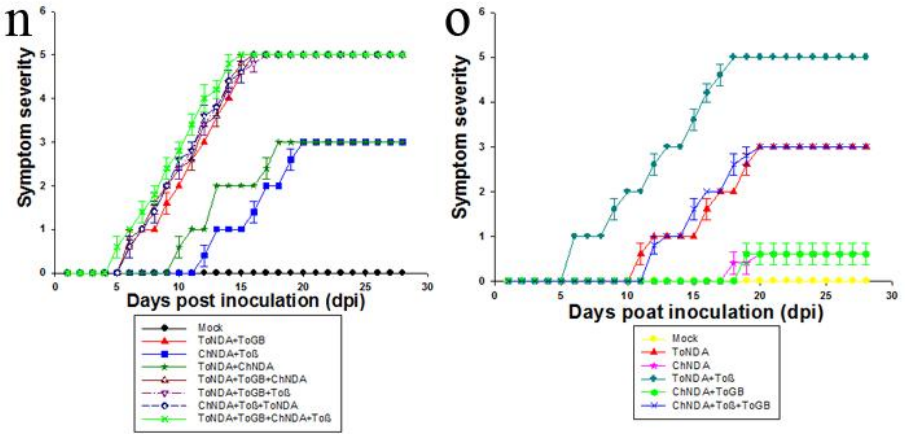
n
Symptom severity
Days post inoculation (dpi)
Mock
ToNDA+ToGB
ChNDA+Toß
ToNDA+ChNDA
ToNDA+ToGB+ChNDA
ToNDA+ToGB+Toß
ChNDA+Toß+ToNDA
ToNDA+ToGB+ChNDA+Toß
o
Symptom severity
Days poat inoculation (dpi)
Mock
ToNDA
ChNDA
ToNDA+Toß
ChNDA+ToGB
ChNDA+Toß+ToGB

Fig. 4.22 Disease development on *N. benthamiana* plants inoculated with different combinations of viral genomic components. (**a)** ToNDA. (**b)** ToNDA+ToGB. **(c)** ToNDA+ToGB+ChNDA. **(d)** ToNDA+ToGB+Toβ **(e)** ChNDA. **(f)** ChNDA+Toβ. **(g)** ChNDA+Toβ+ToNDA. **(h)** ChNDA+Toβ+ToGB. **(i)** ToNDA+ChNDA. **(j)** ToNDA+Toβ. **(k)** ChNDA+ToGB. **(l)** ToNDA+ToGB+ChNDA+Toβ. **(m)** Mock. **(n)** and (**o)** Graphs represents kinetics of symptom severity on *N. benthamiana.*

Table 4.2: Infectivity of genomic components of begomoviruses and betasatellite on *N. benthamiana* plants

Plant Species	Viral combinations	Plants showing Symptoms / inoculated[a]	Symptoms[b]	Symptom Severity[c]	First symptom appeared (in dpi)
N. benthamiana	ToNDA	28/30	LC, VT, SL	+++	10
	ToNDA+ToGB	30/30	LC, VT, St, Pu, YP, SL, Tw	+++++	6
	ChNDA	22/30	LC	+	18
	ChNDA+Toβ	30/30	LC,VT, Pu , SL, Csl	+++	10
	ToNDA+ChNDA	30/30	LC, VT, SL	+++	10
	ToNDA+Toβ	30/30	LC, VT, Pu, Tw,VC	+++	6
	ChNDA+ToGB	23/30	LC	+	19
	ToNDA+ToGB+ChNDA	30/30	LC, VT, St, Pu, YP, SL,Tw, Csl	+++++	6
	ToNDA+ToGB+Toβ	30/30	LC, VT, Pu, YP, Tw, VC	+++++	6
	ChNDA+Toβ+ToNDA	30/30	LC,VT, Pu, Csl, Tw, VC	++++	6
	ChNDA+Toβ+ToGB	30/30	LC,VT, Pu, SL, Csl	++	12
	ToNDA+ToGB+ChNDA +Toβ	30/30	LC, VT, St, Pu, LD, YP, SL, Tw, VC	+++++	5
	Mock	0/30	-	-	-

[a]Number of plants showing symptoms /number of plants inoculated

[b]LC, leaf curling; VT, Vein thickening; St, stunting; LD, leaf distortion; YP, Yellow Patches; ; SL, small leaves; Tw, twisting of petiole; Csl, Cup shaped leaf; Cr, leaf crinkling; Pu, puckering; TL, thickening of leaf lamina; VC, Vein clearing.

[c]Severity of symptoms was scored from mild (+) to severe (++++++).

4.3.4 A comparative analysis of viral DNA accumulation

In order to correlate symptom severity and viral DNA accumulation, the viral titer in *N. benthamiana* plants was analysed by Southern hybridization. The results suggested that plants co-inoculated with all four genomic components (ToNDA, ToGB, ChNDA and Toβ) showed maximum accumulation of viral DNAs. We, therefore, considered viral DNA (of each component) as 100% in the plants inoculated with this combination. Plants inoculated with ToNDA alone showed relatively low accumulation of viral DNA level of which was enhanced when co-inoculated with ToGB (Fig. 4.23a lanes 1 and 2). ToNDA level was marginally enhanced (upto to 10%) in plants co-inoculated with ToNDA and ChNDA as compared to the ToNDA inoculated plants (Fig. 4.23a lane 5). Co-inoculation of plants with both ToNDA and Toβ did not influence accumulation of ToNDA as compared to plants inoculated with ToNDA alone (Fig. 4.23a lane 6). As expected, accumulation of ToNDA was enhanced in plants inoculated with ToNDA, ToGB and ChNDA in comparison to the ToNDA and ToGB inoculated plants (Fig. 4.23a lane 8). Surprisingly, drastic reduction of ToNDA level was observed in plants inoculated with ToNDA, ToGB and Toβ as compared to the ToNDA and ToGB inoculated plants (Fig. 4.23a lane 9). However, ToNDA level was considerably reduced (5%) in plants inoculated with viral components ToNDA, ChNDA and Toβ and was comparable with plants infected with ToNDA alone.

Level of ToGB DNA was approximately 1.6% in ToNDA and ToGB co-inoculated plants (Fig. 4.23b lane 2) as compared to the plants inoculated with the four genomic components. Plants inoculated with ToNDA, ToGB and ChNDA, ToGB titer was estimated to be 2.5% (Fig. 4.23b lane 8). Level of ToGB DNA was below the detection level of Southern blotting in plants inoculated with ChNDA with ToGB; ToNDA, ToGB with Toβ and ChNDA, Toβ with ToGB (Fig. 4.23b lane 7, 9 and 11 respectively).

Although, ChNDA viral DNA accumulation was below the threshold level to be detected by Southern blotting, presence of ChNDA viral DNA could be detected by PCR (data not shown) in ChNDA infected plants (Fig. 4.23c lane 3). There was at least 50% increase in the ChNDA level in plants coinoculated with ChNDA and Toβ than plants inoculated with ChNDA alone (Fig. 4.23c lane 4). Inoculation of ChNDA and ToNDA led to enhanced accumulation of ChNDA (Fig 4.23c lane 5). However, ChNDA DNA titer was found to be below the threshold

level as detected by Southern blotting in ChNDA and ToGB co-inoculated plants similar to that of the ChNDA infected plants (Fig. 4.23c lane 7). Mixed inoculation of ChNDA along with ToNDA and ToGB resulted in 32.6% accumulation of ChNDA DNA (Fig. 4.23c lane 8). Co-infection of ChNDA, Toβ and ToNDA has further enhanced the ChNDA DNA level (Fig. 4.23c lane 10). Plants inoculated with ChNDA, Toβ and ToGB could accumulate 33.8% of ChNDA (Fig. 4.23c lane 11).

Similar to ChNDA, level of Toβ DNA was also found to be very low which could only be detected by PCR in ChNDA and Toβ co-inoculated plants (Fig. 4.23d lane 4). Unlike ChNDA, the association of ToNDA could enhance the Toβ level up to 90% (Fig. 4.23d lane 6). Accumulation of Toβ was found to be 76% in ToNDA, ToGB and Toβ mixed inoculated plants (Fig. 4.23d lane 9). Toβ co-inoculated with ToNDA and ChNDA led to the enhanced level (98%) of Toβ DNA (Fig. 4.23d lane 10). Notably, the titer of Toβ remained below the threshold level of detection in ChNDA, Toβ and ToGB co-inoculated plants.

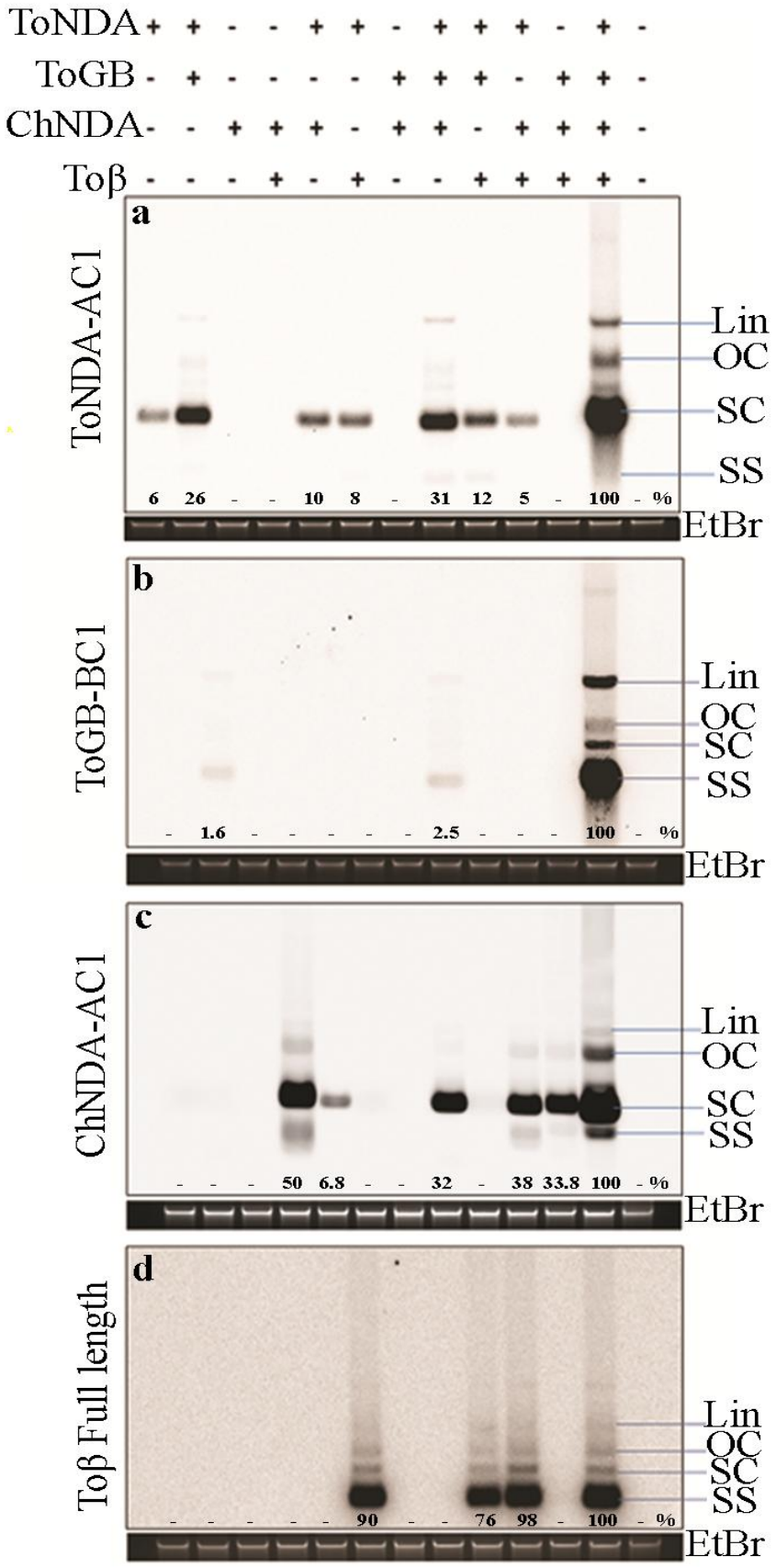
ToNDA + + - - + + - + + + - + -
ToGB - + - - - - + + + - + + -
ChNDA - - + + + - + + - + + + -
Toβ - - - + - + - - + + + + -
a
ToNDA-AC1
Lin
OC
SC
SS
6 26 - - 10 8 - 31 12 5 - 100 - %
EtBr
b
ToGB-BC1
Lin
OC
SC
SS
- 1.6 - - - - - 2.5 - - - 100 - %
EtBr
c
ChNDA-AC1
Lin
OC
SC
SS
- - - 50 6.8 - - 32 - 38 33.8 100 - %
EtBr
d
Toβ Full length
Lin
OC
SC
SS
- - - - - 90 - - 76 98 - 100 - %
EtBr

Fig. 4.23 A comparative analysis of the viral DNA accumulation in *N. benthamiana* plants inoculated with different combinations of begomovirus genomic components at 21dpi. Southern hybridization was performed using viral genome specific probes for detection, **(a)** ToNDA using AC1 probe, **(b)** ToGB using BC1 probe, **(c)** ChNDA using AC1 probe and **(d)** Toβ using βC1 probe. Plant genomic DNA stained with ethidium bromide (EtBr) served as the loading control.

4.3.5 Comparative level of viral DNA in the resistant chilli cultivar Kalyanpur Chanchal

Natural association of four begomoviral genomic components was observed in field grown chilli cv. Kalyanpur Chanchal. Furthermore, these viral genomic components have shown synergistic interaction in the laboratory host, *N. benthamiana*. In addition, the effect of synergistic interaction on symptom severity and viral DNA accumulation in the resistant chilli cultivar Kalyanpur Chanchal was assessed through biolistic inoculation of viral genomic components. Plants inoculated with ToNDA and ToGB showed either no or very mild leaf curling (at 55 dpi) (Fig. 4.24b; Table 4.3). Plants inoculated with ChNDA and Toβ failed to display any noticeable symptom till 60 dpi (Fig. 4.24c; Table 4.3). However, mixed inoculation of ToNDA, ToGB, ChNDA and Toβ led to severe symptoms such as leaf curling, crinkling, puckering and thickening of leaf lamina on the resistant chilli plants. These plants exhibited initial symptoms as early as at 36 dpi and the severity enhanced gradually to the maximum at 60 dpi (Fig. 4.24d; Table 4.3). However, we have failed to detect viral DNA (from 10 µg of total DNA) from these inoculated plants by Southern hybridization. Therefore, the highly sensitive method of polymerase chain reaction (PCR) was used to detect viral DNA accumulation in the chilli plants. For PCR detection, 2 µg of total plant genomic DNA was taken as a template and the reaction was carried out for 23 cycles. A comparative analysis of viral DNA accumulation in inoculated chilli plants indicated correlation with the results observed in the *N. benthamiana* plants. ToNDA, ToGB, ChNDA and Toβ inoculated chilli plants revealed maximum accumulation of viral DNA (Fig. 4.24e). Viral DNA accumulation was very low in the resistant chilli plants inoculated either with ToNDA and ToGB or ChNDA and Toβ.

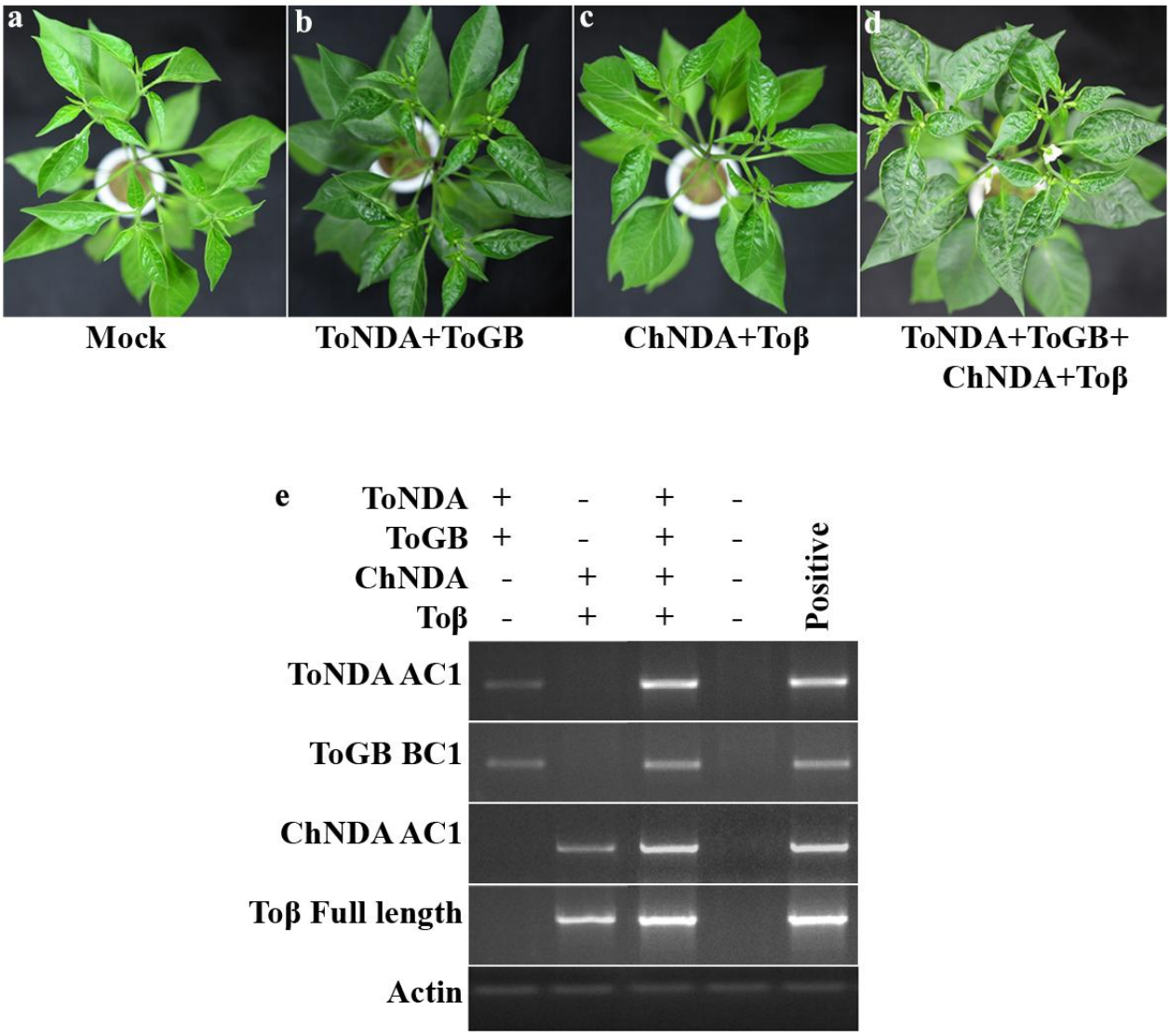

Fig. 4.24 A comparative study of symptom development and viral DNA accumulation in *C. annuum* cv. Kalyanpur Chanchal inoculated with begomoviruses. Symptoms on representative chilli plants inoculated with **(a)** mock, **(b)** ToNDA+ToGB, **(c)** ChNDA+Toβ and **(d)** ToNDA+ToGB+ChNDA+Toβ. (**e)** PCR was carried out using AC1 specific primers to detect ToNDA and ChNDA, BC1 specific primers to detect ToGB. Full-length DNA β specific primers were used to detect Toβ. PCR Amplification of actin using same conditions used for amplification of viral genomes serves as internal control.

Table 4.3: Infectivity of genomic components of begomoviruses and betasatellite on *C. annuum* cv. Kalyanpur Chanchal plants

Plant Species	Viral combinations	Plants showing Symptoms / inoculated[a]	Symptoms[b]	Symptom Severity[c]	First symptom appeared (in dpi)
C. annuum cv. Kalyanpur Chanchal	ToNDA+ToGB	2/8	LC	+	55
	ChNDA+Toβ	0/8*	-	-	-
	ToNDA+ToGB+ChNDA+Toβ	6/8	LC, Cr, Pu, TL	+++	36
	Mock	0/8	-	-	-

[a]Number of plants showing symptoms /number of plants inoculated

[b]LC, leaf curling; Cr, leaf crinkling; Pu, puckering; TL, thickening of leaf lamina.

[c]Severity of symptoms was scored from mild (+) to severe (++++++)

* However, plants have shown presence of ChNDA and Toβ by PCR analysis.

4.3.6 Mixed infection of chilli-begomoviruses suppresses the expression of defense related genes

To protect themselves, plants have developed various defense mechanisms, which can be activated by pathogen infection. One of our previous studies suggested up-regulation of several defense related genes in the resistant chilli cultivar Punjab Lal upon infection with *Chilli leaf curl virus* (Kushwaha et al. 2015a). The expression analysis of these genes was performed to evaluate their plausible role in synergistic interaction noticed in this study. The expression of these defense related genes were analyzed in both the resistant (Kalyanpur Chanchal) and the susceptible (Kashi Anmol) chilli cultivars inoculated with either mock or in combinations of viral DNA (ToNDA+ ToGB, ChNDA+ Toβ, ToNDA+ToGB+ChNDA+Toβ)..

Ascorbate peroxidase (*APX*) functions as a scavenger of excess H_2O_2 and hydroxyl radicals produced following pathogen attack (Mittler et al. 1998). Expression of ascorbate peroxidase (*APX*) gene was found altered in virus inoculated chilli plants. Increased level of *APX* transcript

and as compared to the mock was observed in the susceptible chilli plants inoculated with either ToNDA and ToGB (>400 folds, $p<0.05$) or ChNDA and Toβ (>500 folds, $p=0.006$) as compared to mock inoculated plants (Fig. 4.25a). Reduced level of *APX* expression was observed in ToNDA, ToGB, ChNDA and Toβ inoculated susceptible plants than plants inoculated with either ToNDA and ToGB or ChNDA and Toβ (about 80 folds, $p<0.05$ and 90 folds, $p=0.006$, respectively) (Fig. 4.25a). The resistant chilli plants inoculated with either ToNDA and ToGB or ChNDA and Toβ showed enhanced accumulation of *APX* transcript >6 folds ($p<0.001$) and >~5 folds ($p=0.006$), respectively as compared to the mock inoculated plants (Fig. 4.25e). However, *APX* expression in the resistant plants inoculated with all four viral genomic components revealed reduced level (2.3 folds, $p<0.001$ and 1.7 folds $p<0.05$) as compared to either ToNDA and ToGB or ChNDA and Toβ inoculated plants, respectively) (Fig. 4.25e).

Polyphenol oxidase (PPO) is an oxidorecuctase enzyme known to be involved in plant defense against various pathogens (Li and Steffens, 2002). Level of *PPO* transcript was found to be elevated in the susceptible chilli plants co-inoculated with ToNDA with ToGB (~350 fold, $p=0.025$) and ChNDA with Toβ (~400 fold, $p=0.01$) in comparison with the mock inoculated plants (Fig. 4.25b). Plants of the susceptible cultivar inoculated with ToNDA, ToGB, ChNDA and Toβ showed drastic reduction of PPO expression, even below the detectable level (Fig. 4.25b). PPO transcript accumulation increased more than 6.5 fold ($p<0.001$) and 5.5 fold ($p=0.003$) in the resistant chilli plants inoculated with either ToNDA and ToGB or ChNDA and Toβ, respectively than the mock inoculated plants (Fig. 4.25f). However, plants inoculated with all the four viral components showed reduced level of *PPO* transcripts, 2.3 fold ($p<0.001$) and 2.0 fold ($p=0.019$) than either ToNDA and ToGB or ChNDA and Toβ inoculated plants, respectively (Fig. 4.25f).

Thionin is a copper containing protein which has been characterized as a PR gene product and has been implicated in defense against bacteria, fungi and virus (Epple et al. 1997; Pelegrini and Franco 2005). Thionin expression was analyzed in the susceptible and resistant chilli plants inoculated with different combinations of begomoviruses. Thionin expression was increased in Kashi Anmol plants co-inoculated with either ToNDA and ToGB or ChNDA and Toβ (~290 fold $p=0.03$, and ~310 $p=0.019$, respectively) with respect to the mock plants. Notably, plants inoculated with all four viral genomes showed significant reduction of thionin than either

ToNDA and ToGB or ChNDA and Toβ infected plants (Fig 4.25c). Plants inoculated with either ToNDA and ToGB or ChNDA and Toβ resulted enhanced level (>2 fold) of thionin transcripts as compared to the mock inoculated resistant chilli plants whereas plants inoculated with ToNDA, ToGB, ChNDA and Toβ accumulated reduced level of thionin transcript (~50% less than the mock inoculated plants) (Fig. 4.25g).

Further, expression profile of NBS-LRR gene was analyzed. One of our earlier studies has suggested that ChiLCV inoculation resulted in increased accumulation of NBS-LRR transcript in the resistant chilli plants (Kushwaha et al. 2015a). In the current study, plants (chilli cv. Kashi Anmol) inoculated with either ToNDA and ToGB or ChNDA and Toβ showed enhanced level of NBS-LRR transcript (more than 190 folds, p=0.016 and 110 folds, p=0.035 respectively as compared to the mock plants) (Fig. 4.25d). In mixed inoculated plants NBS-LRR transcript level is significantly reduced than plants inoculated with either ToNDA and ToGB or ChNDA and Toβ alone, which was almost half to that of the mock plant (Fig. 4.25d). The resistant chili cultivar accumulated 2.7 and 4.5 fold enhanced transcripts of NBS-LRR gene inoculated with either ToNDA and ToGB or ChNDA and Toβ with respect to the mock plants (Fig. 4.25h). Further analysis of NBS-LRR in mixed inoculated resistant plants showed reduced level of NBS-LRR transcript than either ToNDA and ToGB or ChNDA and Toβ virus infected plants (about 2.5 fold, p=0.001 and 3.8 fold, p=0.038 respectively) (Fig. 4.25h).

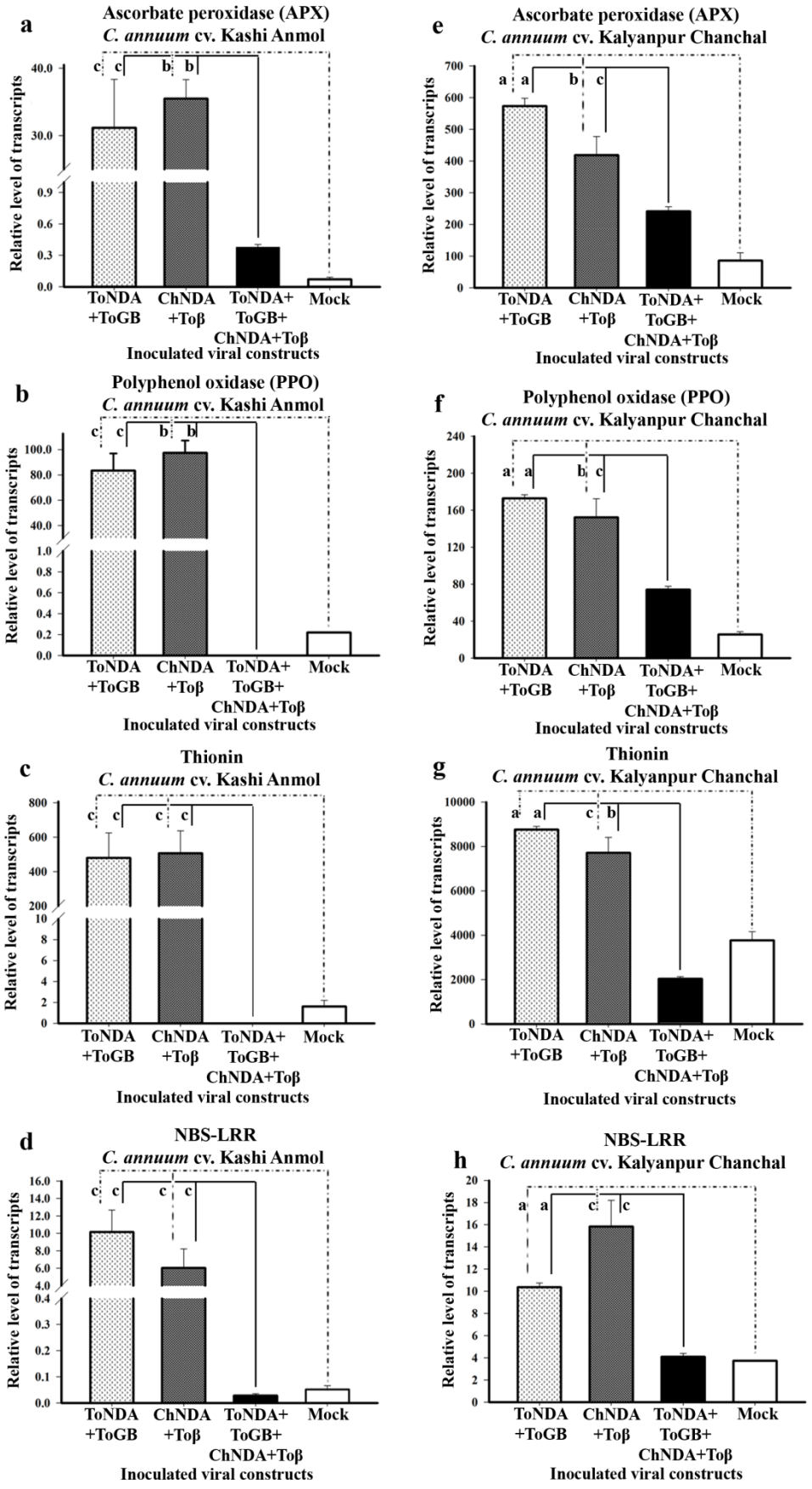
a
Ascorbate peroxidase (APX)
C. annuum cv. Kashi Anmol
Relative level of transcripts
ToNDA +ToGB
ChNDA +Toβ
ToNDA+ ToGB+ ChNDA+Toβ
Mock
Inoculated viral constructs
e
Ascorbate peroxidase (APX)
C. annuum cv. Kalyanpur Chanchal
b
Polyphenol oxidase (PPO)
C. annuum cv. Kashi Anmol
f
Polyphenol oxidase (PPO)
C. annuum cv. Kalyanpur Chanchal
c
Thionin
C. annuum cv. Kashi Anmol
g
Thionin
C. annuum cv. Kalyanpur Chanchal
d
NBS-LRR
C. annuum cv. Kashi Anmol
h
NBS-LRR
C. annuum cv. Kalyanpur Chanchal

Fig. 4.25 Expression profile of host genes during synergistic interaction in the resistant (*C. annuum* cv. Kalyanpur Chanchal) and the susceptible *(C. annuum* cv. Kashi Anmol) chilli plants. To study the host gene expression, qRT PCR was performed using gene specific primers of defense related genes : **(a)** and **(e)** ascorbate peroxidase (APX), **(b)** and **(f)** polyphenol oxidase (PPO), **(c)** and **(g)** thionin, **(d)** and **(h)**. NBS-LRR. Comparison was done between host gene expression in ToNDA and ToGB, ChNDA and Toβ with mock inoculated plants for one data set (indicated as dotted line) ; for another data set gene expression changes among ToNDA and ToGB, ChNDA and Toβ with mixed virus (ToNDA, ToGB, ChNDA and Toβ) inoculated plants (indicated as solid line). Statistical significance is denoted as, a for $p \leq 0.001$ b for $0.001 < p \leq 0.01$ and c for $0.01 < p < 0.05$.

During evolution, plants have developed two strategies that govern innate immunity to prevent invasion of the pathogens. First branch known as "MAMP-triggered immunity" (MTI), employs the receptors which detect nonspecific microbial-associated molecular pattern (MAMPs) such as elongation factor Tu (EF-Tu), peptidoglycan (PGN) and flagellin (Flg) (Kunze et al. 2004; Gust et al. 2007; Felix et al. 1999). To counter the effect of MTI, pathogens have evolved effector molecules that evade host response. If a pathogen is able to evade the MTI, it has to encounter with the second branch of defense known as ''effector-triggered immunity'' (ETI) (Jones and Dangl 2006). ETI employs specific immune receptors known as resistance (R) proteins which recognize pathogen encoded specific effectors. To establish pathogenesis, pathogens need to bypass ETI. Although ETI is totally dependent on the germ-line encoded molecules still it provides remarkable resistance against pathogen. ETI provides resistance through induction of hypersensitivity cell death response (HR). The HR induction restricts the pathogen at the site of infection and ultimately prevents its spread to the adjoining tissues.

R-genes (*NBS-LRR*) are known for providing resistance against several pathogens that include bacteria, viruses, fungi, nematodes and insects (McNeece et al. 2017; Chisholm et al. 2006; Martin et al. 2003). *NBS-LRR* genes always have a basal level of expression and its expression increases in the presence of pathogen (Louis and Rey, 2015; Maiti et al. 2012; Kushwaha et al. 2015b). Nevertheless, expression level of R-gene varies with the pathogen it encounters with. In this study it was found that both sets of primers have amplified R-genes from infected chilli plant's cDNA whereas no amplification was achieved from cDNA isolated from the mock plants. It is possible because the expression of R-genes was higher in the infected chilli plants but below the detection level of PCR in mock-inoculated plants. However, presence of the resistance gene transcripts was detected in the mock-inoculated chilli plants through qRT-PCR.

Plants have numerous R-genes which are evolved through modification (addition, deletion or substitution), recombination and duplication of genes (Dixon et al. 1996; Richter and Ronald 2000). In this study four *NBS-LRR* genes were cloned out of which two were identified as *Capana07g001557* and *Capana07g001559*. Both these genes share 94.9% identity with each other. Phylogenetic analysis showed that both the genes are clustered together in group IV along with *Capana07g001553* and *Capana02g002735*. These results suggest that *Capana07g001557* and *Capana07g001559* may be the outcome of gene duplication followed by specific modification of gene. *Capana07g001553* shares 91.34% and 92.69% identities

with *Capana07g001557* and *Capana07g001559,* respectively. It indicates that the ultimate source of *Capana07g001557* and *Capana07g001559* is probably *Capana07g001553* which might have undergone gene duplication and recombination. During the course of evolution some R-genes may convert to pseudogene (Marone et al. 2013). Remaining two R-genes are *Capana09g000002* and *Capana09g000261* which are clustered together in group I and both the genes have 85.95% identity. *Capana09g000261* have multiple stop codons and nearest R-gene to it in the chromosome 9 is *Capana09g000002*. This information suggests that *Capana09g000261* may be the product of duplication of *Capana09g000002* followed by the modification with insertion of multiple stop codons. The NBS domain of NBS-LRR class of proteins possesses six highly conserved motifs (P-loop, RNBS-A-nonTIR/RNBS-A-nonTIR, kinase-2, RNBS-B, RNBS-C and GLPL) (Wan et al. 2012). Multiple sequence alignment of different *NBS-LRR* proteins has unveiled presence of all the conserved motifs of NBS domains in Capana07g001557, Capana07g001559 and Capana09g000002 but absence of RNBS-C and GLPL motifs in Capana09g000261. This is also an indication that *Capana09g000261* may be a pseudogene.

In the absence of pathogen, NBS-LRR protein remains inactive either due to interaction of internal domains (Moffett et al. 2002) or due to the association of related proteins (Axtell and Staskawicz, 2003). However, in the presence of virulent pathogen (contain avr protein) structural changes occur in the R-protein which leads to its activation (Li et al. 2014). Activation of R-protein is characterized by the induction of hypersensitive cell death response (HR). This study study revealed that Capana07g001557 induces HR in the presence of ChiLCV. Induction of HR exhibits specific interaction between Capana07g001557 and the avr factor of ChiLCV. Other NBS-LRR proteins (Capana07g001559, Capana09g000002 and Capana09g000261) does not induce HR in the presence of ChiLCV is the indication of failure of R-avr interaction. Consequence of R-avr interaction is localized cell death (HR) to prevent the pathogen at the site of infection and ultimately reduced the accumulation of pathogen (Hubert et al. 2003). Southern blotting results of leaf disk samples have also shown reduced titer of ChiLCV in the presence of *Capana07g001557*. Outcome of ChiLCV responsive *NBS-LRR* screening result is that Capana07g001557 works against ChiLCV and reduces the viral DNA accumulation.

Sub-cellular localization is important for achieving optimum function of a protein which provides suitable environment for its activity. Different NBS-LRR proteins have been reported to have dynamic nucleo-cytoplasmic distribution (Shen and Schulze-Lefert 2007;

Bernoux et al. 2011; Meier and Somers 2011). RPM1 is a CC-NBS-LRR which remains on plasma membrane before and after its activation (Boyes et al. 1998; Gao et al. 2011). N-protein of *Nicotiana glutinosa*, RPS4 of *Arabidopsis* and Rx protein of potato are localized at the cytoplasm as well in nucleus (Burch-Smith et al. 2007; Wirthmueller et al. 2007; Tameling et al. 2010). Both N and Rx proteins recognize their related avr factor in cytoplasm but nuclear function is crucial for the activation of downstream signaling (Burch-Smith et al. 2007; Slootweg et al. 2010). RRS1-R of *Arabidopsis* contains WRKY domain at the C-terminal, localizes in the nucleus (Deslandes et al. 2003). Microscopic results identified that Capana07g001557 is present at the cell membrane before and after pathogen infection similar to that of localization of RPM1. It is important to note that both Capana07g001557 and RPM1 are also positioned nearby in the phylogenetic tree. This suggests that Capana07g001557 may function in a manner similar to RPM1.

Activation of plant R-protein starts with the recognition of the specific avr protein of pathogen. Recognition of avr protein by R-protein may involve either the direct or indirect interaction (Caplan et al. 2008; Young and Innes 2006). The simplest way of avr recognition is through physical association of R-avr, however, this phenomenon is not always abundant in nature. Examples of direct interaction are : physical interaction between Pita (CC-NBS-LRR of rice) and AVR-Pita from the fungus *Magnaporthe grisea* (Jia et al. 2000); RRS1-R from *Arabidopsis* with the PopP2 effector of the wilt bacterium (Deslandes et al. 2003) and interaction of L immune receptors (TIR-NBS-LRR) of flax with AvrL567 effectors of the flax rust fungus (Dodds et al. 2006). Second way of avr recognition by R-protein is indirect recognition which is quite common but mechanistically more complex. Most of the effectors modify or alter the particular host protein during pathogenesis. According to the "Guard Hypothesis", specific R-protein monitors modification of the target protein of host and activate the defense response (Van der Biezen and Jones, 1998a; Dangl and Jones, 2001). Another method of avr recognition which is also rare but used by some non-NB-LRR immune receptors is activation of R-protein by effector proteins that act as transcription factors. A pepper resistance protein Bs3 recognizes avrBs3 which localizes to the nucleus (Van den Ackerveken et al. 1996). AvrBs3 binds directly to the promoter of Bs3 gene resulting in the accumulation of Bs3 transcript, ultimately leading to HR (Romer et al. 2007). In the present study ChiLCV avr protein was identified after infiltration of all the viral ORFs in the leaves of chilli plants. Infiltration with the ChiLCV–AC2 ORF has resulted in enhanced accumulation of *Capana07g001557* transcript. From this outcome it is speculated

that ChiLCV-AC2 may be working in similar way as avrBs3. AvrBs3 works a transcription factor (Gurlebeck et al. 2006) and AC2 ORF of different geminiviruses transactivate the host genes (Trinks et al. 2005; Soitamo et al. 2012). Further, to confirm if ChiLCV-AC2 or any other ORF can induce HR lesion in the presence of Capana07g001557, *Capana07g001557* and all viral ORFs infiltrated in *N. benthamiana* leaves. *N. benthamiana* plants were selected for the experiment in place of chilli in order to avoid the bias of expression of internal *Capana07g001557*. Infiltration results showed HR induction by the AC2 ORF only in presence of Capana07g001557 indicates that ChiLCV-AC2 protein function as avr protein for Capana07g001557.

As previously described that avr can interact physically with R-protein, Capana07g001557 interaction with the viral proteins was investigated through Y2H analysis. Y2H results revealed that none of the viral proteins interacts directly with Capana07g001557. This suggests that ChiLCV-AC2 acts as avr protein but does not interact directly with Capana07g001557. According to the "Guard Hypothesis", Capana07g001557 and AC2 might be interacting through another protein (guardee). For identification of guardee prediction of Capana07g001557 interacting proteins was made through STRING software. Capana07g001557 interacting proteins (HSP90, SGT1 and Cyt-c1) predicted by STRING database were analyzed by Y2H analyses. Previous report has shown that RPM1 (CC-NBS-LRR in *Arabidopsis*) function through HSP90 (Liu et al. 2004). Heat shock protein 90 (HSP90) is a highly conserved and essential molecular chaperone involved in various functions such as activation and stabilization of key signaling proteins such as protein kinases, maturation, assembly, transcription factors in eukaryotic cells and hormone receptors (Pearl and Prodromou 2006). HSP90 is involved in innate immunity through a complex formation by HSP90-SGT1-Rar1 and R-protein (Liu et al. 2004; Kadota and Shirasu 2012; Xu et al. 2012). Suppression of HSP90 compromise the N mediated resistance in *N. tabacum* (Liu et al. 2004). SGT1 (suppressor of the G2 allele of skp1) is also a highly conserved protein and involved in R-protein mediated resistance in plants. Interaction of HSP90 and SGT1 is required for the stability and maintenance of steady state level of R-protein (Azevedo et al. 2006; Boter et al. 2007). SGT1 is also a part of HSP90-SGT1-Rar1-Rprotein complex and required for many R-proteins to recognize the viral, bacterial, oomycete or fungal pathogens (Shirasu and Schulze-Lefert 2003; Schulze-Lefert 2004). Y2H results have suggested that neither HSP90 nor SGT1 physically interact with Capana07g001557. It may be possible that HSP90 and SGT1 do not interact directly with Capana07g001557 through

Y2H but it may interact in planta through the formation of whole complex, which needs further experimentation.

STRING database has also predicted Cyt-c1 as an interacting partner of Capana07g001557. NBS-LRR proteins have central conserved NB-ARC domain that is also highly conserved in the human apoptotic regulator Apoptotic protease activating factor 1 (APAF-1) (van der Biezen and Jones 1998b). After binding with Cytochrome C and dATP APAF-1 form an oligomeric apoptosome. Further, apoptosome binds with Procaspase 9 and cleaves it to release its mature and active form. Finally, apoptosome induce the caspase 9 dimerization and subsequent autocatalysis of cell (Pop et al. 2006). Apoptosis and HR both type of programmed cell death are involved in innate immunity of animals and plants (Coll et al. 2011). These information have suggested that plant NBS-LRR can also interact with Cyt-c1. Our Y2H results have indicated that Cyt-c1 does not interact with Capana07g001557.

Previous result of Southern blotting has shown reduction of ChiLCV DNA titer in the presence of Capana07g001557. Further the infiltration of ChiLCV-*EGFP* has also resulted in reduction of ChiLCV accumulation in the presence of Capana07g001557 at the site of infiltration. Again, Southern analysis of ChiLCV infiltration in *Capana07g001557* over expressing *N. tabacum* plants has shown decreased viral DNA accumulation. All these results are concurrent to the previous study which has reported that the expression of R-gene reduces the pathogen accumulation (Mackey et al. 2003). ChiLCV-*EGFP* infiltration results have shown that control infiltrated leaves have higher number of GFP expressing cells and the cells expressing GFP are in the groups (group of 3-4 cells together expressing GFP). ChiLCV-*EGFP* infiltration with *Capana07g001557* shows reduced number of GFP expressing cells and all the GFP expressing cells are discrete (placed distinctly). These results suggest that Capana07g001557 may be impairing the movement of ChiLCV. Southern analysis of *Capana07g001557* over expressing *N. tabacum* plants have demonstrated reduced titer of ChiLCV compared to the wild-type plants. Notably, some transgenic plants have reduced level of ChiLCV DNA compared to the wild type plants. Surprisingly some plants contained linear, open circular and supercoiled forms of viral DNA similar to the wild-type plants inocultade with ChiLCV, however, the single-stranded form of viral DNA was drastically reduced in transgenic plants. Geminivireses have single stranded circular DNA which replicate through double stranded DNA (Vanitharani et al. 2005). Single stranded DNA of geminiviruses is involved in the packaging and movement (Rojas et al. 1998; Jeske et al. 2001; Vanitharani et al. 2005). Reduction of single stranded form of viral DNA is another

evidence of impaired viral movement because of presence of Capana07g001557 in the infected plants. In this study accumulation of viral DNA was also analyzed at the systemic parts of plant and results indicated decreased viral DNA at the systemic part of the plant. Reduction of viral DNA at the systemic part may be the result of disrupted movement of ChiLCV due to the Capana07g001557 or induction of systemic acquired resistance (SAR) by Capana07g001557.

In the absence of avr protein, NBS-LRR protein remains inactive due to the interaction of internal domains (Moffett et al. 2002) or due to the association of related protein (Axtell and Staskawicz, 2003). In the presence of avr protein R-protein get activated, some of the NBS-LRR proteins dimerized through the interaction of N-terminal and NB-ARC domains and starts downstream signaling (Bernoux et al. 2011; Bonardi et al. 2012). Y2H result indicate that Capana07g001557 do not dimerize because NBS-LRR protein cannot dimerize in the absence of avr protein. In planta, BiFC results have also suggested that Capana07g001557 protein molecule does not dimerize in the absence of ChiLCV. BiFC results have also indicated that Capana07g001557 dimerizes only in the presence of ChiLCV.

Taken together, the results suggest that *Capana07g001557* is a CC-NBS-LRR type of R-gene that elicit defense responses against ChiLCV. ChiLCV –AC2 protein acts as the avr protein and gets recognized by Capana07g001557. Capana07g001557 reduces the ChiLCV accumulation may be by hindering the movement of virus (Fig. 5.1). Further experiments need to be performed in order to understand the molecular basis of Capana07g001557 mediated hindrance of ChiLCV movement.

Typical symptoms of severe leaf curl disease caused by begomoviruses were noticed on resistant cultivars of chilli, such as cv. Kalyanpur Chanchal and cv. Bhut Jolokia. Geminiviruses have great potential of recombination and pseudo-recombination. During mixed infection, several genetic interaction occur which leads to development of new viral strain or isolate with improved fitness to overcome host defense machineries for successful pathogenesis. In the present study, four different genomic components of begomoviruses were identified from the symptomatic resistant chilli plants. The association of more than one virus in symptomatic plants has emerged as a common natural incidence and has been reported from previously (Pruss et al. 1997), but the molecular mechanism underlying the resistance breakdown has been largely unknown. In the current study, the nature and kinetics of synergistic interaction among begomoviruses and the molecular mechanism of resistant breakdown in the resistant chilli cultivar Kalyanpur Chanchal has been demonstrated.

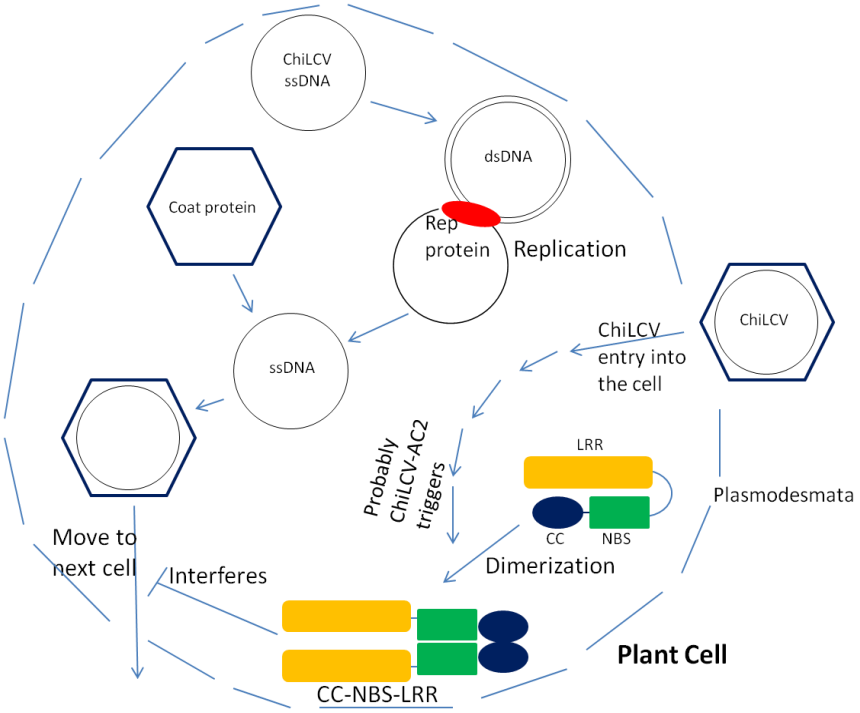

Fig. 5.1 Models for Capana07g001557 working. Capana07g001557 dimerizes in the presence of ChiLCV (probably induced by AC2)and interferes with the movement of ChiLCV.

Interactions between two viruses may result in either positive (synergistic) or negative influence (antagonistic) on their pathogenesis (Méndez-Lozano et al. 2003; Alves-Júnior et al. 2009). Synergistic interaction among the viruses leads to the increased symptom severity and enhanced level of viral titer which has previously been reported among distinct viruses belonging to either same or different family/genera/species (Pruss et al. 1997; Wintermantel, 2005; Chakraborty et al. 2008; Mohamed, 2010; Caracuel et al. 2012). The viral determinants of synergistic interaction among begomoviruses causing severe cassava mosaic disease have also been studied (Vanitharani et al. 2004). Among the RNA viruses, Cucumber mosaic virus (CMV) assists infection of Potato virus Y into internal phloem cells thus PVY could move in the younger leaves and replicates many times higher than single infection (Ryang et al. 2004). Potyvirus encoded P1, helper component proteinase (HC-Pro), and a fraction of P3 enhances the pathogenicity and replication of CMV and *Tobacco mosaic virus* (TMV) by suppression of post transcription gene silencing (PTGS) (Pruss et al. 1997). CMV 2b protein blocks spreading of systemic signal of PTGS and helps in the accumulation of Potato virus Y in the shoot apex (Ryang et al. 2004; Guo and Ding 2002). Similarly, C2 protein of *Beet curly top virus* (Curtovirus) promotes a suitable cell environment to enhance the replication of geminiviruses in Rep assisted manner (Caracuel et al. 2012). Synergistic interaction of geminiviruses may led to recombination, pseudo-recombination or assortment of viral

components, leading to the emergence of new virus species (Fondong et al. 2000; Pita et al. 2001; Rasheed et al. 2006; Chakraborty et al. 2008).

Earlier study has demonstrated the potential of *Tomato leaf curl New Delhi virus* (DNA-A) and *Tomato leaf curl Gujarat virus* (DNA-B) to form a viable supervirulent pseudorecombinant and to develop severe leaf curl disease on tomato (Chakraborty et al., 2008). The present study reports the first natural association of *Tomato leaf curl New Delhi virus* (DNA-A) and *Tomato leaf curl Gujarat virus* (DNA-B) which cause severe leaf curl disease in the resistant chilli cv. Kalyanpur Chanchal. It is well documented that the DNA-B of bipartite virus encodes movement protein and nuclear shuttle protein and faciltates the DNA-A accumulation in systemic parts of the plants. On the other hand, DNA- B depends on DNA-A for replication. Our results indicated that ToNDA and ToGB assist each other and behave like a bipartite begomovirus. According to the southern and PCR results, ChNDA resembles a monopartite virus and therefore, requires Toβ for pathogenesis. However, ToNDA and ChNDA supported each other for multiplication in *N. benthamiana*. DNA β encoded βC1 protein is known to be involved in pathogensis, movement and suppression of host antiviral silencing machineries (Briddon et al. 2003; Yang et al. 2011; Kumari et al. 2010; Shukla et al. 2013).

Our analysis demonstrates that Toβ depends on ToNDA for own replication without influencing helper virus accumulation. The results obtained here indicate that Toβ presumably facilitated interaction between monopartite and bipartite virus. It is relevant to note that ChNDA and ToGB could not form a viable pseudorecombinant between genomic components of a monopartite and bipartite begomovirus. Reduced level of both Toβ and ToGB was observed when both of these components were present together with either ChNDA or ToNDA which suggests competition between Toβ and ToGB for helper virus mediated replication. This result was in concurrent with the previous report which suggested reduced accumulation of DNA-B and betasatellite in plants inoculated with tripartite combination (ToLCV DNA-A, DNA-B and betasatellite) (Joyothsana et al. 2013).

Notably, we failed to detect viral DNA from *Capsicum annuum* plants by Southern hybridization which could be due to low level of viral titer. Furthermore, PCR based detection of the viral genomic components in these plants can be attributed to sensitivity of this technique over Southern hybridization. In *Capsicum annuum* plants monopartite virus failed to induce symptoms while only few plants inoculated with bipartite virus showed mild symptoms. Interestingly, plants inoculated with four genomic components showed severe

symptoms indicating synergistic interaction among the monopartite and bipartite viruses are key for resistant breakdown in chilli.

This breakdown of resistance may be modulated by the differential expression of the host resistance related genes. In a previous study, ChiLCV caused up regulation of several defense related genes, encoding proteins such as nucleotide-binding site leucine-rich repeat (NBS-LRR) domain containing protein, lipid transfer protein, thionin, polyphenol oxidase, and other proteins like ATP/ADP transporter (Kushwaha et al. 2015a). Therefore, we aimed to correlate synergistic interaction among chilli-infecting begomoviruses and expression of host defence genes in both susceptible and resistant chilli cultivars.

Polyphenol oxidase (PPO) is a tetrameric copper containing 52-64 kDa protein (Van Gelder et al. 1997) and catalyses the O-hydroxylation of phenols produced during oxidative burst following pathogen attack. Many reports have revealed that PPO is involved in maintaining the basal defense against fungi, bacteria and viruses (Constabel et al. 1995; Li and Steffens 2002; Thipyapong et al. 2004; Poiatti et al. 2009). In the current study, we observed that PPO transcript level was increased in both monopartite (ChNDA and Toβ) and bipartite virus (ToNDA and ToGB) inoculated plants. This result suggested role of PPO in conferring basal defense mechanism which could be correlated with low viral titer and absence of symptoms on both the cultivars of chilli. Reduced level of PPO transcript in mixed inoculated plants is linked with the suppression of basal defense mechanism and accumulation of higher viral DNA.

APX functions as a scavenger of reactive oxygen species and catalyzes the conversion of H_2O_2 into water (Pignocchi et al. 2003). Thionin, a small cys-rich highly basic protein having antimicrobial activity, involves in eliciting the plant defense against several pathogens (Epple et al. 1997; Pelegrini and Franco 2005). However, no direct evidence of the involvement of thionin in defense against begomoviruses has been elucidated yet. However, the reduced expression of APX and thionin transcripts in mixed inoculated chili plants was in concurrent with the results observed in the case of PPO transcripts. Therefore, in mixed inoculated plants all four molecules of begomoviruses or their encoded products function cooperatively to suppress the defense responsive genes.

NBS-LRR is conserved domain protein that involve in specific resistance to several pathogens including viruses such as *Cucumber mosaic virus* (CMV) (Seo et al. 2006), TMV (Dinesh-Kumar and Baker 2000), *Mungbean yellow mosaic India virus* (MYMIV) (Maiti et

al. 2012). In our previous study, expression of NBS-LRR was found to be up-regulated significantly in ChiLCV inoculated resistant chilli cultivar Punjab Lal (Kushwaha et al. 2015a). The analysis of NBS-LRR expression in mixed inoculation chilli cultivars revealed the suppression of R-gene mediated defence response. There are no available reports on functional characterization of any R-gene leading to the resistance against geminiviruses, moreover few reports have indicated the involvement of R gene mediated resistance against geminiviruses (Maiti et al. 2012). A study reported the upregulation of NBS-LRR gene (namly CRY1) in *V. mungo* which conferred resistance against *Mungabean yellow mosaic Indian virus* (MYMIV). Conversely, NBS-LRR gene's expression has also been suppressed in susceptible plants infected with *Tomato leaf curl New Delhi virus* (Kushwaha et al. 2015b). The present study revealed downregulation of *NBS-LRR* expression in mixed infected plants, whereas plants inoculated with either monopartite or bipartite begomoviruses showed upregulation of *NBS-LRR* transcripts.

In conclusion, qRT PCR result suggested that monopartite and bipartite begomoviruses infection may induce basal (PPO, APX, thionin) and specific (NBS-LRR) defense responses in host plant. However, during mixed infection cumulative effect of all four viral molecules have suppressed both basal and specific defense systems, which was established by reduced expression of defense related genes and higher viral DNA accumulation and symptom severity. Additionally, role of viral suppressors cannot be overlooked during mixed infection. Begomoviruses encode different proteins which function as a suppressor of PTGS, TGS and other host defense machinery. For example, AC1, AC2, AC4, AV2 and βC1 of begomoviruses are known to suppress RNAi machinery (Vanithrani et al. 2004; Trinks et al. 2005; Rodriguez-Negrete et al. 2013). The cumulative effect of these proteins and coordinate action of other viral proteins might help in the down regulation of host basal and specific defense pathways, which eventually leads to symptoms development on resistant chilli cultivar.

Taken together, these results suggest that in mixed infected plants, ToNDA forms cognate pair with ToGB for pathogenesis and also assist replication of ToGB. The enhanced level of ToNDA facilitates the replication of Toβ which in turn favourably influences ChNDA accumulation in plant. The enhanced level of viral genomic components due to synergistic interaction has led to suppress host defense in resistant chilli cultivar. The outcome of the study may assist in the development of efficient antiviral strategies for generating broad-spectrum resistance against chilli-infecting begomoviruses.

Since discovery of the first R-gene isolated by Johal and Briggs in 1992, information became available about numerous R-genes and related pathogens, however, role of any R-gene against the largest family of plant viruses (*Geminiviridae*) has not been characterized till date. To the best of our knowledge, the present study provides information about an R-gene that has been identified and characterized against a geminivirus, *Chilli leaf curl virus*. The present study also underlines the mechanism of breakdown of natural resistance in chilli that was observed for the first time and elucidated role of defense related genes of host during synergistic interaction of viral genomes.

The major outcomes of our study are summarized below:

1. Four different *NBS-LRR* genes were cloned from chilli cv. Punjab Lal out of which a CC-NBS-LRR (*Capana07g001557*) gene showed antiviral response against ChiLCV.
2. ChiLCV-AC2 ORF increases expression of *Capana07g001557* and also triggers hypersensitive response in the presence of Capana07g001557.
3. Capana07g001557 protein is localized on the cell membrane and Capana07g001557 dimerizes in planta in the presence of ChiLCV.
4. Capana07g001557 neither interacts physically with any of the ChiLCV proteins nor with the selected host proteins (HSP90, SGT1, Cyt-c1).
5. Transient overexpression of Capana07g001557 reduces ChiLCV accumulation in local as well distal parts of plant.
6. Reduction of ChiLCV accumulation in the presence of Capana07g001557 may be due to hindrance of ChiLCV movement by Capana07g001557.
7. Breakdown of natural resistance of hitherto known resistant chilli cultivar Kalyanpur Chanchal is due to association of geminivirus genomic components [DNA-A of Tomato leaf curl New Delhi virus (ToNDA), DNA-B of Tomato leaf curl Gujarat virus (ToGB), Chilli leaf curl virus (ChNDA) and Tomato leaf curl betasatellite (Toβ)].
8. Survey of chilli plants grown at the experimental field JNU, New Delhi, showed that more than 60% plants were infected with these viral genomic components.
9. These geminiviral genomic components interact synergistically and produce severe symptoms on model plant (*N. benthamiana*) and natural host chilli.
10. Together, all four geminivirus genomic components (ToNDA, ToGB, ChNDA and Toβ) suppress expression of the defense related genes in resistant chilli plants that leads to breakdown of natural resistance.

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Appendix IA

PREPARATION OF REAGENTS, BUFFERS AND MEDIA

Buffer/Reagent/Media	Method of Preparation
1. For Agarose gel electrophoresis	
i. 50X Tris acetate EDTA (TAE)	242g of Tris base, 57.1 ml of Glacial acetic acid, 0.5M EDTA was added and the volume was made up to 100ml with distilled H_2O. The pH was adjusted to 8.0.
ii. 6X DNA loading dye	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol was dissolved in distilled H_2O and stored at 4ºC.
iii. DNA Molecular Marker	Fermentas DNA marker-(ladder size in bp) 10000, 8000, 6000, 5000, 4000, 3500, 3000,2500 2000, 1500, 1000, 750, 500 and 250.
iv. Ethidium Bromide (10mg/ml)	1g Ethidium Bromide was added to 100ml of distilled H_2O, stirred at magnetic stirrer for several hours, transferred to a dark bottle and stored at room temperature.
2. For Cloning	
i. Ampicillin	Stock solution (100mg/ml) was made in double distilled H_2O, filter sterilized (through 0.22µ filter) and aliquoted in 1.5ml tubes and stored at -20ºC.
ii. Kanamycin	Stock solution (50mg/ml) was made in double distilled H_2O, filter sterilized (through 0.22µ

	filter) and aliquoted in 1.5ml tubes and stored at -20ºC.
iv. Luria broth media	1g of bacto-tryptone, 0.5g of yeast extract and 1g of NaCl was dissolved in 95 ml of H_2 and the pH was adjusted to 7 with 5N NaOH. The volume was adjusted to 100 ml and sterilized by autoclaving.
v. Luria agar media	1g of Bacto-tryptone, 0.5 g of yeast extract and 1g of NaCl was dissolved in 95 ml of water and the pH was adjusted to 7 with 5 N NaOH. The volume was adjusted to 100 ml. Then 1.5g of bacto-agar was added and sterilized by autoclaving.
v. X-gal (5-bromo-4 chloro-3-indolyl-b D-galactopyranoside)	Stock solution (20mg/ml) was prepared by dissolving X-gal in dimethyl foramide, and stored at -20ºC.
vi. IPTG (Isopropyl thio-a-galactoside)	2g of IPTG was dissolved in distilled H_2O, and volume was adjusted to 10ml then filter sterilized through 0.22 disposable filter and was stored at -20ºC.
vii. 100mM $MgCl_2$	2.03g of $MgCl_2.6H_2O$ was dissolved in distilled H_2O, volume adjusted to100ml and sterilized by autoclaving.
viii. 100mM $CaCl_2$	1.47g of $CaCl_2.2H_2O$ was dissolved in distilled H_2O, volume adjusted to100ml and sterilized by autoclaving.
3. For Plasmid Isolation	
i. Solution I (Resuspension buffer)	It was prepared by adjusting final concentration to 1M Tris-HCl (25mM), (pH 8.0)- 2.5ml 20% Glucose (50mM) -4.5 ml 0.5M EDTA (10mM) - 2.0ml and sterile distilled H_2O- upto 100ml.
ii. Solution II	It was prepared freshly by mixing 10N NaOH

(Lysis buffer)	(0.2N)- 2.0ml, 1% SDS- 5.0ml, distilled H_2O- Up to 100ml.
iii. Solution III (pH 4.8) (Neutralization buffer)	40.81g of sodium acetate (3M) was dissolved in minimum volume of distilled H_2O, and pH was adjusted to 4.8 with glacial acetic acid, now volume adjusted to 100ml with H_2O and sterilized by autoclaving.
iv.Phenol:Chloroform: Isoamylalcohol	It was prepared by mixing Tris-saturated Phenol (pH 7.5) 25ml, chloroform 24ml, and Isoamylalcohol 1ml.

4. For Total DNA Extraction

CTAB method	Extraction buffer was prepared by adjusting final concentration to 100mM TrisCl pH 8.0, 100m EDTA pH 8.0, 1.4M NaCl, 2% CTAB (w/v). Solution was incubated this solution at 65C for 30 min - 60 min (till CTAB completely dissolves) and 1% 2-mercapto-ethanol was added just before use.

5. For southern hybridization

i. 20X SSC	It was prepared by adding 175.3g NaCl, 88.2g sodium citrate (pH adjusted to 7.0 by adding 1.4N HCl) in 1 litre of H_2O.
ii. Denauration solution	It was prepared by adjusting final concentration of 1.5M NaCl and 0.5M NaOH in H_2O.
iii. Denhardt's solution (50X)	It was prepared by adding 1% (W/V) ficol, 1% (W/V) polyvinylpyrrolidone, 1% (w/v) BSA and was dissolved in H_2O to prepare a 50X solution.

iv. Depurination solution	It was prepared by adding 2.8ml of HCl in 197.2 ml of sterile H_2O.
v. Neutralization Solution	It was prepared by adding 1.5M NaCl and 1M TrisCl pH 7.2.
vi. Herring sperm	100mg of DNA was dissolved in 10 ml of sterile distilled H_2O and mixed by vortexing, stored at -20C.
vii. Hybridization buffer	It was prepared by adding 0.25M sodium phosphate buffer (pH 7.2), 7% (w/v) SDS, 1mM EDTA.

PREPARATION OF COMMONLY USED STOCK SOLUTION

Calcium chloride (2.5M)

11g of $CaCl_2.6H_2O$ was dissolved in a final volume of 20ml of distilled H2O. The solution was sterilized by passing it through a 0.22 filter. It was then stored in 1ml aliquots at 4C

Deoxyribonucleoside triphosphates (dNTPs)

Each dNTP was dissolved in H_2O at an approximate concentration of 100mM. 0.05M Tris base and a micropipette was used to adjust the pH of each of the solutions to 7.0 (pH paper was used to check the pH). An aliquot of the neutralized dNTP was diluted appropriately and the optical density at the wavelengths as given below in the table, was recorded. The actual concentration of each dNTP was calculated. The solutions were diluted with H_2O to a final concentration of 50mM dNTP. Each was stored separately at -80 C in small aliquots.

Base	Wavelength	Extinction Coefficient (E) (M-1 cm-1)
A	259	1.54 104
G	253	1.37 104
C	271	9.10 103
T	267	9.6 103

EDTA (0.5M, pH 8.0)

186.1g of disodium EDTA-2H_2O was added to 800ml of water. It was stirred vigorously on a magnetic stirrer and pH was adjusted to 8.0with NaOH (20g of NaOH pellets). It was dispensed into aliquots and sterilized by autoclaving.

NaOH (10N)

400g of NaOH pellets were added to 800ml of water, stirring continuously, The volume was adjusted to 1 litre with H_2O once the pellet got dissolved completely. The solution was stored in a plastic container at room temperature.

NaCl (Sodium Chloride, 5M)

292g of NaCl was dissolved in 800ml of H_2O. The volume was adjusted to 1litre with H_2O. It was dispensed into aliquots and then sterilized by autoclaving. The NaCl solution was stored at room temperature.

Potassium acetate (5M)

5M Potassium acetate- 60ml

Glacial acetic acid -11.5ml

H_2O- 28.5 ml

The resulting solution was 3M with respect to potassium and 5M with respect to acetate. The buffer was stored at room temperature.

SDS(20%w/v)

200g of electrophoresis-grade SDS was dissolved in 900ml of H_2O. It was heated to 68C and stirred with magnetic stirrer to assist dissolution. The volume was adjusted to 1 litre with H_2O and stored at room temperature.

Sodium Acetate (3M, pH 5.2 and pH 7.0)

408.3g of sodium acetate 3H_2O was dissolved into 800ml of H_2O. The pH was adjusted to 5.2 with glacial acetic acid. It can also be adjusted to 7.0 with dilute acetic acid. The volume was adjusted to 1 litre with H_2O. It was later dispensed into aliquots and sterilized by autoclaving.

Tris HCl

121.1g of Tris base was dissolved in 800ml of H_2O. The pH was adjusted to desired value by adding concentration HCl, as mentioned below.

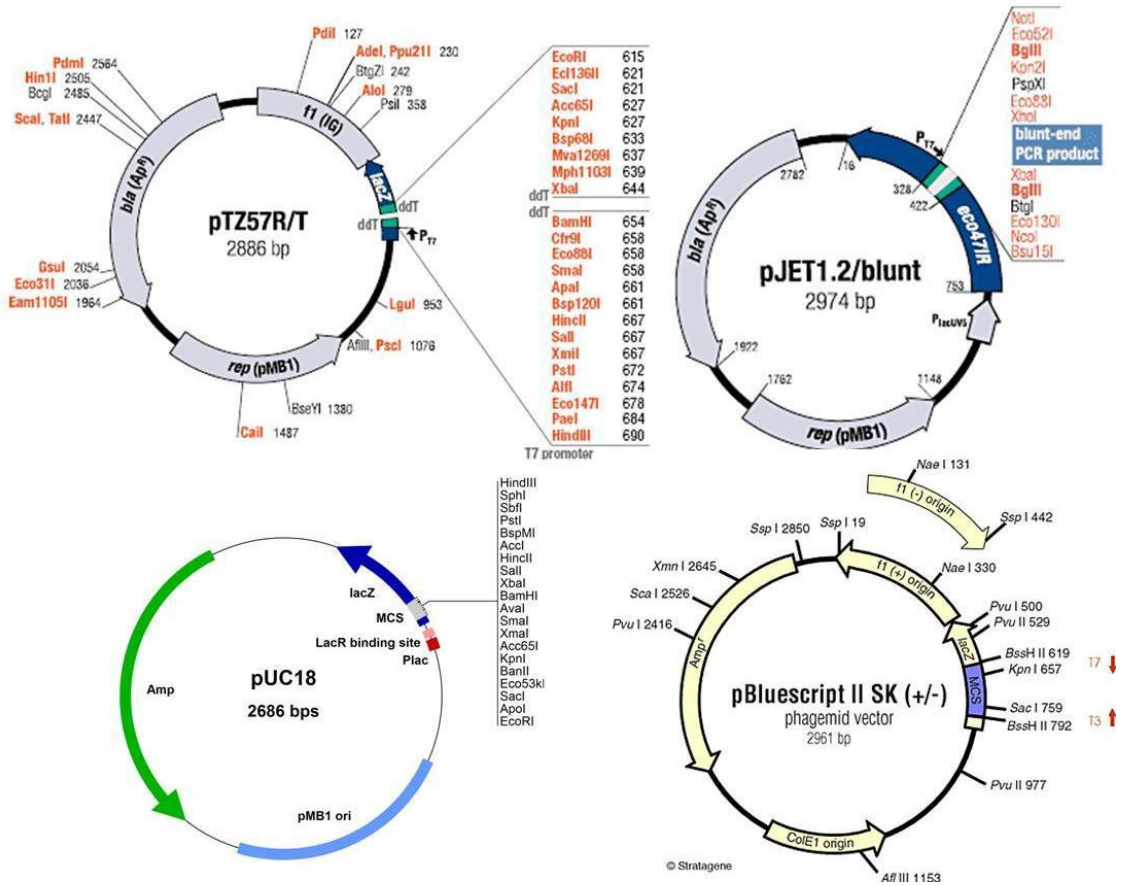
pH	HCl
7.4	70ml
7.6	60ml
8.0	42ml

The solution was allowed to cool at room temperature and the pH was adjusted. Then, volume of solution was adjusted to 1 litre with H_2O. This was then aliquoted and sterilized by autoclaving.

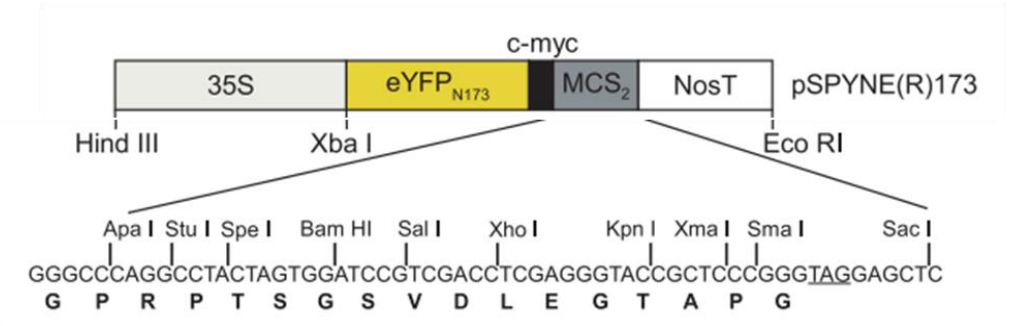
X-gal Solution (2% w/v)

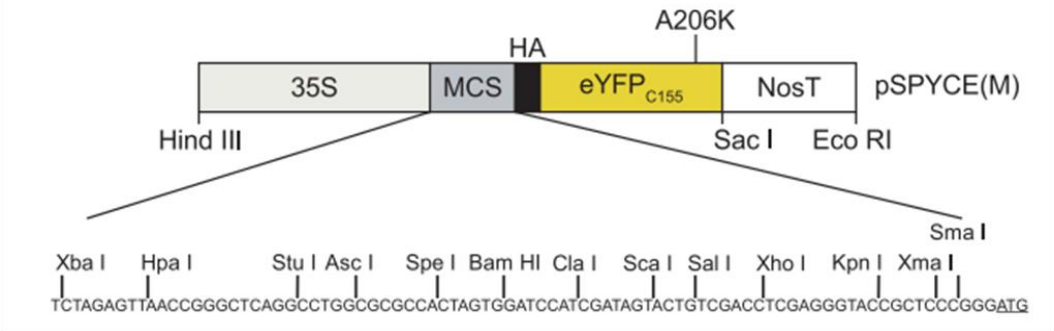
A stock solution was prepared by dissolving X-gal in di-methylformamide at a concentration of 20mg/ml solution. A glass or polypropylene tube was used. The tube containing the solution was wrapped in aluminium foil to prevent damage by light and was stored at -20C. sterilization of X-gal solution by filtration was not required.

Cloning vectors

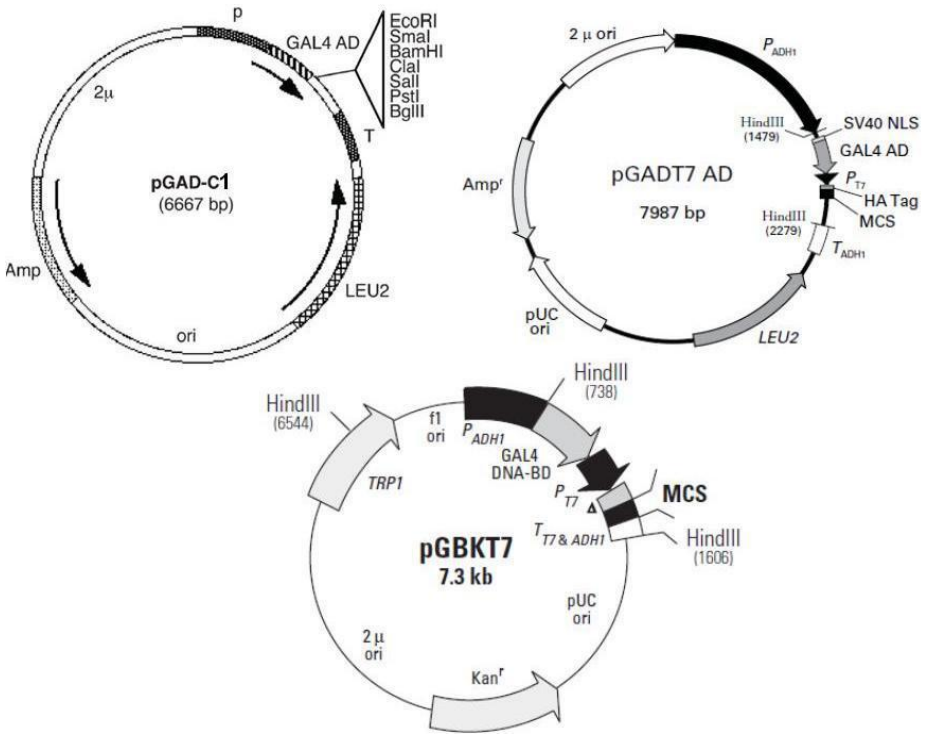


BiFC vectors

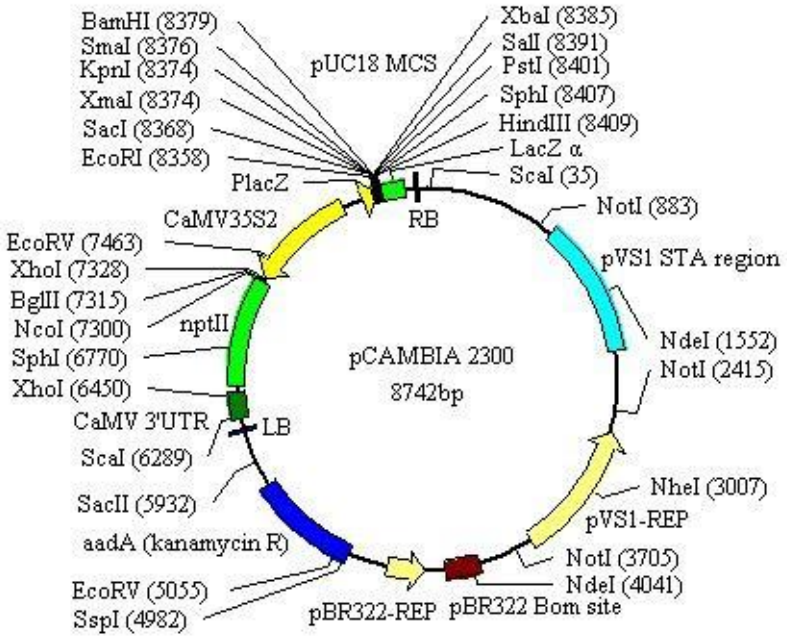


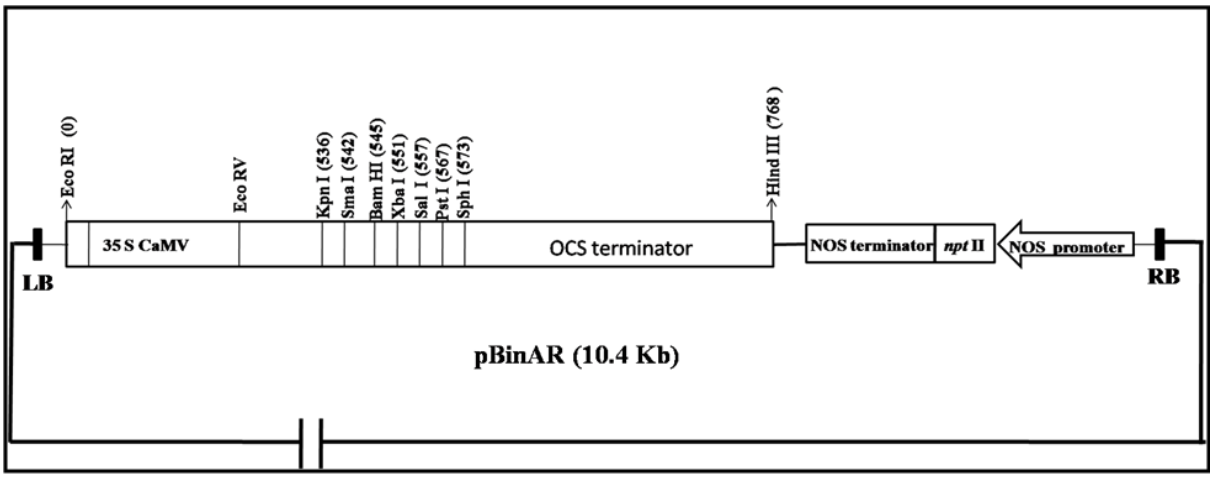


Yeast expression vectors



Plant expression vector




Eco RI (0)
Eco RV
Kpn I (536)
Sma I (542)
Bam HI (545)
Xba I (551)
Sal I (557)
Pst I (567)
Sph I (573)
Hind III (768)
35 S CaMV
OCS terminator
NOS terminator
npt II
NOS promoter
LB
RB
pBinAR (10.4 Kb)

List of Publications

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Synergistic interaction among begomoviruses leads to the suppression of host defense-related gene expression and breakdown of resistance in chilli

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Abstract Chilli (*Capsicum* sp.) is one of the economically important spice and vegetable crops grown in India and suffers great losses due to the infection of begomoviruses. Conventional breeding approaches have resulted in development of a few cultivars of chilli resistant to begomoviruses. A severe leaf curl disease was observed on one such resistant chilli cultivar (*Capsicum annuum* cv. Kalyanpur Chanchal) grown in the experimental field of the Jawaharlal Nehru University, New Delhi. Four different viral genomic components namely, *Chilli leaf curl virus* (DNA A), *Tomato leaf curl Bangladesh betasatellite* (DNA β), *Tomato leaf curl New Delhi virus* (DNA A), and *Tomato leaf curl Gujarat virus* (DNA B) were associated with the severe leaf curl disease. Further, frequent association of these four genomic components was also observed in symptomatic plants of other chilli cultivars (*Capsicum annuum* cv. Kashi Anmol and *Capsicum chinense* cv. Bhut Jolokia) grown in the experimental field. Interaction studies among the isolated viral components revealed that *Nicotiana benthamiana* and chilli plants inoculated with four genomic components of begomoviruses exhibited severe leaf curl disease symptoms. In addition, this synergistic interaction resulted in increased viral DNA accumulation in infected plants. Resistant chilli plants co-inoculated with four genomic components of begomoviruses showed drastic reduction of host basal (ascorbate peroxidase, thionin, polyphenol oxidase) and specific defense-related gene (NBS-LRR) expression. Our results suggested that synergistic interaction among begomoviruses created permissive cellular environment in the resistant chilli plants which leads to breakdown of natural resistance, a phenomenon observed for the first time in chilli.

Keywords Geminivirus · Synergism · Chilli · Resistance breakdown · Host gene expression

Introduction

Plants have developed several layers of defense mechanisms against a wide range of pathogens. For this, either plants produce a myriad of anti-pathogenic compounds or activate specific defense signaling pathways to protect themselves against the pathogens (Heil and Bostock 2002; Veronese et al. 2003). A resistant plant is equipped with specific genes which provide innate and acquired resistance against particular pathogens. In general, an individual pathogen does not succeed to overcome the well-fortified defense barriers and thus, fails to cause disease (Staskawicz 2001; Zhang et al. 2013). However, in nature, plants are continuously exposed to multiple pathogens at a time (Rentería-Canett et al. 2011; Syller 2012), and the cumulative effect of these multiple infection causes exaggerated symptoms on the plant (Pruss et al. 1997). During multiple infection, synergistic interaction promotes either multiplication or movement of pathogens in a host plant, along with the tendency to suppress plant defense mechanisms (Ryang et al. 2004; Burgyan and Havelda 2011; Caracuel et al. 2012). Eventually, the cumulative effect of synergistic interaction leads to the breakdown of defence barriers of the plants

Ashish Kumar Singh and Nirbhay Kushwaha contributed equally to this work.

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(García-Cano et al. 2006). These phenomena of mixed infections are more common in virus-infected plants. Various studies in plant virus interaction have revealed the existence of synergistic effect on several plant species (Pruss et al. 1997; Hu et al. 1998; Rentería-Canett et al. 2011; Caracuel et al. 2012). Nevertheless, exact molecular mechanism underlying resistance breakdown still needs to be identified.

Begomoviruses belong to the family *Geminiviridae* and are characterized by their small geminate-shaped twinned icosahedral capsids (Lazarowitz et al. 1992; Hanley-Bowdoin et al. 2000; Böttcher et al. 2004; Jeske 2009; Shepherd et al. 2010). They are either monopartite or bipartite with 2.5–3.0-kb single-stranded circular DNA genome, which is transcribed bidirectionally and replicate in the host nucleus via double-stranded intermediate (Hanley-Bowdoin et al. 2000; Jeske 2009). Bipartite begomoviruses contain DNA A and DNA B, as genomic components (Hanley-Bowdoin et al. 2000; Jeske 2009). DNA A contains genes necessary for replication, insect transmission, suppression of gene silencing, etc. DNA B depends on DNA A for replication and encodes proteins involved in cell-to-cell and long distance movement of the virus. Begomoviruses are often associated with betasatellites (Briddon et al. 2003; Chattopadhyay et al. 2008; Kumar et al. 2015). Betasatellites depend on helper DNA begomovirus for replication, but play a crucial role during the virus infection and pathogenesis (Briddon et al. 2003; Kumari et al. 2010; Singh et al. 2012; Bhattacharyya et al. 2015).

Chilli is one of the economically important vegetable crops in India. Several begomoviruses are known to infect chilli and cause huge economic losses in India (Kumar et al. 2015). In the present study, we surveyed chilli leaf curl disease occurring on different chilli cultivars grown on field. For the first time, we report the breakdown of resistance in a hitherto resistant chilli cultivar Kalyanpur Chanchal. In the present study, we have isolated the resistance breaking genomic components of begomoviruses infecting chilli. The kinetics of synergistic interaction among the begomoviruses was studied on *Nicotiana benthamiana* and *Capsicum annuum* cv. Kalyanpur Chanchal. Further, cumulative effect of synergistic interaction in host basal and acquired immunity was analyzed.

Materials and methods

Isolation, cloning, and sequencing of full-length genomes

Leaf samples from the resistant chilli cultivars showing severe leaf curling were collected from the experimental field of the Jawaharlal Nehru University (JNU), New Delhi in 2009. To identify the causative virus of chilli leaf curl disease (ChiLCD), total genomic DNA was extracted from the diseased samples according to Dellaporta et al. (1983), and rolling-circle amplification (RCA) was carried out using the TempliPhi 100 amplification kit (GE Amersham, Little Chalfont, UK) following the manufacturer's instruction. The RCA products were digested with *Kpn*I restriction enzyme and subsequently, linearized products were cloned into pUC18 vector at *Kpn*I site. In addition, polymerase chain reaction (PCR)-based amplification of full-length viral components was also carried out using degenerate primers (Briddon et al. 2002; George et al. 2014). Viral clones of 1.4 and 2.8 kb were sequenced commercially at the University of Delhi South Campus, New Delhi.

Phylogenetic analysis of viral sequences

Vector sequence was trimmed from the raw sequence data by dint of VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/), and the remaining sequences were searched for homology at NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For the identification of viral genomic components, Basic Local Alignment Search Tool (BLAST*n*) search was performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis of the sequences was carried out by generating dendrogram using neighbor-joining method with the help of MEGA 6 software using default setting (Tamura et al. 2013).

PCR-based detection of chilli-begomoviruses

A survey was carried out to examine the association of these four genomic DNAs in symptomatic susceptible (*C. annuum* cv. Kashi Anmol) and resistant chilli cultivars (*Capsicum chinense* cv. Bhut Jolokia and *C. annuum* cv. Kalyanpur Chanchal). Leaf samples were collected from all the chilli plants grown in the experimental field, and total genomic DNA was extracted from these samples according to the Dellaporta et al. (1983) with some modifications as described earlier (Kushwaha et al. 2015b) .

For PCR-based detection, 25 cycles were performed with specific primers of ToNDA AC1, ToGB BC1, ChNDA AC1, and Toβ full length (Supplementary Table S1; Briddon et al. 2002). Two micrograms of total genomic DNA isolated from the surveyed samples were served as template (Table 1).

Construction of infectious clone

For construction of all infectious clones, back bone of pCAMBIA2300 binary vector was used. Fragments corresponding to the *Tomato leaf curl New Delhi virus* (ToNDA; KU196750) genome (~1.7 kb) was cloned at *Xba*I (2250 nt) and *Kpn*I (1176 nt) restriction sites followed by insertion of the full-length genome at *Kpn*I site. Likewise, *Tomato leaf curl Gujarat virus* DNA B (ToGB; KU196751) partial clone (1.7 kb) was inserted between *Xba*I (2540 nt) and *Kpn*I (1543 nt) restriction sites followed by insertion of the full-

Table 1 Field occurrence and detection of begomoviruses on symptomatic chilli plants

Chilli cultivars	Symptoms[a]	Total number of plants symptomatic/ surveyed	ToNDA infected plants[a]	ToGB infected plants[a]	ChNDA infected plants[a]	Toβ infected plants[a]	ToNDA+ ToGB+ ChNDA+ Toβ infected plants[a]
C. annuum cv. Kalyanpur Chanchal	ULC, VT, YN, LM, LC, YP, Bsl	10/10	10	8	8	10	7
C. annuum cv. Kashi Anmol	ULC, VT, Ch, SL, LC, YP, Bsl	10/10	8	7	8	10	6
C. chinense cv. Bhut Jolokia	ULC, VT, CS, LR, St	13/13	12	8	11	13	8

ULC upward leaf curling, *VT* vein thickening, *YP* yellow patches on leaves, *SL* small leaves, *Bsl* boat-shaped leaf, *LR* leaf rolling, *LM* leaf mottling, *LC* leaf crinkling, *YN* leaf yellowing and necrotic spots, *Ch* chlorosis, *CS* chlorotic spots, *St* stunting of plants

[a] PCR positive plants out of total number of plants per chilli cultivar surveyed

length genome at *Kpn*I site to develop ToGB construct. Similarly, *Chilli leaf curl virus* DNA A (ChNDA; KR957353) and *Tomato leaf curl Bangladesh betasatellite* (Toβ; KR957354) partial fragments were cloned between *Pst*I (1449) and *Bam*HI (152 nt) sites, *Bam*HI (1149 nt), and *Kpn*I (1100 nt) sites, respectively, and full-length genomes were inserted at *Bam*HI and *Kpn*I restriction sites of the respective clones.

Plant inoculation

Chilli (*C. annuum* cv. Kalyanpur Chanchal) and *N. benthamiana* plants were grown under controlled conditions of 16 h light and 8 h dark period at 25 °C ± 2 °C with relative humidity of 60 %. Seedlings were transferred at two leaf stage in fresh pots, and plants were maintained under the same conditions as mentioned above in an insect-proof glasshouse.

Binary vector pCAMBIA2300 (Hajdukiewicz et al. 1994) having tandem repeat constructs of each viral genome were transformed into *Agrobacterium tumefaciens* strain EHA105 by freeze thaw method. *Agrobacterium* containing infectious viral construct was grown in Luria-Bertani broth medium supplemented with kanamycin (50 μg/ml) and rifampicin (30 μg/ml) at 28 °C for 36 h. *Agrobacterium* cultures were centrifuged and re-suspended in an infiltration buffer containing 10 mM $MgCl_2$ and 100 μM acetosyringone, and optical density was kept 1.00 at 600 nm. For mixed inoculation, *Agrobacterium* suspension harboring the infectious clones of ToNDA, ToGB, ChNDA, or Toβ were mixed in equal volume according to the viral combinations (Table 2). *N. benthamiana* plants were agro-inoculated at 4–5 leaf stage by stem-prick method (Chattopadhyay et al. 2008). *Agrobacterium* harboring only vector (pCAMBIA2300) was used for mock inoculation.

Virus inoculation of chilli plants was carried out by particle bombardment method (Kumar et al. 2015). In brief, gold microcarriers of 0.6 μm diameter coated with viral DNA constructs were used for bombardment. For the coating of viral genome, 25 mg of gold microcarriers were mixed with 100 μl of 0.05 M spermidine, 100 μl of 1 M $CaCl_2$, and viral genome (3 μg/μl, containing equal amount of each viral component) followed by gentle vortexing. Viral DNA-coated gold microcarriers were bombarded on chilli plants (at two leaf stage) at 300 psi with the help of Helios Gene Gun (BioRad, CA, USA). Empty vector (pCAMBIA2300) DNA-coated gold particles were used for mock inoculation. Symptom observation, assessment, and scoring on *N. benthamiana* and chilli were performed following Chakraborty et al. (2008) and Kumar et al. (2006).

Southern blot hybridization

Total DNA was isolated from two uppermost leaves of mock- and virus-inoculated plants. For Southern blotting, 8.0 μg of total plant DNA was resolved on 0.8 % agarose gel at 60 V for 5 h. The gel was treated with depurination solution (0.15 N HCl) followed by denaturation (0.5 N NaOH and 1.5 M NaCl) and neutralization with buffer (0.5 M Tris-HCl and 1.5 M NaCl) for 1 h. Treated DNA was transferred to a positively charged nylon membrane (MDI, Ambala, India) and further subjected to the UV cross-linking. Hybridization was carried out following the protocol as described by Chakraborty et al. (2008) using radiolabeled (αP^{32}-dCTP) specific probes such as, ToNDA AC1 (1499 nt to 2584 nt), ToGB BC1 (1301 nt to 2146 nt), ChNDA AC1 (1526 nt to 2611 nt), and Toβ (full length) regions. Blots were scanned using phosphorimager (GE Amersham, Little Chalfont, UK), and signals were quantified by image analysis software (Quantity One, Bio-Rad, CA, USA). Southern blotting hybridization experiment was repeated three times.

Total RNA isolation and cDNA synthesis

For isolation of total plant RNA, two uppermost leaves from three different plants were harvested from mock- and virus-inoculated plants. Total RNA was extracted using Trizol (SIGMA, St. Louis, USA) following manufacturer's protocol. Harvested samples (100 mg) were ground in liquid nitrogen and homogenized in Trizol reagent. Protein impurities were

Table 2 Infectivity of genomic components of begomoviruses and associated betasatellite on *N. benthamiana* and *C. annuum*

Plant species	Viral combinations used	Symptomic plants/ plants inoculated[a]	Type of symptoms	Symptom severity[b]	Incubation period[c] (in dpi)
N. benthamiana	ToNDA	28/30	LC, VT, SL	+++	10
	ToNDA + ToGB	30/30	LC, VT, St, Pu, YP, SL, Tw	+++++	6
	ChNDA	22/30	LC	+	18
	ChNDA + Toβ	30/30	LC,VT, Pu, SL, Csl	+++	10
	ToNDA + ChNDA	30/30	LC, VT, SL	+++	10
	ToNDA + Toβ	30/30	LC, VT, Pu, Tw,VC	+++	6
	ChNDA + ToGB	23/30	LC	+	19
	ToNDA + ToGB + ChNDA	30/30	LC, VT, St, Pu, YP, SL,Tw, Csl	+++++	6
	ToNDA + ToGB + Toβ	30/30	LC, VT, Pu, YP, Tw, VC	+++++	6
	ChNDA + Toβ + ToNDA	30/30	LC,VT, Pu, Csl, Tw, VC	++++	6
	ChNDA + Toβ + ToGB	30/30	LC,VT, Pu, SL, Csl	++	12
	ToNDA + ToGB + ChNDA + Toβ	30/30	LC, VT, St, Pu, LD, YP, SL, Tw, VC	+++++	5
	Mock	0/30	–	–	–
C. annuum cv. Kalyanpur Chanchal	ToNDA + ToGB	2/8	LC	+	55
	ChNDA + Toβ	0/8[d]	–	–	–
	ToNDA + ToGB + ChNDA + Toβ	6/8	LC, Cr, Pu, TL	+++	36
	Mock	0/8	–	–	–

LC leaf curling, *VT* vein thickening, *St* stunting, *LD* leaf distortion, *YP* yellow patches, *SL* small leaves, *Tw* twisting of petiole, *Csl* cup-shaped leaf, *Cr* leaf crinkling, *Pu* puckering, *TL* thickening of leaf lamina, *VC* vein clearing

[a] Number of plants showing symptoms/number of plants inoculated

[b] Severity of symptoms was scored from mild (+) to severe (++++++) according to Chattopadhyay et al. (2008) and Kumar et al. (2015)

[d] Time taken for the first symptom appearance

[e] Plants indicated presence of ChNDA and Toβ by PCR analysis

removed by treating with chloroform at 12,000 g at 4 °C. RNA was precipitated with 0.8 % isopropanol and subsequently washed three times with 70 % ethanol. The pellet was air dried and dissolved in DEPC (0.1 %)-treated autoclaved sterile distilled water. The complementary DNA (cDNA) was synthesized from 1.0 μg of DNase-treated total RNA by oligodT method.

Quantitative real-time PCR

Primers for the expression analysis of host genes were designed with the help of Primer Express v3.0 software (Applied Biosystem, California, USA) (Supplementary Table S1). Thionin (accession no JK522901), ascorbate peroxidase (JK523038), polyphenol oxidase (JK523006), and NBS-LRR (accession no JK523032) genes were selected for expression analysis in the present study. Actin (FP 5′ GAAGCTCAATCCAAACGTGGTATT 3′, RP 5′ CTCAAACATGATTTGTGTCATC 3′) was considered as internal control. cDNA from the virus- and mock-inoculated plants was subjected to the qRT-PCR analysis. For qRT-PCR 1X SYBR, Green master mix (Applied Biosystem, CA, USA) and 1:10 dilution of cDNA were used as template. qRT-PCR was performed on Applied Biosystem 7500 real-time machine (Applied Biosystem, CA, USA). PCR program comprises of initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 35 s, and extension at 72 °C for 35 s. Three biological and three technical replicates were taken for each case. The ΔCt of genes was normalized with internal control actin. The ΔΔCt values were used to plot graph using Sigma-plot 11.0 software (Post-hoc Holm-Sidak method) (Singh et al. 2013). To compare the relative expression level of host genes, the Student's *t* test was performed.

Results

Breakdown of natural resistance in chilli

Severe leaf curl disease was observed on the hitherto-known resistant chilli cultivar Kalyanpur Chanchal during 2009 in the experimental field of JNU, New Delhi. All the experimental plants of this cultivar showed typical symptoms of begomovirus infection such as severe leaf curling and stunted plant growth. This study reports for the first time breakdown of natural resistance in chilli. We further extended the survey to other chilli cultivars grown in the same field. It was noticed

that cultivars like "Bhut Jolokia" and "Kashi Anmol" also displayed severe leaf curl disease symptoms. The characteristic disease symptoms were upward leaf curling, vein thickening, chlorosis, small leaves, leaf crinkling, yellow patches, and boat-shaped leaf structure (Fig. 1a, Table 1). Additionally, symptomatic plants of Bhut Jolokia cultivar also displayed leaf rolling and chlorotic spots (Fig. 1a, Table 1). Notably, in all three cases, 100 % disease incidence was observed (Table 1).

Cloning and detection of begomovirus genomic components associated with breakdown of resistance in chilli

Cloning and sequencing of the viral genomic components revealed the presence of four different types of begomovirus molecules (Supplementary Fig. S1). From the diseased resistant chilli plants, two types of DNA-A molecules and one molecule each of DNA-B and betasatellite were isolated. Two sequences of viral DNA (GenBank accession nos. KR957353 and KU196750) showed maximum homology of 97.45 and 99 % with New Delhi isolate of *Chilli leaf curl virus* and *Tomato leaf curl New Delhi virus*, respectively. Similarly, another sequence of 2.7 kb in size shared 99 % nucleotide identity with *Tomato leaf curl Gujarat* virus DNA B component (GenBank accession no KU196751). In addition, a viral nucleotide sequence (GenBank accession no KR957354) possessed 97.45 % identity with *Tomato leaf curl Bangladesh betasatellite* (Supplementary Fig. S1). According to the revised species demarcation threshold for begomoviruses (Brown et al. 2015) and betasatellites (Briddon et al. 2008), the cloned viral molecules were considered to be the isolates

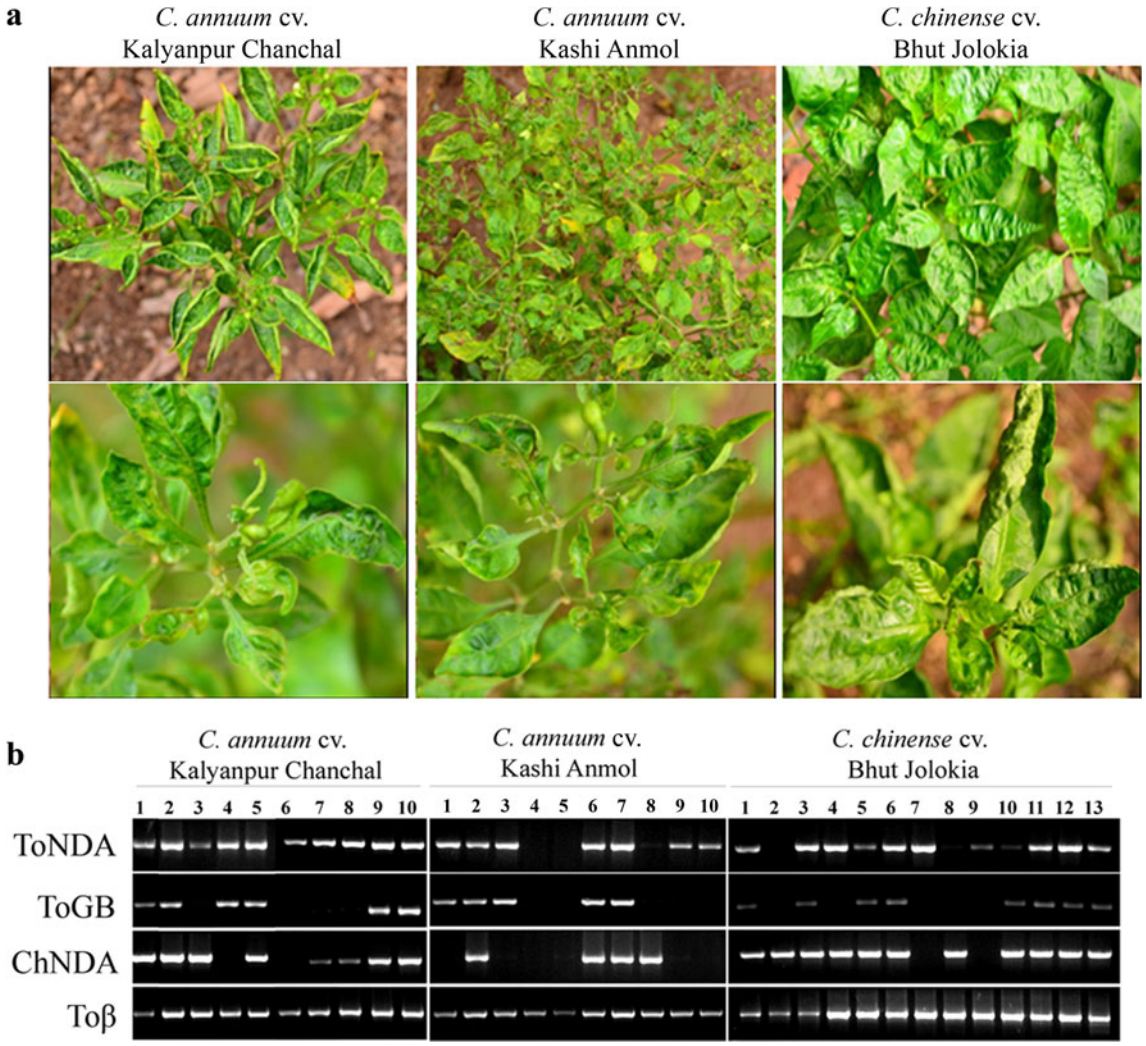
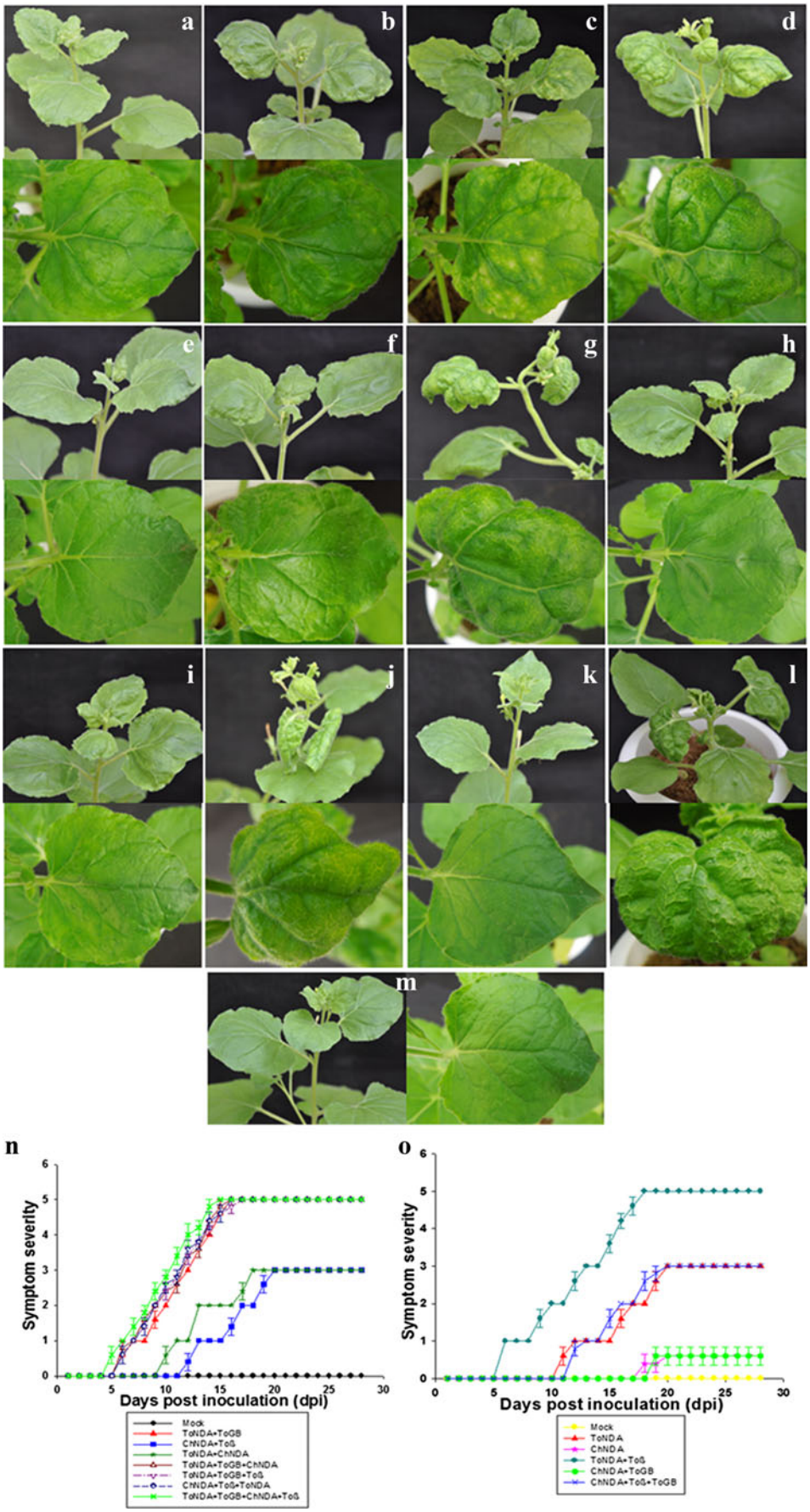


Fig. 1 Typical symptoms of leaf curl disease on plants belonging to *Capsicum* spp. and detection of begomoviruses and associated betasatellite. Mixed infection of diverse begomoviruses and betasatellite resulted in breakdown of resistance in chilli cultivars. **a** A survey was carried out and symptomatic chilli plants belonging to three cultivars (as indicated) showing severe leaf curl symptoms were observed in natural condition. **b** Total plant DNA was used to isolate the resistant-breaking strains of viruses. Detection of begomovirus was carried out by polymerase chain reaction using ToNDA-, ToGB-, ChNDA-, and Toβ-specific primers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m
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Symptom severity
Days post inoculation (dpi)
Mock
ToNDA+ToGB
CNDA+ToG
ToNDA+ChNDA
ToNDA+ToGB+ChNDA
ToNDA+ToGB+ToG
ChNDA+ToG+ToNDA
ToNDA+ToGB+ChNDA+ToG
o
Symptom severity
Days post inoculation (dpi)
Mock
ToNDA
ChNDA
ToNDA+ToG
ChNDA+ToGB
ChNDA+ToG+ToGB

◀ **Fig. 2** Disease development on *N. benthamiana* plants inoculated with different combinations of viral genomic components. **a** ToNDA. **b** ToNDA + ToGB. **c** ToNDA + ToGB + ChNDA. **d** ToNDA + ToGB + Toβ. **e** ChNDA. **f** ChNDA + Toβ. **g** ChNDA + Toβ + ToNDA. **h** ChNDA + Toβ + ToGB. **i** ToNDA + ChNDA. **j** ToNDA + Toβ. **k** ChNDA + ToGB. **l** ToNDA + ToGB + ChNDA + Toβ. **m** Mock. **n** and **o** *Graphs* represent kinetics of symptom severity on *N. benthamiana*

of *Chilli leaf curl virus* (ChNDA), *Tomato leaf curl New Delhi virus* (ToNDA), *Tomato leaf curl Gujarat virus* (ToGB), and *Tomato leaf curl Bangladesh betasatellite* (Toβ). Based on the nucleotide sequence identity, the cloned viral genomic components were positioned along with their respective begomovirus species and the betasatellite group in the respective phylogenetic trees (Supplementary Fig. S3).

In order to test the presence of these genomic components among all the symptomatic plants of the resistant cultivar Kalyanpur Chanchal grown in the experimental field, PCR using specific primers was carried out (Supplementary Table S1). Results revealed that almost each chilli plant contained ToNDA and Toβ molecules, while 80 % plants showed the infection of ToGB and ChNDA (Fig. 1b, Table 1). Overall, 70 % of the infected plants of this cultivar showed the presence of all the four viral genomic components. We further studied the presence of these four components in other two chilli cultivars, i.e., Bhut Jolokia and Kashi Anmol (Fig. 1b). In the case of Bhut Jolokia, all plants were found to be infected with Toβ, while 92.3, 61.5 and 84.6 % of the symptomatic plants contained ToNDA, ToGB, and ChNDA, respectively. Similarly, Kashi Anmol plants showed infection of ToNDA in 80 %, ToGB in 70 %, ChNDA 80 %, and Toβ in 100 % plants (Table 1). Presence of all the four genomic components was ascertained in 61.5 and 60 % of the symptomatic plants of the chilli cultivars Bhut Jolokia and Kashi Anmol, respectively (Table 1).

Interaction among chilli-infecting begomoviruses in *N. benthamiana*

Infectious tandem repeats of all the isolated viral genomes and the betasatellite were constructed and were initially used to study the interaction in *N. benthamiana*. *N. benthamiana* plants infected with ToNDA alone showed initial symptom appearance at 10 dpi, and the symptoms gradually increased upto level 3 at 20 dpi. ToNDA-inoculated plants showed characteristics symptoms such as mild downward leaf curling, thickening of veins and small leaves (Fig. 2a, Table 2). However, plants co-inoculated with ToNDA and ToGB showed the first symptom at 6 dpi and exhibited maximum symptom severity (level 5) at 15 dpi (Fig. 2n, Table 2). ToNDA- and ToGB-inoculated plants developed severe symptoms such as leaf curling, vein thickening, stunting, leaf puckering, yellow patches on leaf lamina, small leaves, and twisting of petiole (Fig. 2b, Table 2, Supplementary Fig. S2). Notably, plants inoculated with ChNDA alone produced very mild symptoms on *N. benthamiana* (Fig. 2e; Supplementary Fig. S2); however, severe symptoms were observed on plants inoculated with ChNDA and Toβ (Fig. 2f; Supplementary Fig. S2). ChNDA- and Toβ co-inoculated plants developed initial symptoms as downward leaf curling, small leaves, vein thickening with leaf puckering, and cup-shaped leaves at 10 dpi while symptom severity enhanced persistently and reached up to level 3 (Fig. 2f, n, Table 2). On the other hand, plants co-inoculated with ToNDA and ChNDA displayed symptoms similar to ToNDA-inoculated plants (Fig. 2i). ChNDA- and ToGB-inoculated plants exhibited very mild symptoms similar to plants inoculated with ChNDA alone (Fig. 2k). Evaluation of the ToNDA and Toβ co-inoculated plants revealed that the earliest symptoms appeared on 6 dpi, and the severity gradually increased to the highest level on 16 dpi (Fig. 2j, o).

Initial symptom was recorded at 6 dpi on the plants co-inoculated with ToNDA, ToGB, and ChNDA, and the severity progressed to the maximum level 5 at 14 dpi. Symptoms appeared were similar to the ToNDA and ToGB co-inoculated plants, although with increased severity (Fig. 2c, n, Table 2, Supplementary Fig. S2). Plants co-inoculated with ToNDA, ToGB, and Toβ also showed severe leaf curl diseases which appeared initially at 6 dpi and attained maximum severity (symptom level 5) at 15 dpi (Fig. 2d, n, Table 2). Co-inoculation of ChNDA, Toβ, and ToNDA displayed comparable symptoms and disease progression similar to the ToNDA and Toβ co-inoculated plants (Fig. 2g, Fig. 2n, Table 2). Co-inoculation of ChNDA, Toβ, and ToGB induced similar symptoms as ChNDA and Toβ co-inoculated plants (Fig. 2h, o). *N. benthamiana* plants co-inoculated with all the four infectious molecules exhibited the most severe symptom (level-5) such as downward leaf curling, vein thickening, stunting, leaf puckering, leaf distortion veinal chlorosis, small leaves, and twisting of petiole. On these plants, initial symptom appeared at 5 dpi, which subsequently reached maximum severity level 5 at 14 dpi (Fig. 2l, n, Table 2, Supplementary Fig. S2l).

A comparative analysis of viral DNA accumulation

In order to correlate symptom severity and viral DNA accumulation, the viral titer in *N. benthamiana* plants was analyzed by Southern hybridization. The results suggested that plants co-inoculated with all four genomic components (ToNDA, ToGB, ChNDA, and Toβ) showed maximum accumulation of viral DNAs. We, therefore, considered viral DNA (of each component) as 100 % in the plants inoculated with this combination. Plants inoculated with ToNDA alone showed relatively low level of viral DNA which was further enhanced when co-inoculated with ToGB (Fig. 3a lanes 1 and 2). ToNDA level was marginally enhanced (upto to 10 %) in

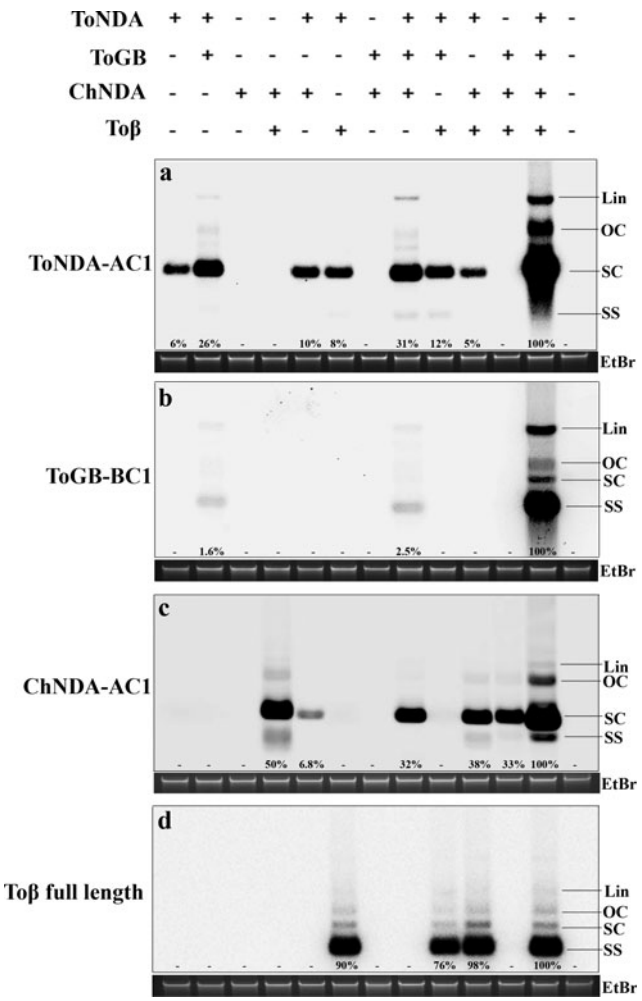


Fig. 3 A comparative analysis of the viral DNA accumulation in *N. benthamiana* plants inoculated with different combinations of begomovirus genomic components at 21 dpi. Southern hybridization was performed using viral genome-specific probes for detection. **a** ToNDA using AC1 probe. **b** ToGB using BC1 probe. **c** ChNDA using AC1 probe. **d** Toβ using βC1 probe. Plant genomic DNA stained with ethidium bromide (EtBr) served as the loading control

plants co-inoculated with ToNDA and ChNDA as compared to the ToNDA-inoculated plants (Fig. 3a lane 5). Co-inoculation of Toβ with ToNDA did not influence accumulation of ToNDA (Fig. 3a lane 6). As expected, accumulation of ToNDA was enhanced in plants inoculated with ToNDA, ToGB, and ChNDA in comparison to ToNDA- and ToGB-inoculated plants (Fig. 3a lane 8). Surprisingly, drastic reduction of ToNDA level was observed in plants inoculated with ToNDA, ToGB, and Toβ as compared to the ToNDA- and ToGB-inoculated plants (Fig. 3a lane 9). However, ToNDA level was considerably reduced (5 %) in plants inoculated with the viral components ToNDA, ChNDA, and Toβ, and were comparable with plants infected with ToNDA alone.

Level of ToGB DNA was approximately 1.6 % in ToNDA and ToGB co-inoculated plants (Fig. 3b lane 2) as compared to the plants inoculated with the four genomic components. Plants inoculated with ToNDA, ToGB, and ChNDA, ToGB titer was estimated to be 2.5 % (Fig. 3b lane 8). Level of ToGB DNA was below the detection level of Southern blotting in plants inoculated with either ChNDA and ToGB or ToNDA, ToGB and Toβ or ChNDA, Toβ and ToGB (Fig. 3b lane 7, 9, and 11, respectively).

Although, ChNDA viral DNA accumulation was below the threshold level of detection by Southern blotting, the presence of ChNDA could be detected by PCR (data not shown) in the infected plants (Fig. 3c lane 3). There was at least 50 % increase in the ChNDA level in plants co-inoculated with ChNDA and Toβ than plants inoculated with ChNDA alone (Fig. 3c lane 4). Inoculation of ChNDA and ToNDA led to enhanced accumulation of ChNDA than only ChNDA-inoculated plants (Fig. 3c lane 5). However, ToGB did not influence the accumulation of ChNDA, when co-inoculated with ToGB. (Fig. 3c lane 7). Mixed inoculation of ChNDA along with ToNDA and ToGB resulted in 32 % accumulation of ChNDA DNA (Fig. 3c lane 8). Co-infection of ChNDA, Toβ, and ToNDA has further enhanced the ChNDA DNA level (Fig. 3c lane 10). Plants inoculated with ChNDA, Toβ, and ToGB could accumulate 33 % of ChNDA (Fig. 3c lane 11).

Similar to ChNDA, level of Toβ DNA could only be detected by PCR in ChNDA and Toβ co-inoculated plants (Fig. 3d lane 4). Unlike ChNDA, ToNDA could enhance the Toβ level up to 90 % (Fig. 3d lane 6). Accumulation of Toβ was found to be 76 % in ToNDA, ToGB, and Toβ mixed inoculated plants (Fig. 3d lane 9). Toβ co-inoculated with ToNDA and ChNDA led to the enhanced level (98 %) of Toβ DNA (Fig. 3d lane 10). Notably, the titer of Toβ remained below the threshold level of detection by Southern blotting in ChNDA, Toβ, and ToGB co-inoculated plants.

Comparative level of viral DNA in the resistant chilli cultivar Kalyanpur Chanchal

Further, the effect of synergistic interaction on symptom severity and viral DNA accumulation in the resistant chilli cultivar Kalyanpur Chanchal was assessed through biolistic inoculation of viral genomic components. Plants inoculated with ToNDA and ToGB showed either no or very mild leaf curling (at 55 dpi) (Fig. 4b, Table 2). Plants inoculated with ChNDA and Toβ failed to display any noticeable symptom until 60 dpi (Fig. 4c, Table 2). However, mixed inoculation of ToNDA, ToGB, ChNDA, and Toβ led to severe symptoms such as leaf curling, crinkling, puckering, and thickening of leaf lamina on the chilli plants (Fig. 4d, Table 2). However, we have failed to detect viral DNA (from 10 μg of total DNA) from these inoculated plants by Southern hybridization. Therefore, the highly sensitive method of polymerase chain reaction (PCR) was used to detect viral DNA accumulation in the chilli plants. A comparative analysis of viral DNA

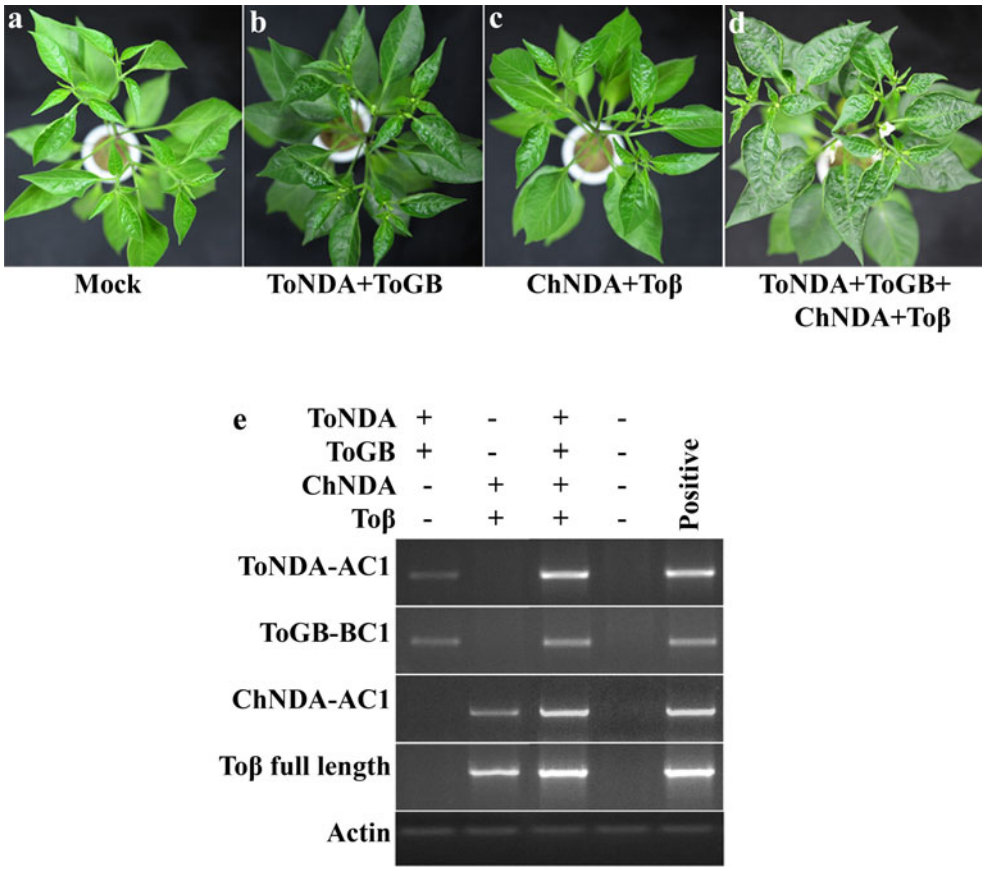


Fig. 4 A comparative study of symptom development and viral DNA accumulation in *C. annuum* cv. Kalyanpur Chanchal inoculated with begomoviruses. Symptoms on representative chilli plants inoculated with **a** mock. **b** ToNDA + ToGB. **c** ChNDA + Toβ. **d** ToNDA + ToGB + ChNDA + Toβ. **e** PCR was carried out using AC1-specific primers to detect ToNDA and ChNDA, BC1-specific primers to detect ToGB. Full-length DNA β-specific primers were used to detect Toβ. PCR amplification of actin using same conditions used for amplification of viral genomes serves as internal control

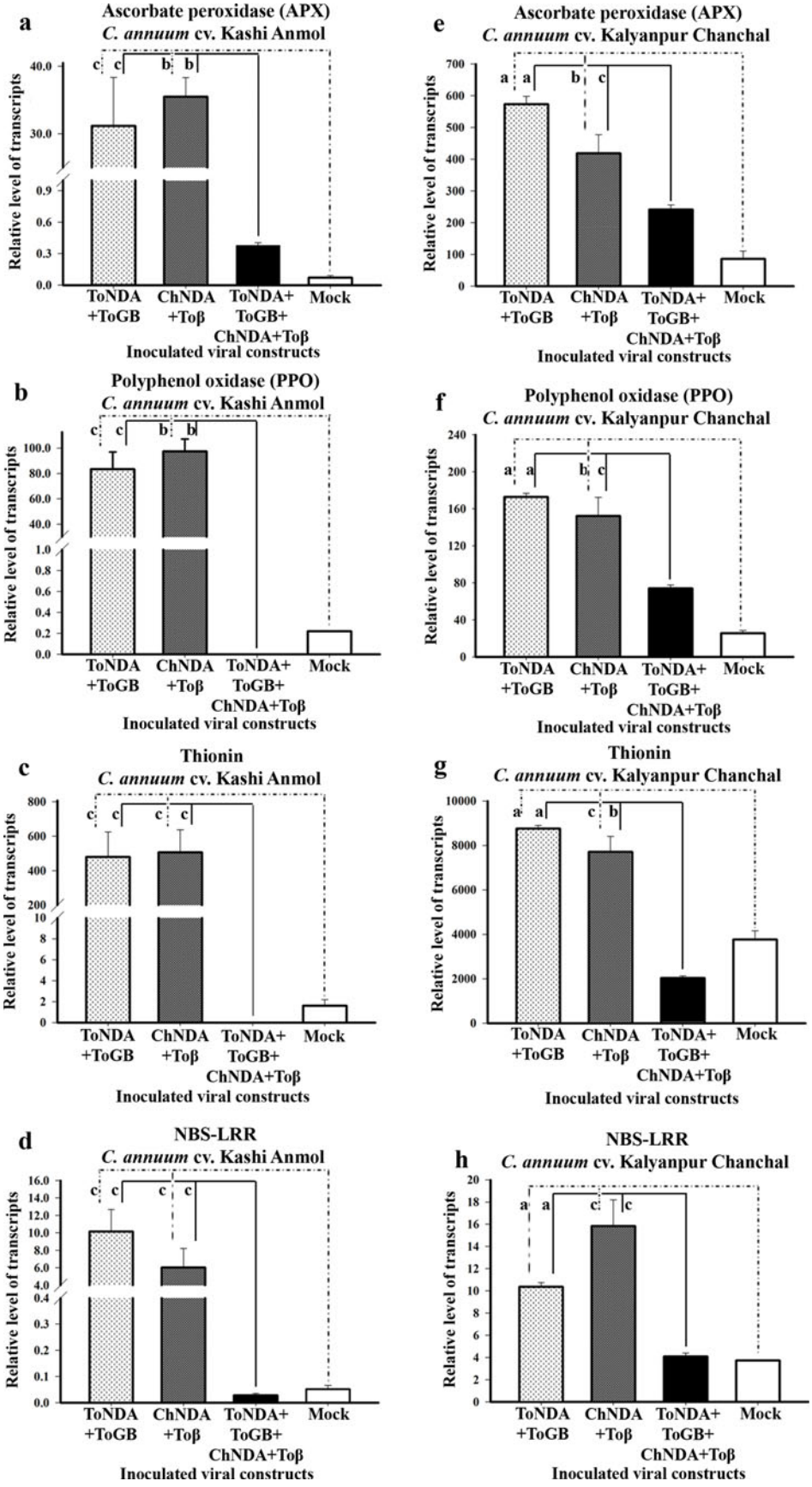
accumulation in the inoculated chilli plants indicated correlation with the results observed in the *N. benthamiana* plants. ToNDA-, ToGB-, ChNDA-, and Toβ-inoculated chilli plants revealed maximum accumulation of viral DNA (Fig. 4e). Viral DNA accumulation was very low in the resistant chilli plants inoculated either with ToNDA and ToGB or ChNDA and Toβ.

Mixed infection of chilli begomoviruses suppresses the expression of defense-related genes

Expression analysis of the defense-related genes was performed to evaluate their plausible role in synergistic interaction noticed in this study. The expression of these defense-related genes were analyzed in both the resistant (Kalyanpur Chanchal) and the susceptible (Kashi Anmol) chilli cultivars inoculated with either mock or in combinations (ToNDA + ToGB, ChNDA + Toβ, ToNDA + ToGB + ChNDA + Toβ).

Expression of ascorbate peroxidase (*APX*) gene was found altered in virus-inoculated chilli plants. Increased level of *APX* transcript was observed in the susceptible chilli plants inoculated with either ToNDA and ToGB (>400 folds, $p < 0.05$) or ChNDA and Toβ (>500 folds, $p = 0.006$) as compared to mock-inoculated plants (Fig. 5a). Reduced level of *APX* expression was observed in ToNDA-, ToGB-, ChNDA-, and Toβ-inoculated susceptible plants than plants inoculated with either ToNDA and ToGB or ChNDA and Toβ (about 80 folds, $p < 0.05$ and 90 folds, $p = 0.006$, respectively) (Fig. 5a). The resistant chilli plants inoculated with either ToNDA and ToGB or ChNDA and Toβ showed enhanced accumulation of *APX* transcript >sixfold ($p < 0.001$) and > fivefold ($p = 0.006$), respectively, as compared to the mock-inoculated plants (Fig. 5e). However, *APX* expression in the resistant plants inoculated with all four viral genomic components revealed reduced level (2.3-fold, $p < 0.001$ and 1.7-fold $p < 0.05$) as compared to either ToNDA and ToGB or ChNDA and Toβ inoculated plants, respectively) (Fig. 5e).

Level of *PPO* transcript was found to be elevated in the susceptible chilli plants co-inoculated with ToNDA with ToGB (~350-fold, $p = 0.025$) and ChNDA with Toβ (~400-fold, $p = 0.01$) in comparison with the mock-inoculated plants (Fig. 5b). Plants of the susceptible cultivar inoculated with ToNDA, ToGB, ChNDA, and Toβ showed drastic reduction of *PPO* expression, even below the detectable level (Fig. 5b). *PPO* transcript accumulation increased more than 6.5-fold ($p < 0.001$) and 5.5-fold ($p = 0.003$) in the resistant chilli plants inoculated with either ToNDA and ToGB or ChNDA and Toβ, respectively, than the mock-inoculated plants (Fig. 5f). However, plants inoculated with all the four viral components showed reduced level of *PPO* transcripts, 2.3-fold ($p < 0.001$)


a
Ascorbate peroxidase (APX)
C. annuum cv. Kashi Anmol
Relative level of transcripts
ToNDA +ToGB
ChNDA +Toβ
ToNDA+ ToGB+ ChNDA+Toβ
Mock
Inoculated viral constructs
e
Ascorbate peroxidase (APX)
C. annuum cv. Kalyanpur Chanchal
b
Polyphenol oxidase (PPO)
C. annuum cv. Kashi Anmol
f
Polyphenol oxidase (PPO)
C. annuum cv. Kalyanpur Chanchal
c
Thionin
C. annuum cv. Kashi Anmol
g
Thionin
C. annuum cv. Kalyanpur Chanchal
d
NBS-LRR
C. annuum cv. Kashi Anmol
h
NBS-LRR
C. annuum cv. Kalyanpur Chanchal

◀ **Fig. 5** Expression profile of host genes during synergistic interaction in the resistant (*C. annuum* cv. Kalyanpur Chanchal) and the susceptible (*C. annuum* cv. Kashi Anmol) chilli plants. To study the host gene expression, qRT-PCR was performed using gene-specific primers of defense-related genes—a and e ascorbate peroxidase (APX), b and f polyphenol oxidase (PPO), c and g thionin, d and h NBS-LRR. Comparison was done between host gene expression in ToNDA and ToGB, ChNDA and Toβ with mock-inoculated plants for one dataset (indicated as *dotted line*). For another dataset, gene expression changes among ToNDA and ToGB, ChNDA and Toβ with mixed virus (ToNDA, ToGB, ChNDA, and Toβ) inoculated plants (indicated as solid line). Statistical significance is denoted as, a for $p \leq 0.001$, b for $0.001 < p \leq 0.01$ and c for $0.01 < p < 0.05$

and 2.0-fold ($p = 0.019$) than either ToNDA- and ToGB- or ChNDA- and Toβ-inoculated plants, respectively (Fig. 5f).

Thionin expression was analyzed in the susceptible and resistant chilli plants inoculated with different combinations of begomoviruses. *Thionin* expression was increased in Kashi Anmol plants co-inoculated with either ToNDA and ToGB or ChNDA and Toβ (~290-fold $p = 0.03$, and ~310-fold $p = 0.019$, respectively) with respect to the mock plants. Notably, plants inoculated with all four viral genomes showed significant reduction of *thionin* than either ToNDA- and ToGB- or ChNDA- and Toβ-infected plants (Fig. 5c). Plants inoculated with either ToNDA and ToGB or ChNDA and Toβ resulted enhanced level (>2 fold) of *thionin* transcripts as compared to the mock-inoculated resistant chilli plants whereas plants inoculated with ToNDA, ToGB, ChNDA, and Toβ accumulated reduced level of *thionin* transcript (~50 % less than the mock-inoculated plants) (Fig. 5g).

Further, expression profile of *NBS-LRR* gene was analyzed. Plants (chilli cv. Kashi Anmol) inoculated with either ToNDA and ToGB or ChNDA and Toβ showed enhanced level of *NBS-LRR* transcript (more than 190-fold, $p = 0.016$- and 110-folds, $p = 0.035$, respectively, as compared to the mock plants) (Fig. 5d). In mixed-inoculated plants, *NBS-LRR* transcript level is significantly reduced than plants inoculated with either ToNDA and ToGB or ChNDA and Toβ alone, which was almost half to that of the mock plant (Fig. 5d). The resistant chili cultivar accumulated 2.7- and 4.5-fold enhanced transcripts of *NBS-LRR* gene inoculated with either ToNDA and ToGB or ChNDA and Toβ with respect to the mock plants (Fig. 5h). Further analysis of NBS-LRR in mixed inoculated resistant plants showed reduced level of *NBS-LRR* transcript than either ToNDA and ToGB or ChNDA and Toβ virus infected plants (about 2.5-fold, $p = 0.001$ and 3.8-fold, $p = 0.038$, respectively) (Fig. 5h).

Discussion

Based on our knowledge, the present study reports the breakdown of resistance in chilli cultivars under natural condition for the first time. Typical symptoms of severe leaf curl disease caused by begomoviruses were noticed on resistant cultivars of chilli, such as cv. Kalyanpur Chanchal and cv. Bhut Jolokia. Geminiviruses have great potential of recombination and pseudo-recombination. During mixed infection, several genetic interactions occur which leads to development of new viral strain or isolate with improved fitness to overcome host defense machineries for successful pathogenesis. In the present study, four different genomic components of begomoviruses were identified from the symptomatic resistant chilli plants. The association of more than one virus in symptomatic plants has emerged as a common natural incidence and has been reported from previously (Pruss et al. 1997), but the molecular mechanism underlying the resistance breakdown has been largely unknown. In the current study, the nature and kinetics of synergistic interaction among begomoviruses and the molecular mechanism of resistant breakdown in the resistant chilli cultivar Kalyanpur Chanchal have been demonstrated.

Interactions between two viruses may result in either positive (synergistic) or negative influence (antagonistic) on their pathogenesis (Méndez-Lozano et al. 2003; Alves-Júnior et al. 2009). Synergistic interaction among the viruses leads to the increased symptom severity and enhanced level of viral titer which has previously been reported among distinct viruses belonging to either same or different family/genera/species (Pruss et al. 1997; García-Cano et al. 2006; Chakraborty et al. 2008; Mohamed 2010; Caracuel et al. 2012). The viral determinants of synergistic interaction among begomoviruses causing severe cassava mosaic disease have also been studied (Vanitharani et al. 2004). Among the RNA viruses, cucumber mosaic virus (CMV) assists infection of potato virus Y into internal phloem cells; thus, PVY could move in the younger leaves and replicate many times higher than single infection (Ryang et al. 2004). Potyvirus encoded P1, helper component proteinase (HC-Pro), and a fraction of P3 enhances the pathogenicity and replication of CMV and *Tobacco mosaic virus* (TMV) by suppression of post-transcription gene silencing (PTGS) (Pruss et al. 1997). CMV 2b protein blocks spreading of systemic signal of PTGS and helps in the accumulation of potato virus Y in the shoot apex (Ryang et al. 2004; Guo and Ding 2002). Similarly, C2 protein of *Beet curly top virus* (Curtovirus) promotes a suitable cell environment to enhance the replication of geminiviruses in Rep-assisted manner (Caracuel et al. 2012). Synergistic interaction of geminiviruses may led to recombination, pseudo-recombination or assortment of viral components, leading to the emergence of new virus species (Fondong et al. 2000; Pita et al. 2001; Rasheed et al. 2006; Chakraborty et al. 2008).

Earlier study has demonstrated the potential of *Tomato leaf curl New Delhi virus* (DNA-A) and *Tomato leaf curl Gujarat virus* (DNA-B) to form a viable supervirulent pseudorecombinant and to develop severe leaf curl disease on tomato (Chakraborty et al. 2008). The present study reports

the first natural association of *Tomato leaf curl New Delhi virus* (DNA-A) and *Tomato leaf curl Gujarat virus* (DNA-B) which causes severe leaf curl disease in the resistant chilli cv. Kalyanpur Chanchal. It is well documented that the DNA-B of bipartite virus encodes movement protein and nuclear shuttle protein and facilitates the DNA-A accumulation in systemic parts of the plants. On the other hand, DNA-B depends on DNA-A for replication. Our results indicated that ToNDA and ToGB assist each other and behave like a bipartite begomovirus. According to the southern and PCR results, ChNDA resembles a monopartite virus and therefore, requires Toβ for pathogenesis. However, ToNDA and ChNDA supported each other for multiplication in *N. benthamiana*. DNA β-encoded βC1 protein is known to be involved in pathogensis, movement, and suppression of host antiviral silencing machineries (Briddon et al. 2003; Kumari et al. 2010; Yang et al. 2011; Shukla et al. 2013).

Our analysis demonstrates that Toβ depends on ToNDA for own replication without influencing helper virus accumulation. The results obtained here indicate that Toβ presumably facilitated interaction between monopartite and bipartite virus. It is relevant to note that ChNDA and ToGB could not form a viable pseudorecombinant between genomic components of a monopartite and bipartite begomovirus. Reduced level of both Toβ and ToGB was observed when both of these components were present together with either ChNDA or ToNDA which suggests competition between Toβ and ToGB for helper virus-mediated replication. This result was in concurrent with the previous report which suggested reduced accumulation of DNA-B and betasatellite in plants inoculated with tripartite combination (ToLCV DNA-A, DNA-B and betasatellite) (Joyothsana et al. 2013).

Notably, we failed to detect viral DNA from *C. annuum* plants by Southern hybridization which could be due to low level of viral titer. Furthermore, PCR-based detection of the viral genomic components in these plants can be attributed to sensitivity of this technique over Southern hybridization. In *C. annuum* plants, monopartite virus failed to induce symptoms while only few plants inoculated with bipartite virus showed mild symptoms. Interestingly, plants inoculated with four genomic components showed severe symptoms indicating synergistic interaction among the monopartite and bipartite viruses are key for resistance breakdown in chilli.

Plants trigger various defense mechanisms, subsequent to pathogen infection. In a previous study, ChiLCV caused upregulation of several defense-related genes, encoding proteins such as nucleotide-binding site leucine-rich repeat (NBS-LRR) domain containing protein, lipid transfer protein, thionin, polyphenol oxidase, and other proteins like ATP/ADP transporter (Kushwaha et al. 2015a). Therefore, we aimed to correlate synergistic interaction among chilli-infecting begomoviruses and expression of host defense genes in both susceptible and resistant chilli cultivars.

Polyphenol oxidase (PPO) is a tetrameric copper containing 52–64 kDa protein (van Gelder et al. 1997) and catalyzes the O-hydroxylation of phenols produced during oxidative burst following pathogen attack. Many reports have revealed that PPO is involved in maintaining the basal defense against fungi, bacteria and viruses (Constabel et al. 1995; Li and Steffens 2002; Thipyapong et al. 2004; Poiatti et al. 2009). In the current study, we observed that PPO transcript level was increased in both monopartite (ChNDA and Toβ) and bipartite virus (ToNDA and ToGB) inoculated plants. This result suggested role of PPO in conferring basal defense mechanism which could be correlated with low viral titer and absence of symptoms on both the cultivars of chilli. Reduced level of PPO transcript in mixed inoculated plants is linked with the suppression of basal defense mechanism and accumulation of higher viral DNA.

APX functions as a scavenger of reactive oxygen species and catalyzes the conversion of H_2O_2 into water (Mittler et al. 1998; Pignocchi et al. 2003). Thionin, a small cys-rich highly basic protein having antimicrobial activity, involves in eliciting the plant defense against several pathogens (Epple et al. 1997; Pelegrini and Franco 2005). However, no direct evidence of the involvement of thionin in defense against begomoviruses has been elucidated yet. However, the reduced expression of *APX* and *thionin* transcripts in mixed inoculated chili plants was in concurrent with the results observed in the case of *PPO* transcripts. Therefore, in mixed-inoculated plants, all four molecules of begomoviruses or their encoded products function cooperatively to suppress the defense-responsive genes.

NBS-LRR is conserved domain protein that involve in specific resistance to several pathogens including viruses such as *Cucumber mosaic virus* (CMV) (Seo et al. 2006), TMV (Dinesh-Kumar and Baker 2000), and *Mungbean yellow mosaic India virus* (MYMIV) (Maiti et al. 2012). In our previous study, expression of *NBS-LRR* was found to be upregulated significantly in ChiLCV-inoculated resistant chilli cultivar Punjab Lal (Kushwaha et al. 2015a). The analysis of *NBS-LRR* expression in mixed inoculation chilli cultivars revealed the suppression of *R*-gene-mediated defense response. There are no available reports on functional characterization of any *R*-gene leading to the resistance against geminiviruses; moreover, few reports have indicated the involvement of *R*-gene-mediated resistance against geminiviruses (Maiti et al. 2012). A study reported the upregulation of *NBS-LRR* gene (namly CRY1) in *V. mungo* which conferred resistance against *Mungabean yellow mosaic Indian virus* (MYMIV). Conversely, *NBS-LRR* gene's expression has also been suppressed in susceptible plants infected with *Tomato leaf curl New Delhi virus* (Kushwaha et al. 2015b). The present study revealed downregulation of *NBS-LRR* expression in mixed-infected plants; whereas, plants inoculated with either monopartite or bipartite begomoviruses showed upregulation of *NBS-LRR* transcripts.

In conclusion, qRT-PCR result suggested that monopartite and bipartite begomoviruses infection may induce basal (PPO, APX, thionin) and specific (NBS-LRR) defense responses in host plant. However, during mixed infection, cumulative effect of all four viral molecules have suppressed both basal and specific defense systems, which was established by reduced expression of defense related genes and higher viral DNA accumulation and symptom severity. Additionally, role of viral suppressors cannot be overlooked during mixed infection. Begomoviruses encode different proteins which function as a suppressor of PTGS, TGS, and other host defense machineries. For example, AC1, AC2, AC4, AV2, and βC1 of begomoviruses are known to suppress RNAi machinery (Vanithrani et al. 2004; Trinks et al. 2005; Rodríguez-Negrete et al. 2013). The cumulative effect of these proteins and coordinate action of other viral proteins might help in the downregulation of host basal and specific defense pathways, which eventually leads to symptoms development on resistant chilli cultivar.

Taken together, our results suggest that in mixed-infected plants, ToNDA forms cognate pair with ToGB for pathogenesis and also assists replication of ToGB. The enhanced level of ToNDA facilitates the replication of Toβ which in turn favorably influences ChNDA accumulation in plant. The enhanced level of viral genomic components due to synergistic interaction has led to suppress host defense in resistant chilli cultivar. The outcome of the study may assist in the development of efficient antiviral strategies for generating broad-spectrum resistance against chilli-infecting begomoviruses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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Complexity of begomovirus and betasatellite populations associated with chilli leaf curl disease in India

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Chilli, which encompasses several species in the genus *Capsicum*, is widely consumed throughout the world. In the Indian subcontinent, production of chilli is constrained due to chilli leaf curl disease (ChiLCD) caused by begomoviruses. Despite the considerable economic consequences of ChiLCD on chilli cultivation in India, there have been scant studies of the genetic diversity and structure of the begomoviruses that cause this disease. Here we report on a comprehensive survey across major chilli-growing regions in India. Analysis of samples collected in the survey indicates that ChiLCD-infected plants are associated with a complex of begomoviruses (including one previously unreported species) with a diverse group of betasatellites found in crops and weeds. The associated betasatellites neither enhanced the accumulation of the begomovirus components nor reduced the incubation period in *Nicotiana benthamiana*. The ChiLCD-associated begomoviruses induced mild symptoms on *Capsicum* spp., but both the level of helper virus that accumulated and the severity of symptoms were increased in the presence of cognate betasatellites. Interestingly, most of the begomoviruses were found to be intra-species recombinants. The betasatellites possess high nucleotide variability, and recombination among them was also evident. The nucleotide substitution rates were determined for the AV1 gene of begomoviruses (2.60×10^{-3} substitutions site^{-1} year^{-1}) and the βC1 gene of betasatellites [chilli leaf curl betasatellite (ChiLCB), 2.57×10^{-4} substitution site^{-1} year^{-1}; tomato leaf curl Bangladesh betasatellite (ToLCBDB), 5.22×10^{-4} substitution site^{-1} year^{-1}]. This study underscores the current understanding of Indian ChiLCD-associated begomoviruses and also demonstrates the crucial role of betasatellites in severe disease development in *Capsicum* spp.

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INTRODUCTION

The family *Geminiviridae* includes a group of twinned icosahedral plant viruses containing circular, single-stranded DNA (ssDNA) and is divided into seven genera based on host range, vector transmission and genome organization: *Becurtovirus*, *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Mastrevirus*, *Topocuvirus* and *Turncurtovirus* (Varsani *et al.*, 2014). Begomoviruses are widely distributed geographically, are transmitted through the arthropod vector *Bemisia tabaci*, and are further classified as monopartite with a DNA-A-like component or as bipartite containing DNA-A and DNA-B (Brown *et al.*, 2012). The majority of the monopartite begomoviruses have been reported from the 'Old World' while the begomoviruses of the 'New World' are mostly bipartite, along with a true monopartite begomovirus (Melgarejo *et al.*, 2013; Nawaz-ul-Rehman & Fauquet, 2009). Generally, DNA-A encodes

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Seven supplementary figures and six supplementary tables are available with the online Supplementary Material.

six ORFs necessary for virus replication, transcription activation and encapsidation, while the proteins encoded by the DNA-B component facilitate cell–cell and nucleocytoplasmic trafficking of the viral genome. In addition, intracellular movement is controlled by proteins encoded in the DNA-A-like component of monopartite begomoviruses (Hanley-Bowdoin *et al.*, 2013).

Begomoviruses have been reported to be associated with ssDNA satellite molecules and/or satellite-like molecules, namely betasatellites and alphasatellites, respectively (Mansoor *et al.*, 1999; Nawaz-ul-Rehman & Fauquet, 2009; Saunders *et al.*, 2000). The majority of the Old World monopartite begomoviruses co-exist with betasatellites. Betasatellites typically contain a satellite conserved region (SCR), an adenine-rich region and a βC1 ORF. The βC1 ORF has been characterized as a pathogenicity determinant that suppresses host antiviral silencing and is involved in virus movement (Cui *et al.*, 2005; Li *et al.*, 2014; Saunders *et al.*, 2004; Yang *et al.*, 2011). A class of autonomously replicating satellite-like molecules identified as alphasatellites require helper begomoviruses for their intra- and intercellular movement and are reported to ameliorate symptoms in a few cases (Idris *et al.*, 2011; Nawaz-ul-Rehman *et al.*, 2010).

Capsicum spp. are cultivated as vegetables and spice crops throughout the world. Chilli leaf curl disease (ChiLCD) causes severe crop loss in the tropical and subtropical regions of the Indian subcontinent and has emerged as a major factor limiting chilli cultivation in this part of the world (Chattopadhyay *et al.*, 2008; Mishra *et al.*, 1963; Senanayake *et al.*, 2007, 2013; Varma & Malathi, 2003). The symptoms of ChiLCD-infected plants are leaf curling, leaf rolling, puckering and reduced leaf size; in severe cases, the infected plants remain stunted and fail to bear any fruit, resulting in complete crop loss. Although the first incidence of ChiLCD was reported in the 1960s, its association with begomoviruses was confirmed only recently (Chattopadhyay *et al.*, 2008; Khan *et al.*, 2006; Kumar *et al.*, 2012; Senanayake *et al.*, 2007). During the past few years, monopartite begomoviruses and betasatellites associated with ChiLCD have spread to major chilli-growing regions of the Indian subcontinent. To date, in India, chilli leaf curl virus (ChiLCV), chilli leaf curl India virus (ChiLCINV), chilli leaf curl Vellanad virus (ChiLCVV), tomato leaf curl Joydebpur virus and tomato leaf curl New Delhi virus (ToLCNDV) are known to be associated with ChiLCD (Khan *et al.*, 2006; Kumar *et al.*, 2011, 2012; Senanayake *et al.*, 2007; Shih *et al.*, 2007).

Despite the severe losses due to ChiLCD, a systematic study of the begomoviruses associated with ChiLCD in different chilli-growing regions of India is lacking. Here, we have carried out a comprehensive survey to analyse the genetic diversity and the phylogeographical distribution of ChiLCD-associated begomoviruses. This study reveals the association of seven distinct begomovirus species with five different groups of betasatellites with ChiLCD. Most of the ChiLCD-associated begomoviruses and betasatellites were recombinants. The isolated viral components could infect *Nicotiana benthamiana* and *Capsicum annuum*. Furthermore, our study also highlighted the indispensable role of betasatellites in inducing severe leaf curl disease on *Capsicum* spp.

RESULTS

Cloning of ChiLCD-associated viral components across India

Leaf samples from chilli plants exhibiting leaf curling, crinkling and stunting of plants were collected from 28 major chilli-growing regions of India (Fig. S1, Table S1, available in the online Supplementary Material). The symptoms were observed to vary among the samples surveyed (Table S1). Total DNA was extracted from symptomatic leaves, and PCR amplification with abutting primers for DNA-A, DNA-B and betasatellite indicated the association of begomoviruses and betasatellites with the field samples (data not shown). PCR-based amplification of full-length viral components and/or rolling circle amplification (RCA) using φ29 polymerase yielded DNA fragments of either $\sim$2.8 kb or $\sim$1.3 kb, which were cloned (Table 1). Clones from respective samples were digested with several restriction enzymes to identify the diversity based on RFLP and were subsequently partially sequenced. We obtained the complete nucleotide sequences of 41 molecules of $\sim$2.8 kb and 33 molecules of $\sim$1.3 kb in size from samples representing the 28 chilli-growing regions. Cloning method and accession numbers are provided in Table 1.

Diversity and phylogenetic analysis of ChiLCD-associated begomoviruses

Our analysis showed that 35 out of the 41 molecules were $\sim$2.8 kb in size with a DNA-A/DNA-A-like begomovirus genome arrangement, whereas six molecules had a DNA-B-like genomic organization. Betasatellites were present in all the samples irrespective of whether the begomovirus genome detected was either monopartite or bipartite (Table 1).

Based on 91 % nucleotide sequence identity as the species demarcation threshold for DNA-A (Brown *et al.*, 2015) and by pairwise sequence comparison, these DNA-A/DNA-A-like isolates were categorized into seven distinct species including one newly identified species (HM007119). When compared with the genomic sequences available in GenBank, the majority of these sequences had high similarity with previously reported begomoviruses associated with either chilli or tomato (Fig. S2a).

The viral sequences represented in clones O1A and K1A showed maximum identities of 95.8 % and 94.8 % with a 'Croton' strain of papaya leaf curl virus (PaLCuV-Cro;

Table 1. Details of viral components identified from ChiLCD-infected *Capsicum* spp. in India

Location		Year	GPS coordinates	DNA-A/DNA-A-like*				DNA-β				DNA-B				Nature of genome
State	Area			Clone name	Accession number	Cloning method	Isolate†	Clone name	Accession number	Cloning method	Isolate†	Clone name	Accession number	Cloning method	Isolate†	
Andhra Pradesh	Guntur	2009	16.30° N 80.45° E	A1A	HM007100	RCA	ChiLCV-Gun	A1β	JN663859	PCR	ToLCBDB					Monopartite
Assam	Jorehat	2009	26.44° N 94.10° E	A2A	JN663861	PCR	ChiLCV-PK	A2β	JN663862	PCR	ToLCJoB					Monopartite
Bihar	Chhapra	2010	25.78° N 84.72° E	B1A	JN663852	RCA	ChiLCV-Chi[1]	B1β	JN663854	RCA	ToLCBDB[1]					Monopartite
				B2A	JN663853	RCA	PepLCBV-BD[1]	B2β	JN663855	RCA	ToLCBDB[1]					
	Patna	2008	25.36° N 85.7° E	B3A	HM007117	RCA	ChiLCV	B3β	HM007118	RCA	ToLCBDB					Monopartite
Goa	Keri	2011	26.67° N 73.18° E	G1A	KP235539	PCR	ChiLCV-Gun	G1β	JN663858	PCR	RaLCB					Monopartite
Gujarat	Ahmedabad	2009	23.03° N 72.58° E	G2A	JN663846	RCA	ChiLCV-IN	G2β	JN663847	PCR	ToLCBDB					Monopartite
Haryana	Pataudi	2007	28.35° N 76.75° E	H1A	HM007116	PCR	ChiLCV-IN	H1β	EU582020	RCA	ToLCBDB					Monopartite
Himachal Pradesh	Palampur	2009	32.11° N 76.53° E	H2A	JN663870	PCR	ChiLCV-PK[2]	H2β	JN663872	PCR	ToLCRnB[2]	H2B	JN663871	PCR	ToLCNDV[2]	Monopartite & bipartite
				H3A	KP235540	PCR	ToLCNDV[2]									
Karnataka	Bengaluru	2010	13.81° N 77.29° E	K1A	HM007094	RCA	ToLCV-Ban	K1β	JN663849	PCR	ToLCBDB	K1B	JN663848	PCR	ToLCNDV	Monopartite
	Tumkur	2008	13.20° N 77.08°E	K2A	HM007120	RCA	ToLCNDV	K2β	JN663875	RCA	ToLCBDB					
Kerala	Vellanad	2008	8.34° N 77.30° E	K3A	HM007121	RCA	ChiLCVV[3]	K3β	JN663877	PCR	RaLCB[3]					Monopartite
								K4β	JN663876	PCR	ToLCBDB[3]					
Madhya Pradesh	Jabalpur	2008	23.10° N 79.59° E	M1A	HM007101	RCA	PepLCBV-PK	M1β	JN663860	PCR	ToLCBDB					Monopartite
Maharashtra	Nagpur	2009	21.8° N 79.5° E	M2A	JN663864	PCR	PepLCBV-IN[4]	M2β	JN663863	PCR	ToLCJoB[4]					Monopartite
				M3A	JN663865	PCR	ChiLCV-Gun[4]									
	Pune	2009	18.31° N 73.51° E	M4A	KP235542	PCR	ToLCNDV	M4β	JN663878	PCR	CroYVMB	M4B	KP235543	PCR	ToLCNDV	Bipartite
New Delhi	New Delhi	2010	28.36° N 77.12° E	N1A	HM007113	RCA	ToLCNDV[5]	N1β	JN663868	PCR	ToLCBDB[5]	N2B	JN663867	PCR	ToLCNDV[5]	Monopartite & bipartite
				N2A	JN663866	RCA	ChiLCV-IN[6]	N2β	JN663869	PCR	ToLCBDB[6]	N3B	KP235538	PCR	ToLCGV[5]	
Orissa	Bhubaneswar	2009	20.14° N 85.50° E	O1A	JN663850	RCA	PaLCuV-Cro	O1β	JN663851	PCR	ToLCRnB					Monopartite

Table 1. cont.

Location		Year	GPS coordinates	DNA-A/DNA-A-like*				DNA-β				DNA-B				Nature of genome
State	Area			Clone name	Accession number	Cloning method	Isolate†	Clone name	Accession number	Cloning method	Isolate†	Clone name	Accession number	Cloning method	Isolate†	
Rajasthan	Jodhpur	2009	26.18° N 73.04° E	R1A	HM007104	RCA	ChiLCV-IN	R1β	HM007105	RCA	ToLCBDB					Monopartite
Tamil Nadu	Coimbatore	2008	11.0° N 76.58° E	T1A	HM007096	RCA	PepLCBV-IN[7]	T1β	JN663856	PCR	ToLCBDB[7]					Monopartite
								T2β	JN663857	PCR	CroYVMB[7]					
	Salem	2008	11.44° N 78.4° E	T2A	HM007119	RCA	ChiLCSV[8]	T3β	JN663873	PCR	RaLCB[8]	T2B	KF471060	PCR	ToLCNDV[8]	Monopartite & bipartite
				T3A	KP235541	PCR	ToLCNDV[8]	T4β	JN663874	PCR	CroYVMB[8]					
Uttar Pradesh	Jaunpur	2007	25.44° N 82.41° E	U1A	HM007102	PCR	ChiLCV	U1β	HM007103	RCA	ToLCJoB					Monopartite
	Ghazipur	2010	25.35° N 83.34° E	U2A	HM007097	RCA	PepLCBV-BD[9]	U2β	HM007099	PCR	ToLCBDB[9]					Monopartite
				U3A	HM007098	RCA	ChiLCV-Gaz[9]									
	Kanpur	2008	26.28° N 80.21° E	U4A	HM007106	RCA	PepLCBV-Kan	U4β	HM007107	RCA	ToLCBDB					Monopartite
	Noida	2007	28.57° N 77.32° E	U5A	HM007114	PCR	ChiLCV-IN	U5β	HM007115	RCA	ToLCBDB					Monopartite
	Varanasi	2006	25.20° N 83.0° E	U6A	EF190217	PCR	ChiLCV-PK	U6β	EF190215	PCR	ToLCBDB					Monopartite
West Bengal	Baruipur	2008	22.20° N 88.26° E	W1A	HM007095	PCR	ChiLCV-Chi	W1β	JN704344	PCR	ToLCJoB					Monopartite
	Kalyani	2006	22.59° N 88.29° E	W2A	EF194765	PCR	ChiLCV	W2β	EF190216	RCA	ToLCJoB					Monopartite
	Kolkata	2008	22.34° N 88.22° E	W3A	HM007108	RCA	ChiLCV[10]	W3β	HM007110	RCA	ToLCJoB[10]					Monopartite
				W4A	HM007109	RCA	ChiLCV[10]									
	Magrahat	2007	21.41° N 88.4° E	W5A	HM007111	RCA	PepLCBV-BD	W5β	HM007112	RCA	ToLCJoB					Monopartite

*DNA-A-like and DNA-A refer to monopartite and bipartite begomoviruses, respectively.
†Clones followed by the same numbers (in superscript) indicate that they were obtained from the same plant in that particular location.

AJ507777) and a 'Bangalore' strain of tomato leaf curl virus (ToLCV-Ban; FJ514798), respectively, and were thus identified as isolates of the aforesaid respective species (Fig. S2a). With 92.8–95.8 % nucleotide identity among them, B2A, M1A, U2A and W5A were classified as isolates of the species *Pepper leaf curl Bangladesh virus* (PepLCBV), based on their nucleotide identity (93.1–96.8 %) with the isolates from either Bangladesh or Pakistan, and formed a separate clade in the phylogenetic tree (Fig. 1a). Since U4A shared maximum identity of 91.9 % with PepLCBV-PK (DQ116881), and M2A and T1A displayed a maximum identity between 90.3 % and 92.3 % with PepLCBV (AM404179), they were, according to ICTV guidelines, considered to be new strains of the respective species and were named 'PepLCBV-IN' and 'PepLCBV-Kan', respectively (Fig. S2a). The complete nucleotide sequences of H3A, K2A, M4A, N1A and T3A were found to have 94.2–95.1 % sequence identity with ToLCNDV (AJ875157). Thus, they were considered as isolates of ToLCNDV and clustered as a separate clade in the phylogenetic tree (Fig. 1a). The sequences of B3A, U1A, W2A, W3A and W4A shared maximum nucleotide identity between 94 % and 96.9 % with ChiLCV (AJ875159). The sequences of five isolates (G2A, H1A, N2A, R1A and U5A) displayed 96.3–96.9 % nucleotide identity with the 'Indian' strain of ChiLCV (ChiLCV-IN; GU136803)

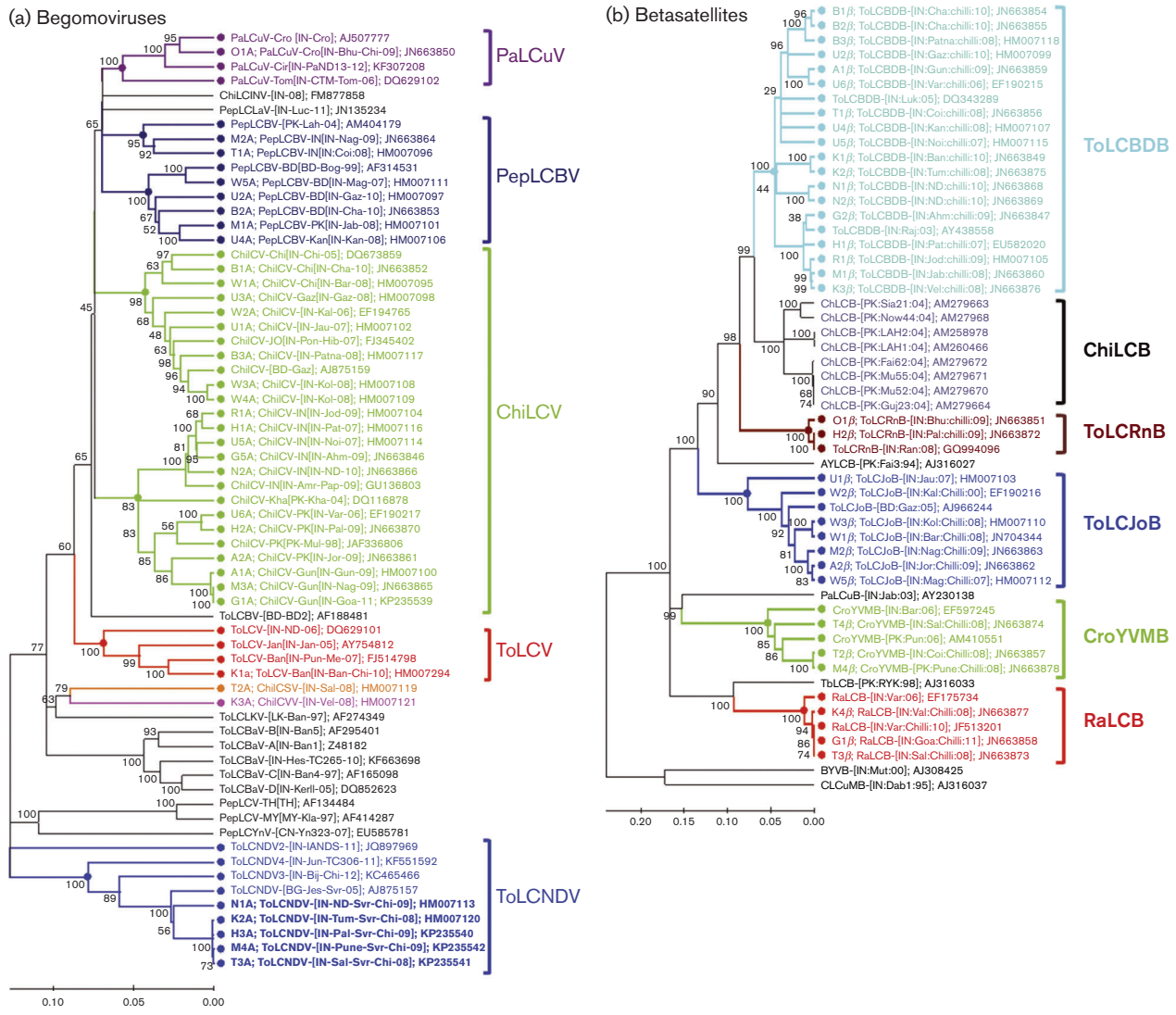

Fig. 1. Neighbour-joining phylogenetic dendrogram of ChiLCD-associated begomoviruses (a) and betasatellites (b) obtained in this study (indicated in bold type) with the most closely related representative genomes available in the database. GenBank accession numbers for all these sequences are shown. Prior to tree generation, all the sequences were aligned using CLUSTAL W. Percentage bootstrap values of 1000 replicates are given at nodes. Major clades in the same colour belong to a particular species, as indicated at the right side of each tree. The scale bars represent the rate of nucleotide substitutions per site.

(Fig. S2a). The sequences of A2A, H2A and U6A shared 94.8–95.5 % nucleotide identity with the 'Pakistan' strain of ChiLCV (ChiLCV-PK; AF336806), and 95.9–98.6 % identity among them. Since U3A shared maximum identity (92.8 %) with ChiLCV (AJ875159), according to ICTV guidelines, it was considered as a new strain of ChiLCV (ChiLCV-Gaz). Likewise, A1A, G1A and M3A were found to share a maximum identity of 91.9 % with ChiLCV-PK (AF336806) and hence considered as new strains of ChiLCV (Fig. S2a). Consistent with the nucleotide identities, all the strains/isolates belonging to ChiLCV formed a distinct clade in the phylogenetic dendrogram (Fig. 1a). Results indicated that ChiLCVs were not confined to any single geographical territory of India.

Based on the species demarcation criteria, the isolate from the Vellanad region belongs to chilli leaf curl Vellanad virus (ChiLCVV). A sequence from the Salem region exhibited a nucleotide identity of 82.2 % with tomato leaf curl Sri Lanka virus (AF274349) and we propose the name 'chilli leaf curl Salem virus' (ChiLCSV) for the species. Also note that ChiLCVV and ChiLCSV occupy unique positions in the phylogenetic dendrogram (Fig. 1a).

All five DNA-B sequences identified with these begomoviruses, i.e. H2B, K1B, M4B, N2B and T2B, were 2696 nt in length (Table 1), and shared >99 % nucleotide identity with ToLCNDV DNA-B (U15017). Hence, these DNA-Bs are considered as components of the isolates of ToLCNDV. Similarly, a sequence (N3B) isolated from New Delhi was shown to have maximum sequence identity with tomato leaf curl Gujarat virus (AY190291) DNA-B component. In the phylogenetic analysis, all these isolates grouped with their respective DNA-A (Fig. S3).

Distribution and phylogenetic relationship among betasatellites

Betasatellites 1350–1379 nt in size were isolated from the samples that harboured either monopartite (DNA-A-like) or bipartite (DNA-A and DNA-B) begomoviruses. According to the 78 % identity of species demarcation threshold for betasatellites (Briddon *et al.*, 2008) and pairwise sequence identity, these betasatellites may be broadly grouped as croton yellow vein mosaic betasatellite (CroYVMB), radish leaf curl betasatellite (RaLCB), tomato leaf curl Bangladesh betasatellite (ToLCBDB), tomato leaf curl Joydebpur betasatellite (ToLCJoB) and tomato leaf curl Ranchi betasatellite (ToLCRnB) (Fig. S2b). The sequences of G1β, K4β and T3β showed >99 % identity with RaLCB (EF175734), whereas those of M4β, T2β and T4β shared maximum identities of 90.3–91.5 % with CroYVMB (AM410551). All these weed-associated betasatellites were obtained from samples collected from southern India (Table 1). In the phylogenetic dendrogram, CroYVMB-, RaLCB-, tomato- and chilli-associated betasatellite clades were distinctly placed (Fig. 1b).

Of the 33 betasatellites isolated here, 27 were grouped into three clusters: ToLCJoB, ToLCBDB and ToLCRnB. The ToLCRnB cluster comprised the H2β and O1β sequences, sharing maximum sequence identity of 91.5–99.4 % with ToLCRnB (GQ994096), while the isolates A2β, M2β, U1β, W1β, W2β, W3β and W5β displayed 93–94 % identity to ToLCJoB (AJ966244) (Fig. S2b). The remaining 18 isolates shared 88.4–98.2 % nucleotide sequence identity with ToLCBDB (DQ343289, AY438558). The nucleotide sequence identity among ToLCJoBs ranged between 80.6 % and 99.6 %, whereas it ranged between 87.7 % and 99.7 % for ToLCBDB isolates. In the phylogenetic tree, ToLCBDB and ToLCJoB were grouped as distinct clades separated from chilli leaf curl betasatellite (ChiLCB) reported from Pakistan (Fig. 1b). Betasatellites identified from Assam and West Bengal belonged to ToLCJoB, but ToLCBDBs were distributed throughout the country.

Detection of recombination among ChiLCD-associated begomoviruses and betasatellites

Recombination is known to play a major role in the emergence and evolution of geminiviruses (George *et al.*, 2015; Lefeuvre & Moriones, 2015; Padidam *et al.*, 1999). In recombination analysis of begomoviral components, we detected significant evidence of recombination in the vast majority of isolates (26/35) (Table 2). Among them, nine isolates showed evidence of recombination events in both the AC1 and AV1 regions, whereas in three isolates, the breakpoints were located within the common region (CR) and AV1 (Table 2). In two isolates, recombination breakpoints were detected in the CR and AC1 regions. Furthermore, ChiLCV isolates (Nagpur and Goa) had recombination breakpoints in both the CR and AC2/AC3 regions. Similarly, the Ahmedabad isolate possessed breakpoints in AV1 and AC2/AC3 regions (Table 2). The recombination events located either in AC1 or in AV1 only were identified in five and three isolates, respectively (Table 2). However, no evidence of a recombination event was observed in ChiLCVV. In the phylogenetic tree of AV1 and AC1 ORFs, the sequences from recombinant molecules clustered with their respective contributors (Fig. S4). These results are in line with previous observations of AV1 and AC1 regions as the recombination hotspots (George *et al.*, 2015; Lefeuvre *et al.*, 2007; Lefeuvre & Moriones, 2015). In addition to interspecific recombination events, most of these ChiLCD-associated begomoviruses are obvious intra-species recombinants (Table 2).

Among the 33 betasatellites identified in this study, 17 showed evidence of recombination (Table 2). The recombination breakpoints were located around the A-rich region of CroYVMB (two isolates) and ToLCBDB (seven isolates), the SCR (six ToLCJoB isolates) or the βC1 ORF (sequences from Bhubaneswar and Jaunpur).

Table 2. Recombination analysis of ChiLCD-associated begomoviruses and betasatellites

Recombinant sequence	Event no.	Breakpoint positions			Putative minor parent	Putative major parent	Methods†	*P* value‡
		Begin	End	Location in the genome*				
ChiLCSV-[IN-Sal-08]	1	454	1190	AV1	PepLCBV-[PK-Lah-04]	ToLCLKV-[LK-Ban-97]	RMC	7.28×10^{-11}
	2	2146	2712	AC1	PaLCuV-PK[PK-Cot-02]	ToLCSKV-[LK-Ban-97]	GBMC	1.94×10^{-24}
ChiLCV-PK[IN-Jor-09]	1	1997	2591	AC1	ChiLCV-[BD-Gaz]	PepLCBV-[PK-Lah-04]	RGBMCS	3.78×10^{-27}
	2	2592	2742	CR	ChiLCV-PK[PK-Mul-98]	PepLCBV-[PK-Lah-04]	RGBMC	4.56×10^{-11}
ChiLCV-PK[IN-Pal-09]	1	2083	2582	AC1	ChiLCV-[BD-Gaz]	PepLCBV-[PK-Lah-04]	RGBMCS	1.51×10^{-26}
ChiLCV-IN[IN-Ahm-09]	1	512	1084	AV1	ChiLCV-PK[PK:Mul:98]	ChiLCV-Chi[IN-Chi-05]	RGBMC	1.34×10^{-21}
	2	1230	1790	AC2/AC3	PepLCBV-BD[BD-Bog-99]	ChiLCV-Chi[IN-Chi-05]	RGBMCS	1.76×10^{-8}
ChiLCV-Gun[IN-Gun-08]	1	502	1151	AV1	ToLCV-Ban[IN-Ban-93]	ChiLCV-PK[PK-Mul-98]	RBMCS	7.17×10^{-7}
	2	2031	2595	AC1	ChiLCV-[BD-Gaz]	ChiLCV-PK[PK-Mul-98]	RGBMCS	3.65×10^{-24}
ChiLCV-IN[IN-Jod-09]	1	323	940	AV1	PepLCBV-[PK-Lah-04]	ChiLCV-PK[PK-Mul-98]	RGBS	8.36×10^{-33}
	2	2031	2695	AC1	ChiLCV-Chi[IN-Chi-05]	ChiLCV-PK[PK-Mul-98]	RGBMCS	3.06×10^{-16}
ChiLCV-Gun[IN-Nag1-09]	1	1090	1359	AC2/AC3	PepLCBV-[PK-Lah-04]	ChiLCV-PK[PK-Mul-98]	RGBMS	4.92×10^{-12}
and ChiLCV-Gun[IN-Goa-11]	2	2412	1	CR	PepLCBV-BD[BD-Bog-99]	ChiLCV-PK[PK-Mul-98]	RGBMS	1.89×10^{-9}
ChiLCV-IN[IN-ND-10]	1	7	1438	AV1	PepLCBV-[PK-Lah-04]	ChiLCV-Chi[IN-Chi-05]	RGBMCS	2.37×10^{-19}
	2	1680	2123	AC1	ChiLCV-PK[PK-Mul-98]	ChiLCV-Chi[IN-Chi-05]	RGBMCS	7.95×10^{-9}
ChiLCV-IN[IN-Noi-07]	1	47	1086	AV1	ChiLCV-PK[PK-Mul-98]	ChiLCV-Chi[IN-Chi-05]	RGBMCS	6.34×10^{-23}
	2	1786	7	AC1/CR	ChiLCV-Chi[IN-Chi-05]	ChiLCV-PK[PK-Mul-98]	RGBMCS	7.28×10^{-28}
ChiLCV-IN[IN-Pat-07]	1	441	1076	AV1	ToLCV-Ban[IN-Ban-93]	ChiLCV-Chi[IN-Chi-05]	RGBMC	8.80×10^{-8}
	2	2006	23	AC1/CR	ChiLCV-Chi[IN-Chi-05]	ChiLCV-PK[PK-Mul-98]	RGBMCS	1.83×10^{-27}
PepLCBV-BD[IN-Mag-07]	1	543	1153	AV1	ChiLCV-[BD-Gaz]	PepLCBV-PK[PK-Kha-04]	RGBS	1.72×10^{-16}
	2	1483	1642	AC1	Unknown species	PepLCBV-PK[PK-Kha-04]	GMCS	1.29×10^{-60}
PepLCBV-BD[IN-Gha1-10]	1	541	928	AV1	ChiLCV-[BD-Gaz]	PepLCBV-PK[PK-Kha-04]	RGBMCS	4.82×10^{-11}
	2	963	1165	AV1	Unknown species	PepLCBV-PK[PK-Kha-04]	GMC	1.77×10^{-4}
PepLCBV-PK[IN-Jab-07]	1	2136	2675	AC1	PepLCBV-PK[PK-Kha-04]	ChiLCV-Chi[IN-Chi-05]	RGBMCS	2.20×10^{-28}
PepLCBV-BD[IN-Chp2-10]	1	8	691	AV1	ChiLCV-Chi[IN-Chi-05]	PepLCBV-PK[PK-Kha-04]	RGBMCS	2.31×10^{-14}
	2	2401	2689	AC1	PepLCBV-PK[PK-Kha-04]	Unknown species	RGBMCS	5.70×10^{-22}
PepLCBV-Kan[IN-Kan-08]	1	1854	2689	AC1	PepLCBV-[PK-Lah-04]	PepLCBV-PK[PK-Kha-04]	RGBMC	9.09×10^{-11}
PepLCBV-IN[IN-Coi-08]	1	2357	2699	AC1	PaLCuV-IN[IN:Luc]	PepLCBV-[PK-Lah-04]	RGBMC	4.11×10^{-10}
	2	2724	407	CR	PepLCBV-BD[BD-Bog-99]	PepLCBV-[PK-Lah-04]	RGBMCS	7.06×10^{-10}
PepLCBV-IN[IN-Nag1-09]	1	414	1092	AV1	PaLCuV-PK[PK-Cot-02]	PepLCBV-[PK-Lah-04]	RBMCS	2.44×10^{-5}
	2	2619	2702	CR	PepLCBV-PK[PK-Kha-04]	PepLCBV-[PK-Lah-04]	GBMC	4.71×10^{-8}
ChiLCV-Chi[IN-Chp1-10]	1	2713	8	CR	PepLCBV-BD[BD-Bog-99]	ChiLCV-PK[PK-Mul-98]	RGBMCS	5.45×10^{-24}
ChiLCV-Chi[IN-Bar-08]	1	508	883	AV1	ChiLCV-[BD-Gaz]	ChiLCV-Chi[IN-Chi-05]	RGBMCS	1.67×10^{-16}
	2	2410	2691	AC1	ChiLCV-Chi[IN-Chi-05]	PepLCBV-PK[PK-Kha-04]	GBMCS	2.60×10^{-27}
ChiLCV-[IN-Kal-06]	1	546	962	AV1	ChiLCV-[BD-Gaz]	Unknown species	RGBMCS	3.07×10^{-14}
	2	2115	2588	AC1	ChiLCV-PK[PK-Mul-98]	PepLCBV-PK[PK-Kha-04]	RGBMCS	1.45×10^{-24}
ChiLCV[IN-Kol-08]	1	354	1160	AV1	ChiLCV-[BD-Gaz]	Unknown species	GMCS	1.62×10^{-31}
ChiLCV-[IN-Patna-08]	1	613	1262	AV1	PepLCBV-PK[PK-Kha-04]	Unknown species	RGBMCS	6.77×10^{-25}
	2	2097	2586	AC1	ChiLCV-PK[PK-Mul-98]	PepLCBV-PK[PK-Kha-04]	RGBMCS	3.99×10^{-24}

Table 2. cont.

Recombinant sequence	Event no.	Breakpoint positions			Putative minor parent	Putative major parent	Methods†	*P* value‡
		Begin	End	Location in the genome*				
ChiLCV-[IN-Jau-07]	1	2564	2721	CR	ChiLCV-[BD-Gaz]	Unknown species	GBMC	4.17×10^{-9}
ChiLCV[IN-Gha2-10]	1	2045	2240	AC1	ChiLCV-PK[PK-Mul-98]	ChiLCV-[BD-Gaz]	RGBS	3.55×10^{-8}
ToLCV-Ban[IN-Ban- Chilli-10]	1	2238	2694	AC1	ToLCBaV-D[IN-KerII-05]	ChiLCV-PK[PK-Mul-98]	RGBMCS	4.18×10^{-15}
CroYVMB-[IN-Sal-Chilli-08]	1	779	963	A-rich	ToLCB-[IN-Var-06]	PaLCuB-[IN-Jab-03]	RGBMC	9.49×10^{-10}
CroYVMB-[IN-Pune-Chilli-09]	1	832	953	A-rich	ToLCB-[IN-Var-06]	PaLCuB-[IN-Jab-03]	RGBMC	3.25×10^{-9}
ToLCBDB-[IN-Gha1-Chilli-10]	1	519	1161	A-rich	ToLCBDB-[BD-Gaz-01]	ChLCB-[PK-Sia21-04]	RGBMCS	3.08×10^{-6}
ToLCBDB-[IN-Var-Chilli-06]	1	984	1193	A-rich	ToLCBDB-[IN-Raj-03]	Unknown species	RGBMC	8.36×10^{-5}
ToLCBDB-[IN-Coi1-Chilli-08]	1	521	1308	A-rich	ToLCBDB-[BD-Gaz-01]	ChLCB-[PK-Sia21-04]	RGBMCS	5.45×10^{-7}
ToLCBDB-[IN-Kan-Chilli-08]	1	472	1366	A-rich	ToLCBDB-[BD-Gaz-01]	ChLCB-[PK-Sia21-04]	RGBMCS	1.17×10^{-10}
ToLCBDB-[IN-Ahm-Chilli-09]	1	765	955	A-rich	ToLCBDB-[BD-Gaz-01]	Unknown species	GBMCS	3.78×10^{-15}
ToLCBDB-[IN-Jod-Chilli-09]	1	518	1036	A-rich	ToLCBDB-[BD-Gaz-01]	ChLCB-[PK-Sia21-04]	RGBMCS	1.06×10^{-5}
ToLCBDB-[IN-Vel2-Chilli-08]	1	716	1077	A-rich	ToLCBDB-[BD-Gaz-01]	ChLCB-[PK-Sia21-04]	RGBMCS	4.83×10^{-7}
ToLCJoB-[IN-Kal-Chilli-06]	1	1140	95	SCR	ChLCB-[PK-Guj23-04]	AYLCB-[PK-Fai3-94]	RGBMCS	6.83×10^{-13}
ToLCJoB-[IN-Kol-Chilli-08]	1	1123	89	SCR	ChLCB-[PK-Guj23-04]	AYLCB-[PK-Fai3-94]	RGBMCS	5.06×10^{-12}
ToLCJoB-[IN-Bar-Chilli-08]	1	1075	1261	A-rich/SCR	ToLCBDB-[IN-Raj-03]	Unknown species	RGBMCS	1.12×10^{-14}
ToLCJoB-[IN-Nag-Chilli-09]	1	1065	1251	A-rich/SCR	ToLCBDB-[IN-Raj-03]	Unknown species	RGBMCS	3.80×10^{-15}
ToLCJoB-[IN-Jor-Chilli-09]	1	1123	92	SCR	ChLCB-[PK-Guj23-04]	AYLCB-[PK-Fai3-94]	RGBMCS	1.99×10^{-14}
ToLCJoB-[IN-Mag-Chilli-07]	1	1123	92	SCR	ChLCB-[PK-Guj23-04]	AYLCB-[PK-Fai3-94]	RGBMCS	3.02×10^{-14}
ToLCJoB-[IN-Jau-Chilli-07]	1	1276	521	βC1	ToLCBDB-[IN-Raj-03]	ToLCJoB-[IN-Kenaf-07]	RGBMCS	4.54×10^{-30}
ToLCRnB-[IN-Bhu-Chilli-09]	1	146	499	βC1	ToLCBDB-[BD-Gaz-01]	Unknown species	GMCS	4.43×10^{-31}

*CR, common region of a begomovirus genome; SCR, satellite conserved region of betasatellites.
†R, RDP; G, GeneConv; B, Bootscan; M, MaxChi; C, Chimaera; S, SiScan.
‡The lowest *P* value calculated for the underlined method in the penultimate column.

Table 3. Nucleotide diversity of ChiLCD-associated Indian begomoviruses and betasatellites

Group	N*	Size	s	η	π	k	θ−w	θ−η
ChiLCV	19	2790	708	849	0.0934	253.5	0.0749	0.0997
PepLCBV	7	2743	576	690	0.0962	263.9	0.0857	0.1160
ToLCNDV	5	2740	134	134	0.0198	53.8	0.0236	0.0243
CroYVMB	4	1396	218	228	0.0913	121.2	0.0896	0.1053
ToLCBDB	18	1402	407	500	0.0855	112.4	0.0901	0.1262
ToLCJoB	7	1394	302	340	0.0829	110.0	0.0929	0.1184

*Details of the abbreviations used are described in Methods.

Genetic structure and substitution rate of begomoviruses and betasatellites

From an evolutionary perspective, recombination during the replication of begomoviruses alone may not lead to the genetic variation *de novo*. Nucleotide substitutions that occur during evolution also contribute to genetic variation. ChiLCD-associated begomoviruses and betasatellites were determined to have a high degree of genetic variability ($\pi > 0.08$). Our analyses showed ChiLCV and PepLCBV to be the most diverse begomoviruses, and CroYVMB and ToLCBDB to possess much higher genetic variability than the other betasatellites (Table 3). To assess the presence of any subpopulations within begomoviruses, a cluster-based method (STRUCTURE) was employed. The results suggest the presence of seven subpopulations among ChiLCV and five subpopulations were noticed among PepLCBV isolates (Fig. S5a, b). However, low nucleotide diversity and the absence of any subpopulations among chilli isolates of ToLCNDV suggest their recent association with this host (Fig. S5c, Table 3). Furthermore, to verify the randomness of nucleotide variability throughout the genome, we analysed the nucleotide diversity of all the ORFs encoded by ChiLCD-associated begomoviruses and betasatellites. Higher nucleotide diversity was observed in all the ORFs encoded by the begomoviruses. The nucleotide diversity patterns of the complete genomes of betasatellites and βC1 ORFs were observed to be highly congruent (Fig. S6). The selection pressure on the ORFs was analysed using single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL) and random-effects likelihood (REL) methods. A large proportion of the sites were found to have evolved under purifying/negative selection (Table S2).

We also estimated the nucleotide substitution rate of AV1 and βC1 ORFs using the parameters listed in Table 4. The mean nucleotide substitution rate was estimated to be 2.60×10^{-3} substitutions site^{-1} year^{-1} for the AV1 gene of ChiLCV, 2.57×10^{-4} substitutions site^{-1} year^{-1} for the βC1 gene of ChiLCB and 5.22×10^{-4} substitutions site^{-1} year^{-1} for the βC1 gene of ToLCBDB (Table 4). Since codon degeneracy could play a vital role in the selection pressure leading to genetic variation, we estimated the mutation rate of all three nucleotide positions in the codon using the BSP method. Strikingly, the mutation rate of the AV1 ORF in codon position 1 was seen to be higher than the mutation rates of the other positions (Table 4). In βC1, however, the mutation rate was found to be highest in the wobble position of the codon; this position of the codon affects the amino acid sequence less often than do the other positions.

Infectivity analysis of newly emerging begomoviruses

Partial tandem repeats of the newly identified begomoviruses NgA (PepLCBV-IN) and VeA (ChiLCVV) infected *N. benthamiana* plants. Notably, the incubation period remained unaltered upon co-inoculation of either NgA or VeA with their cognate betasatellites (Ngβ and Veβs) (Table 5). *N. benthamiana* plants co-inoculated with either NgA + Ngβ or VeA + Veβ1 exhibited symptoms such as downward leaf curling and enations (Fig. 2a). However, agroinoculation of VeA + Veβ2 failed to induce downward leaf curling in *N. benthamiana*, a characteristic symptom induced by betasatellites. In Southern analysis, all these begomoviruses could trans-replicate their cognate betasatellites, but these

Table 4. Mean substitution rate and codon position mutation rate for AV1 and βC1 ORFs encoded by ChiLCD-associated begomoviruses and betasatellites

Parameters used	AV1				βC1		
	ChiLCV	CLCuMuV[a]*	TYLCV[b]	EACMV[c]	ChiLCB	ToLCBDB	CLCuMuB[a]
Number of sequences analysed	52	19	54	71	25	31	39
Chain length (millions)	20	40	20	15	40	40	40
Mean substitution rate (nucleotides site^{-1} year^{-1})	2.60×10^{-3}	4.24×10^{-4}	4.63×10^{-4}	1.37×10^{-3}	2.57×10^{-4}	5.22×10^{-4}	3.51×10^{-3}
Codon position 1 mutation rate	1.690	1.640	–	–	0.800	1.067	0.850
Codon position 2 mutation rate	0.366	0.449	–	–	0.622	0.679	0.730
Codon position 3 mutation rate	0.944	0.909	–	–	1.578	1.256	1.430

*Estimated by: *a*, Nawaz-ul-Rehman *et al.* (2012); *b*, Duffy & Holmes (2008); *c*, Duffy & Holmes (2009).

Table 5. Infectivity of cloned viral components on *N. benthamiana* and *C. annuum* plants

Host plant	Viral construct	Number of symptomatic plants/number of inoculated plants	Incubation period (days)*	Type of symptoms on inoculated plants†	PCR‡	
					DNA-A	DNA-β
Nicotiana benthamiana	NgA	10/10	8	LC, LR, St	+	−
	NgA + Ngβ	10/10	8	DLC, LCr, St	+	+
	VeA	10/10	6	LC, St, SL, MY	+	−
	VeA + Veβ1	10/10	6	DLC, LCr, St, SL, MY	+	+
	VeA + Veβ2	6/6	7	LC, LCr, St, SL, MY	+	+
Capsicum annuum	NgA	10/20	35	MLC	+	−
var. Sulekha	NgA + Ngβ	15/21	25	LC, LR, LCr, LB, Vt, SL, St	+	+
	VeA	9/20	35	MLC	+	−
	VeA + Veβ1	14/22	27	LC, LR, LCr, LB, Vt, SL, St	+	+
	ChA	5/20	55	MLC	+	−
	ChA + Chβ	11/20	40	LC, LCr, Vt	+	+
	JoA	7/20	51	MLC	+	−
	JoA + Joβ	9/19	37	LC, LCr, LR, LB	+	+
	MgA	5/20	50	MLC	+	−
	MgA + Mgβ	12/21	38	LC, LCr, LR, LB	+	+

*Incubation period is defined as the time taken for the first symptom appearance in days post-inoculation.
†LC, leaf curling; DLC, downward leaf curling; MLC, mild leaf curling; SL, small leaves; MY, mild yellowing; LB, leaf blistering; LR, leaf rolling; St, stunting; LCr, leaf crinkling; Vt, vein thickening.
‡Primers specific for DNA-A and DNA-β; + indicates the presence of viral components.

betasatellites did not influence the accumulation of begomovirus in *N. benthamiana* (Fig. 2b). *C. annuum* plants inoculated with begomoviruses alone (ChA, JoA, MgA, NgA, VeA) developed mild symptoms and the presence of these begomoviruses was ascertained by PCR (Fig. S7; Table 5). However, when these begomoviruses were inoculated along with their cognate betasatellites (ChA + Chβ, JoA + Joβ, MgA + Mgβ, NgA + Ngβ, VeA + Veβ1), symptoms such as stunting, leaf curling, leaf crinkling, blistering and vein thickening were observed on infected *C. annuum* var. Sulekha plants (Fig. 2a). In Southern analysis, no viral DNA accumulation could be detected from the chilli plants inoculated with diverse begomoviruses in the absence of their cognate betasatellites. However, when chilli plants were inoculated with begomoviruses and cognate betasatellites, high levels of viral DNA were detected (Fig. 2b). Collectively, our results suggest that these begomoviruses require betasatellites for development of severe ChiLCD in *Capsicum* spp. (Figs 2b and S7, Table 5).

DISCUSSION

ChiLCD is one of the most devastating diseases of chillies in India and has become a major constraint for production of *Capsicum* (Maruthi *et al.*, 2007). The viruses causing ChiLCD were identified initially based on the type of symptoms induced on the host and later by either partial or complete nucleotide sequencing of the viral genome (Chattopadhyay *et al.*, 2008; Khan *et al.*, 2006; Kumar *et al.*, 2012; Mishra *et al.*, 1963; Senanayake *et al.*, 2007). The present study provides the first comprehensive survey and analysis to our knowledge of begomoviruses associated with ChiLCD in India. Our effort has resulted in the isolation and characterization of 74 genomic components (35 DNA-A/DNA-A-like, 6 DNA-B and 33 betasatellites). The existence of newly identified and previously reported begomoviruses associated with ChiLCD clearly indicates the high degree of species diversity and genetic variability of these pathogens. In addition, the newly emerged begomoviruses have been shown to infect *N. benthamiana* and *Capsicum* spp. Our results demonstrate that the viruses tested are not only associated with but are the causal agents of ChiLCD. Furthermore, the betasatellites are found to be a prerequisite for the induction of severe leaf curl symptoms in *Capsicum* spp.

Our study has identified ChiLCV as the most widely distributed begomovirus associated with ChiLCD across India, followed by PepLCBV and ToLCNDV (Fig. 3). In the eastern region (encompassing states such as West Bengal, Bihar and the eastern part of Uttar Pradesh), ChiLCV and PepLCBV-BD predominate, with a notable exception of ChiLCV-PK in Jorehat and Varanasi. Mixed infections of ChiLCV and PepLCBV were also noticed in eastern Uttar Pradesh (Ghazipur) and the Bihar (Chhapra) region. The isolates from the northern and western regions, including the states of Haryana, Delhi, Rajasthan, Himachal

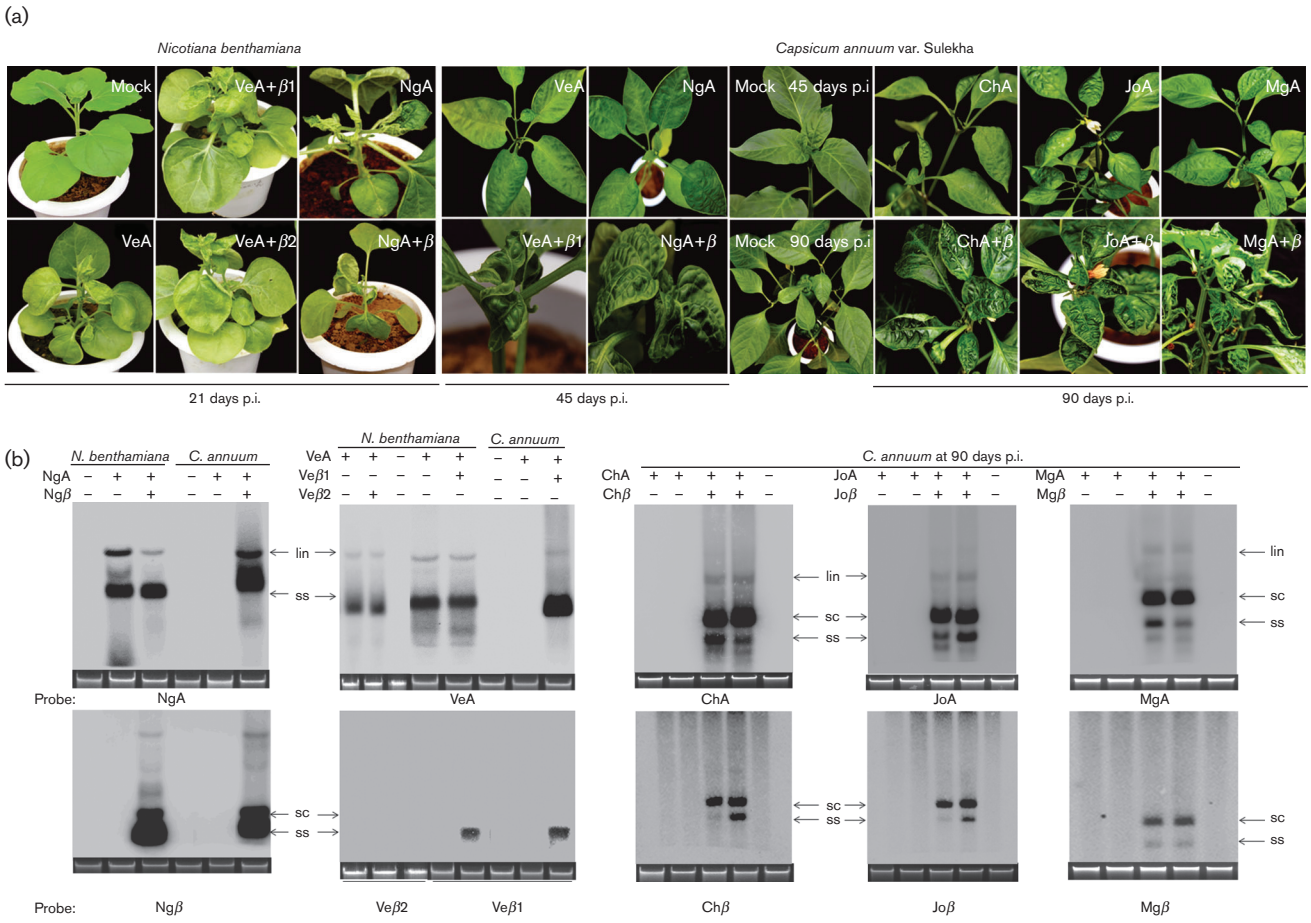

Fig. 2. Infectivity and viral DNA accumulation of cloned components. (a) Appearance of symptoms on *N. benthamiana* and *C. annuum* plants infected with various begomoviruses alone or with their respective cognate betasatellites as indicated. (b) Relative DNA accumulation of various begomoviruses and betasatellites in *N. benthamiana* [at 21 days post-inoculation (p.i.)] and *C. annuum* plants (at 90 days p.i.), as analysed by Southern hybridization for each combination mentioned. Probes used for hybridization are given at the bottom of each blot. The replicative DNA forms are abbreviated as linear (lin), super-coiled (sc) and single-stranded (ss). Ethidium-bromide-stained DNA serves as a loading control. VeA, ChiLCVV; NgA, PepLCBV-IN; ChA, ChiLCV-PK; JoA, ChiLCV; MgA, PepLCBV-BD; Veβ and Chβ, ToLCBDB; Joβ, Mgβ and Ngβ, ToLCJoB.

Pradesh, Goa, Gujarat, Maharashtra and Uttar Pradesh (western part) belong to ChiLCV. Taken together, these data indicate that ChiLCD in these regions is associated with the begomoviruses previously reported from Pakistan, whereas viruses identified in the eastern part of India are predominantly present in Bangladesh (Fig. 3). It is relevant to mention that states adjoining the border region follow similar cropping patterns, which might have facilitated the migration of the viruses through whiteflies.

In southern India, various distinct begomoviruses such as ChiLCV-Gun, ChiLCVV, PaLCuV-Cro, PepLCBV-IN, ToLCV-Ban and ToLCNDV were found to be associated with ChiLCD. In addition, the one previously unreported begomovirus (ChiLCSV) was also identified in this region. Taken altogether, our analysis from southern India indicates that diverse begomoviruses and betasatellites are associated with ChiLCD (Fig. 3). Under laboratory conditions, an isolate of tomato leaf curl Gujarat virus caused systemic infection in chilli (Chakraborty *et al.*, 2003), and hence the association of diverse tomato-infecting begomoviruses with ChiLCD-affected plants is not surprising.

Recombination has been reported to be the major factor in the emergence of begomoviruses infecting crops and weeds in India (Kumar *et al.*, 2011; Kumari *et al.*, 2010; Singh *et al.*, 2012). Begomoviruses associated with chilli and tomato contributed to 32 out of the 44 recombination events detected in ChiLCD-associated begomoviruses, and the remaining recombination events (12/44) were associated with yet undescribed or non-solanaceous-crop-infecting begomoviruses. Our results indicate that the chilli- and tomato-associated begomoviruses have a complex history of recombination in this part of the world, with a relevant contribution of interspecific recombination. It is noteworthy that ChiLCSV may have emerged after multiple recombination events. A non-recombinant

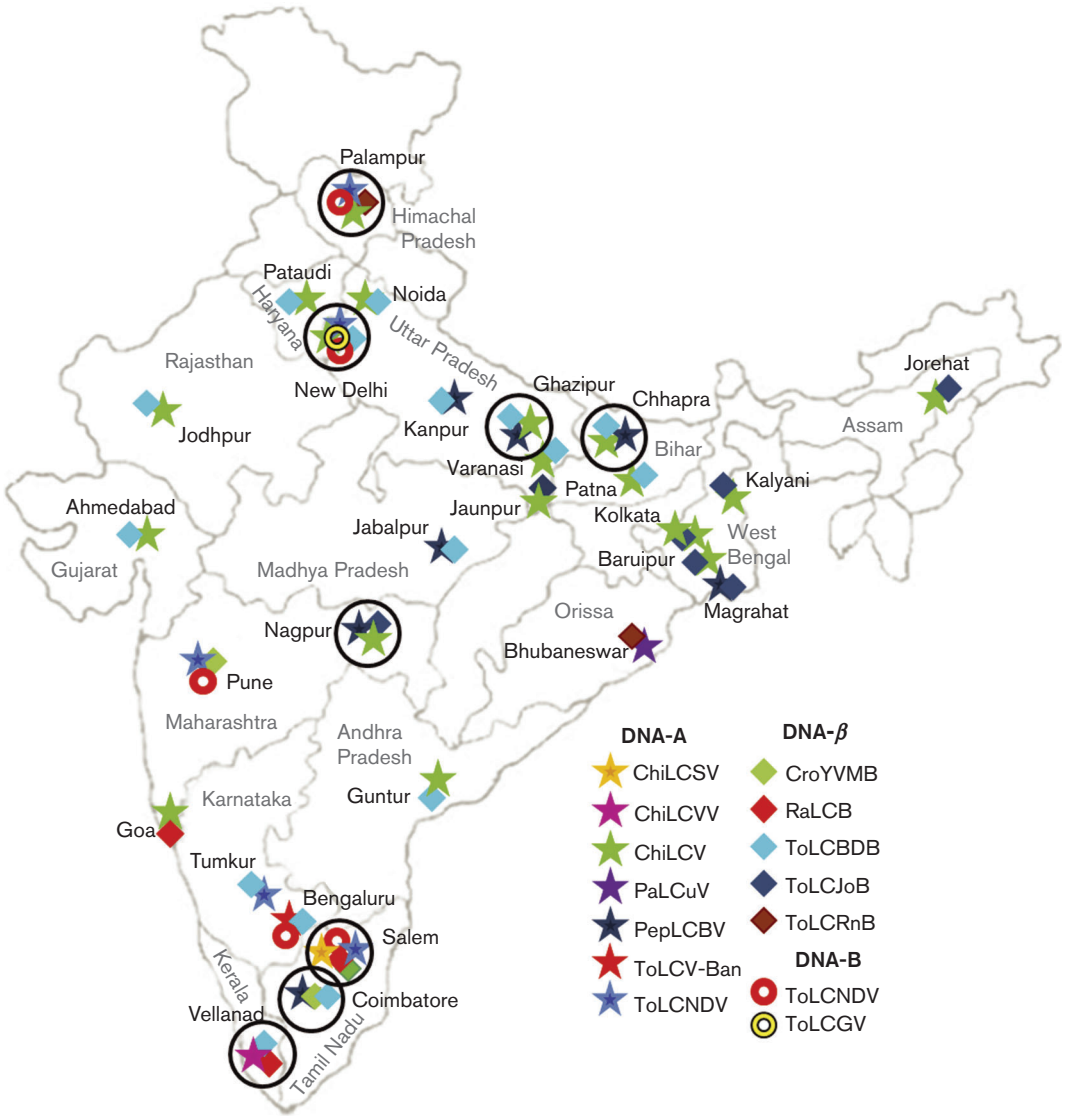

Fig. 3. Distribution of ChiLCD-associated begomoviruses and betasatellites in India identified in this study. Diverse components of DNA-A, DNA-B and betasatellites are depicted in different shapes and colours. Black circles indicate mixed infections.

ChiLCVV could have evolved quite separately after the earlier recombination event(s) and resulted in the present genome organization. These results pinpoint the significant contribution of solanaceous as well as non-solanaceous-infecting begomoviruses in intra- and inter-species recombination leading to the emergence of ChiLCD-associated begomoviruses. Recombination among ChiLCD-associated begomoviruses could provide high evolutionary potential for rapid multiplication, expansion of geographical territory and, more importantly, invasion of new hosts.

Mixed infections of distinct begomoviruses were detected at six locations, viz. Chhapra, Ghazipur, Nagpur, New Delhi, Palampur and Salem (Table 1, Fig. 3). Notably, ChiLCV-PK and PepLCBV-IN have emerged through intra- as well as inter-species recombination. This observation emphasizes that mixed infection, a pre-requisite for recombination, might have facilitated their emergence. Interestingly, New Delhi, Palampur and Salem samples were found to be infected with a monopartite begomovirus (either ChiLCVs or ChiLCSV) and a bipartite begomovirus (ToLCNDV). The presence of a bipartite begomovirus together with a monopartite one could further promote the evolution of severe disease-causing complexes. Possible synergistic interactions, if any, between these monopartite and bipartite begomoviruses in chilli have not been studied so far.

Betasatellites, being important pathogenicity determinants for monopartite begomoviruses, have been the subject of many scientific investigations (Bhattacharyya *et al.*, 2015;

Cui *et al.*, 2005; Li *et al.*, 2014; Saunders *et al.*, 2004; Yang *et al.*, 2011). At the time of sampling, only two betasatellite groups, ToLCBDB and ChiLCB, were known to be associated with ChiLCD in India (Chattopadhyay *et al.*, 2008; Kumar *et al.*, 2011). In India, the most prevalent betasatellites associated with ChiLCD have been reported from tomato, rather than from weeds. Among these, ToLCBDB has co-existed with diverse begomoviruses predominantly in the western part of India, whereas ToLCJoB is mostly located in the eastern and western regions (Fig. 3). In spite of the prevalence of begomoviruses associated with ChiLCD identified from Pakistan (ChiLCV, PepLCBV), their cognate ChiLCB could not be detected here. It is interesting to note the association of betasatellites with both bipartite and monopartite begomoviruses from New Delhi, Palampur, Pune and Salem. Recently, diverse betasatellites have been shown to enhance the accumulation of ToLCNDV DNA-A and antagonistically interact with the ToLCNDV DNA-B component (Jyothsna *et al.*, 2013). However, the significance of this association for the establishment of ChiLCD needs to be explored.

Mixed infection of betasatellites also persists in the southern region of India (Salem, Coimbatore, Vellanad). Both betasatellites from Vellanad (ToLCBDB and RaLCB) could be trans-replicated by ChiLCVV in *N. benthamiana*. However, they could neither influence symptom severity nor reduce the incubation period (Fig. 2, Table 5). This observation is in agreement with earlier reports suggesting that the betasatellites are dispensable for disease development in *N. benthamiana* (Ranjan *et al.*, 2014; Saunders *et al.*, 2000). Chilli plants inoculated with DNA-A-like sequences alone produced mild symptoms; however, the accumulation of viral DNA as well the severity of the symptoms was increased in the presence of cognate betasatellites (Fig. 2, Table 5). This is the first experimental evidence to our knowledge demonstrating the necessity of betasatellites for development of severe leaf curl disease in chilli, thereby explaining their ubiquitous presence with ChiLCD. Furthermore, their dependence on betasatellites for severe disease development supports the notion that these monopartite begomoviruses require betasatellites for ChiLCD, similar to the complexes reported with ageratum yellow vein and cotton leaf curl diseases (Mansoor *et al.*, 2006; Saunders *et al.*, 2000).

Geminiviruses are known to have high rates of nucleotide substitution, similar to those of RNA viruses (Drake, 1993; Duffy & Holmes, 2008, 2009). ChiLCD-associated begomoviruses possess higher genetic variability than observed in other begomoviruses (Melgarejo *et al.*, 2013; Rocha *et al.*, 2013; Silva *et al.*, 2012). As described by Lima *et al.* (2013), the nucleotide diversity in these begomoviruses was noticed to be non-uniformly distributed throughout the genome. Our results emphasize that purifying selection is the major evolutionary determinant leading to the emergence of these begomoviruses and betasatellites, in agreement with other studies (Lima *et al.*, 2013; Rocha *et al.*, 2013; Silva *et al.*, 2012).

In conclusion, the information generated through our studies demonstrates that ChiLCD is caused by a large group of monopartite begomoviruses and a few bipartite begomoviruses in association with diverse betasatellites. Diverse species of begomoviruses and betasatellites were found at every geographical location in India and their evolution is largely driven by intra- and inter-species recombination and by nucleotide substitution. It is evident from the infectivity analysis that the betasatellites are essential for disease development in the chilli host. As chilli-associated begomoviruses and betasatellites are expanding their host range and geographical distribution (George *et al.*, 2014), further epidemiological studies of the impact of the high genetic variability of these viruses are required.

METHODS

Isolation, cloning and sequencing of full-length genomes. An extensive survey was carried out in all major chilli-growing regions across India. For each region, a total of nine samples (from at least three individual fields) were collected from naturally infected chilli plants exhibiting typical symptoms of ChiLCD (Table S1). Total genomic DNA was extracted from all samples (Dellaporta *et al.*, 1983) and subjected to RCA using φ29 DNA polymerase (Inoue-Nagata *et al.*, 2004). Association of begomovirus with these symptomatic samples was verified by PCR using abutting primers specific for DNA-A and DNA-B components (Rojas *et al.*, 1993; Wyatt & Brown, 1996). RCA products of all the samples were digested with single-cutting restriction endonucleases, viz. *Bam*HI, *Kpn*I, *Hin*dIII or *Pst*I, and cloned into pBluescript II KS (+) vector (Stratagene), previously linearized with the respective restriction enzymes. Simultaneously, PCR with primers specific for DNA-A and DNA-B (George *et al.*, 2014) and betasatellite (Briddon *et al.*, 2002) were used to amplify the full-length genomic components. Details of the primers used to clone viral components are provided in Table S3. PCR products of either ~2.8 kb or ~1.3 kb in size were cloned into the pTZ57R/T vector (Fermentas). All the recombinant clones obtained from each of the samples were digested with various restriction enzymes and were sequenced partially (using M13 primers) to identify the variability among them, if any. On the basis of restriction variability and partial sequencing of the viral genome, one of the representative cloned viral genomes was sequenced commercially at the University of Delhi South Campus.

Phylogenetic and recombination analysis. Using the CLUSTAL W method in SDT v. 1.0 (Muhire *et al.*, 2014) and MEGA5 (Tamura *et al.*, 2011), neighbour-joining phylogenetic dendrograms and percentage pairwise identity of the identified sequences and the representative sequences from the database were generated. For RDP analysis, the begomoviruses were selected according to the criteria followed by George *et al.* (2015) and the sequence accession numbers are listed in Table S4. Recombination breakpoints and their putative parental sequences were detected by the RDP3 program with the Bonferroni-corrected *P* value cut-off of 0.01 as described by George *et al.* (2015) and Martin *et al.* (2010).

Estimation of population structure and nucleotide substitution rates. To assess the genetic variability in the virus populations, parameters in DnaSP v. 5.10 used were total number of segregating sites (s), total number of mutations (η), average number of nucleotide differences between sequences (k), nucleotide diversity (π), Watterson's estimate of the population mutation rate based on the total number of segregating sites ($\theta - w$) and the total number of mutations ($\theta - \eta$). Begomovirus population structure was studied using STRUCTURE v. 2.3.3 as described by Prasanna *et al.* (2010) and Pritchard

et al. (2000). The number of subpopulations (*K* value) was estimated by using 100 000 Markov chain Monte Carlo steps after a burn-in period of 100 000 steps. The best-supported *K* values were determined based on ln*P*(*D*) after three independent runs.

Neutrality tests, Tajima's *D*, and Fu and Li's D^* and F^*, were employed to test the hypothesis of what type of selection was occurring in the ORFs encoded by the viral components (Rozas *et al.*, 2003). The detection of sites evolved under positive and negative selection was performed by the methods implemented in the Data-Monkey server (http://www.datamonkey.org): SLAC, FEL and REL (Kosakovsky Pond & Frost, 2005). Bayes factors >50 or *P* value <0.1 were used as thresholds for the analysis. NEXUS files of AV1 and βC1 regions were generated using CLUSTAL W in MEGA5, and nucleotide substitution rate was estimated using the GTR nucleotide substitution model, uncorrelated lognormal relaxed clock model and BSP co-alescence model in the BEAST v. 1.7 package (Drummond *et al.*, 2012; Nawaz-ul-Rehman *et al.*, 2012). The begomovirus and betasatellite sequences used to estimate population structure and nucleotide substitution rate are provided in Table S5.

Generation of infectious clones. For infectivity analysis, newly identified begomoviruses and their associated betasatellites were cloned as partial tandem repeats in plant transformation vectors. For PepLCBV-IN (Nagpur isolate), the *Eco*RI (1653)–*Bam*HI (155) fragment was cloned into pCAMBIA1300, followed by the mobilization of the monomer to generate a partial tandem repeat, designated NgA. For ChiLCVV, a 2.4 kb *Sal*I (1856)–*Kpn*I (1620) fragment containing the CR was cloned into pCAMBIA2301. The full-length monomer linearized with *Kpn*I was then ligated to generate the ChiLCVV tandem repeat construct (designated VeA). For ToLCBDB- and RaLCB-Vellanad, 1.2 kb (*Bam*HI and *Kpn*I) and 0.4 kb (*Eco*RI and *Kpn*I) fragments were cloned into pCAMBIA1300, followed by ligation of the respective monomer to yield partial tandem repeats of ToLCBDB- and RaLCB-Vellanad (referred as Veβ1 and Veβ2, respectively). For ToLCJoB-Nagpur, the 470 bp *Kpn*I (1297)–*Bam*HI (411) fragment was cloned in pCAMBIA1300, followed by insertion of the full-length *Kpn*I fragment to produce the tandem repeat of the ToLCJoB-Nagpur (as Ngβ). Similarly, partial tandem repeat constructs of ChiLCV-PK (ChA-EF190217), PepLCBV-BD (MgA-HM007111), ToLCBDB (Chβ-EF190215), ToLCJoB (Mgβ-HM007112, Joβ-EF190216) and ChiLCV (JoA-EF194765) were also generated and the cloning details are shown in Table S6.

Plant inoculation and Southern blot hybridization. Infectivity of the cloned components was analysed in *N. benthamiana* plants through the *Agrobacterium*-mediated inoculation method (Singh *et al.*, 2012). *C. annuum* var. Sulekha plants were bombarded using a HELIOS Gene Gun apparatus (Bio-Rad) following the manufacturer's instructions. Twenty-five milligrams of gold microcarriers (0.6 μm diameter) were coated with plasmid mixture (3 μg mg^{-1}) of each of these infectious clones and delivered at 300 p.s.i. (pounds per square inch). These plants were maintained in an insect-free growth chamber to monitor the symptom induction. Total genomic DNA (10 μg) from the systemic leaves was resolved on an agarose gel (0.8 %) and transferred onto nylon membrane (Singh *et al.*, 2012). The blots were hybridized separately with the [α^{32}P]dCTP-radiolabelled AV1 region of DNA-A or the monomer of DNA-β specific to the species concerned and the viral DNA was detected using a phosphor image analyser.

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ORIGINAL ARTICLE

Differential response of diverse solanaceous hosts to tomato leaf curl New Delhi virus infection indicates coordinated action of NBS-LRR and RNAi-mediated host defense

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Abstract Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite begomovirus (family *Geminiviridae*) that infects a wide range of plants. ToLCNDV has emerged as an important pathogen and a serious threat to tomato production in India. A comparative and molecular analysis of ToLCNDV pathogenesis was performed on diverse solanaceous hosts (*Capsicum annuum*, *Nicotiana benthamiana, N. tabacum*, and *Solanum lycopersicum*). *N. benthamiana* was found to be the most susceptible host, whereas *C. annuum* showed resistance against an isolate of ToLCNDV collected in New Delhi from tomato (GenBank accession no. U15015 and U15017). *S. lycopersicum* and *N. tabacum* developed conspicuous symptoms and allowed virus to accumulate to significantly high titers. The viral DNA level was concurrent with symptom severity. ToLCNDV-specific siRNA levels were directly proportional to the amount of viral DNA. To investigate the basis for the differences in response of these hosts to ToLCNDV, a comparative expression analysis of selected defense-related genes was carried out. The results indicated differences in expression levels of genes involved in the posttranscriptional gene silencing machinery (RDR6, AGO1 and SGS3) as well as basal host defense responses (nucleotide-binding site and leucine-rich repeat [NBS-LRR] proteins and lipid transfer protein [LTP]). Among these, expression of NBS-LRR genes was found to be significantly higher in *C. annuum* following ToLCNDV infection. Our analyses suggest that the expression of host defense responses determines the level of ToLCNDV accumulation and degree of symptom development.

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Introduction

Geminiviridae is the largest plant virus family, which currently includes 325 species (www.ictvonline.org/virusTaxonomy). Members of this family collectively infect a wide variety of plant hosts. Members of the genus *Begomovirus,* the largest genus of the family, are transmitted by whiteflies (*Bemisia tabaci*) and possess either monopartite or bipartite genomes. Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite begomovirus that, like other geminiviruses, contains a circular, single-stranded DNA genome and replicates via double-stranded DNA intermediates in the nucleus of the host cell [17, 34]. Although ToLCNDV was initially isolated and characterized from tomato, this virus can infect other hosts as well [19, 21]. However, symptom severity might vary from one host to another and even between different genotypes of a particular host. The genetic and biological diversity among ToLCNDV isolates can also result in differences in virus-host interactions. ToLCNDV has emerged as a major constraint to crop production in India [4]. Several studies have been performed to understand geminivirus pathogenesis and to identify host factors for developing an antiviral defense strategy [7, 45, 46]. However, an analysis of host-specific responses at the molecular level for plants of various species inoculated with ToLCNDV has not yet been performed.

Induction of symptoms on a susceptible host is the outcome of expression of specific sets of genes and dynamic interactions between host- and pathogen-encoded proteins [18, 30, 31]. In most cases, a susceptible host provides all necessary factors required for virus replication, transcription

and movement. In a non-permissive host, viruses cannot survive due to the lack of a favourable cellular environment and incompatible interactions between host and viral factors that restrict viral multiplication and spread [9].

Plants have evolved several specific and nonspecific defense mechanisms. Nonspecific defense responses usually involve production of thionin, ascorbate peroxidase, polyphenol oxidase, salicylic acid, jasmonic acid, ethylene, and lipid transfer proteins [30]. Specific defense machineries are also activated against pathogen invasion [32]. R-gene- and RNAi-mediated defense machineries are two extensively studied responses in plants against viral infection. Several R genes have been identified and characterized with respect to their role in defense against bacterial and viral pathogens, and most of them contain nucleotide binding site and leucine-rich repeat (NBS-LRR) domains. Lipid transfer protein (LTP) is also known to be upregulated following pathogen attack and is involved in imparting basal defense [42]. Posttranscriptional and transcriptional gene silencing (PTGS and TGS) are endogenous antiviral defenses operating in plants [35]. Due to constant pressure exerted by hosts, viruses have evolved to encode multifunctional proteins that play key roles in regulating their life cycle as well as suppressing host defenses. Viral suppressors of RNAi have been identified and characterized from several families of plant viruses [20]. AC1, AC2, AC4 and AV2 of geminiviruses, 2b of cucumber mosaic viruses (CMV), p19 of tombusviruses and HC-Pro of potyviruses are some well-characterized examples of viral suppressor proteins [26, 37, 38].

In the present study, we aimed to explore the molecular mechanism of pathogenesis and host defense responses against a ToLCNDV isolate collected in New Delhi from tomato [34] in four different solanaceous hosts (*Capsicum annuum, Nicotiana benthamiana, Nicotiana tabacum and Solanum lycopersicum*). Our study indicated host-specific roles of both innate and acquired resistant factors against ToLCNDV.

Materials and methods

Plant growth

N. benthamiana, *S. lycopersicum* cv. Punjab Chhuhara, *N. tabacum* cv. Xanthi and *C. annuum* cv. Punjab Lal plants were grown in an insect-proof glass house with a 16/8 h (light/dark) photoperiod and relative humidity of 60 %.

Plant inoculation

Infectious clones (tandem repeat constructs) of ToLCNDV DNA-A (GenBank accession no. U15015) and DNA-B (U15017) [34] available in our laboratory were used to transform *Agrobacterium tumefaciens* strain EHA105 by a freeze-thaw method [41]. *Agrobacterium* cells harboring ToLCNDV infectious constructs were grown for 36 h on selection media supplemented with kanamycin (50 μg/ml) and rifampicin (30 μg/ml). The culture was then centrifuged at 5000 rpm, and the pellet was resuspended in inoculation buffer (100 μM acetosyringone, 10 mM $MgCl_2$ and 10 mM MES). Petioles of test plants at the 4- to 5-leaf stage were pierced, and 20 μl of bacterial suspension containing both DNA-A and DNA-B was applied. Inoculated plants were maintained in an insect-proof glasshouse until 30 days post-inoculation (dpi) for assessing symptom severity. In each case, four plants were inoculated following standard procedures [5, 23] and the experiment was repeated three times. Mock-infected plants were inoculated with *A. tumefaciens* strain EHA105 carrying the empty vector pCAMBIA2300.

Analysis of symptom severity

Symptom development on inoculated plants was recorded as described by Chakraborty et al. [4]. Severity of symptoms was scored based on phenotypic expression, which ranged from no visible symptoms (0) to severe symptoms (5).

Total plant DNA isolation

Total plant DNA was isolated from either virus- or mock-inoculated plants of the four species according to Dellaporta et al. [10] with some modifications. DNA was isolated from various plant parts, including uninoculated uppermost leaves, stems above the inoculation level, and root pieces, at 21 days post-inoculation (dpi). Plant samples were homogenized in extraction buffer (100 mM Tris-Cl, pH 8.0, 50 mM EDTA, pH 8.0, 200 mM NaCl, 0.5 % β-mercaptoethanol). Subsequently, sodium dodecyl sulphate (SDS, 1 %) was added, followed by incubation of the samples at 65 °C for 15 min. Subsequently, 332 μl of 5 M potassium acetate was added and samples were centrifuged at 13,000 rpm for 10 min. The supernatant was mixed with chloroform:isoamyl alcohol solution (24:1), followed by centrifugation at 13,000 rpm for 15 min. Total DNA was precipitated with 0.8 volumes of isopropanol, and the DNA pellet was washed three times with 70 % ethanol. The pellet was air dried and dissolved in 500 μl of sterile water. DNA was quantified using a UV visible spectrophotometer (GE Healthcare). Three biological replicates and two technical replicates were taken for the experiment.

Southern hybridization and quantification of the viral DNA

Total DNA (8 μg) was separated on 0.8 % agarose gels in the presence of ethidium bromide and transferred to nylon membranes. Viral DNA was detected by hybridizing blots using either AC1- or BC1-specific radiolabelled probes for detection of DNA A and DNA B, respectively, according to Chakraborty et al. [4]. Virus-specific bands were detected using a phosphorimager analysis system (Typhoon, Amersham, GE Healthcare).

Isolation and detection of siRNAs

Total RNA was isolated from the two uppermost leaves (1 g) using TRI Reagent (Sigma, USA) following the manufacturer's protocol. Enrichment of low-molecular-weight RNAs was performed using 5 % polyethylene glycol and 0.5 M NaCl following the procedure of Lu et al. [28], and siRNAs were separated on 15 % tris-borate-EDTA-urea acrylamide gels and transferred to Hybond-N^+ membranes (Amersham, GE Healthcare) using a semi-dry electroblotter (Amersham, GE Healthcare). For detection of virus-specific siRNAs, α-P^{32} labeled DNA probes of the overlapping region between AC2/AC3 of ToLCNDV (nt 1148-1701) were used. Hybridization was carried out in hybridization buffer containing 0.1 % SDS, 5X SSC, 50 mM sodium phosphate (pH 6.8), 1x Denhardt's solution and 100 μg/ml herring sperm DNA at 40 °C overnight. Washing was performed twice with 0.1 % SDS and 2X SSC at room temperature for 20 min each. ToLCNDV-specific siRNAs were detected using a phosphorimager analysis system.

Detection of viral DNA by PCR

PCR was carried out using ToLCNDV AC1-specific primers (FP 5′-ATG GCT CCG CCA CGT CGT TTC AG-3′ and RP 5′-TCA ACT CGC CTC CTG CGA ATG CTC TTC-3′) and 200 ng of plant DNA as template. The programme included an initial denaturation step at 94 °C for 4 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min 20 s. The samples were resolved on 1 % agarose gels containing ethidium bromide.

qRT-PCR

Total RNA (1 μg) was used to prepare cDNA from mock- and virus-inoculated plants. The two uppermost inoculated leaves from three different plants were analyzed separately to study host gene expression. Only plants that tested positive for the presence of viral DNA by PCR were considered for qRT-PCR analysis. SYBR Green (1X) master mix (Applied Biosystems, USA) and 2 μl of diluted cDNA (1:10) were used for qRT-PCR. In one of our earlier experiments, enhanced levels of NBS-LRR and LTP transcripts were detected in chilli plants inoculated with the monopartite begomovirus chilli leaf curl virus [24]. We therefore selected these two genes for our study. Sequences of RNA-dependent RNA polymerase 6 (RDR6) (*Nicotiana tabacum*, GenBank accession number FJ966891), Argonaute 1 (AGO1) (*Nicotiana tabacum*, AB542739), Suppressor of Gene Silencing 3 (SGS3) (*Solanum lycopersicum*, NM_001247782.1), NBS-LRR (*Capsicum annuum*, JK523032), and LTP (*Capsicum annuum*, JK523040) were retrieved from the NCBI database. The following primer pairs were used for host gene expression: SGS3, 5'-AGA TGT GGG TGA AAT GAG TTA TGA GA-3' and 5'-GCC TGG ACC ACC TTT GCA T-3'; RDR6, 5'-AGT GGA GCC ATT GGT AGG TGT AA-3' and 5-CTG ACA TGG ACA CCC CAA AAT-3'; AGO1, 5'-CCG CAT CAG CCT GTA CCA TA-3' and 5'-GGT TGT ATT GCT TGG GTT GCA-3'; NBS-LRR, 5'-G GAT CCT AAT GGG AGA TGC AAT CAT GGA-3' and 5'-GTC GAC TCA GTG CAT GTC ATC AAA TTC TC-3'; LTP, 5'-CGG CCG AGG TCA ACT ACG T-3' and 5'-GAC GAT CAG GAG TTG TGG TAG CT-3'. Actin sequences of *N. benthamiana* (Niben.v0.3.Ctg25058627), *C. annuum* (CA11g11900), *S. lycoperiscum* (SL1.03sc00395) and *N. tabacum* (EU938079.1) were retrieved from Sol Genomics Network (http://solgenomics.net) and NCBI. A conserved primer pair (5'-GAA GCT CAA TCC AAA CGT GGT ATT-3' and 5'-CTC AAA CAT GAT TTG TGT CAT C-3') was designed. The following programme was used: initial denaturation at 94 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. PCR amplification of the actin gene was performed as follows: initial denaturation at 94 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s and extension at 72 °C for 45 s. qRT-PCR was performed on an Applied Biosystems qRT 7500 thermocycler. For relative quantification analysis, ΔCt values were normalized to an internal control (actin), and the obtained ΔΔCt was used to plot graphs using PRIZM software. Student's *t*-test was performed to compare the relative expression levels.

Results

Differential infectivity of ToLCNDV

The results of agroinoculation of ToLCNDV onto three solanaceous hosts (*N. benthamiana*, *N. tabacum* and

S. lycopersicum) revealed distinct dynamics of leaf curl disease development, while *C. annuum* failed to exhibit any visible symptoms. *N. benthamiana* exhibited the most severe symptoms, while the other two hosts (*N. tabacum* and *S. lycopersicum*) showed a less severe but clearly visible phenotype (Fig. 1A-D). The earliest symptoms appeared on *N. benthamiana* (5 dpi), followed by *N. tabacum* (8 dpi) and *S. lycopersicum* (10 dpi) (Fig. 1E). Notably, none of the *C. annuum* plants inoculated with ToLCNDV produced any visible symptoms (Fig. 1C). *N. benthamiana* plants showed leaf curling, stunting, yellowing of leaf lamina, small leaves, and chlorosis and reached maximum symptom severity (grade 5) by 16-17 dpi (Fig. 1D and E; Table 1). *N. tabacum* plants showed severe leaf curling, vein banding, stunting, yellowing, chlorosis and crumpling of leaf lamina, which gradually became severe (grade 4) by 16 dpi (Fig. 1A and E; Table 1). Tomato plants exhibited symptoms of downward leaf curling, stunting and yellowing, and the plants attained maximum symptom severity (grade 4) at 22 dpi (Fig. 1B and E; Table 1).

Differential accumulation of ToLCNDV in plant parts

A comparison of the accumulation of ToLCNDV DNA-A and DNA-B in inoculated plants was done by Southern blotting using AC1 (replication-associated protein)- and BC1 (movement protein)-specific probes, respectively, at

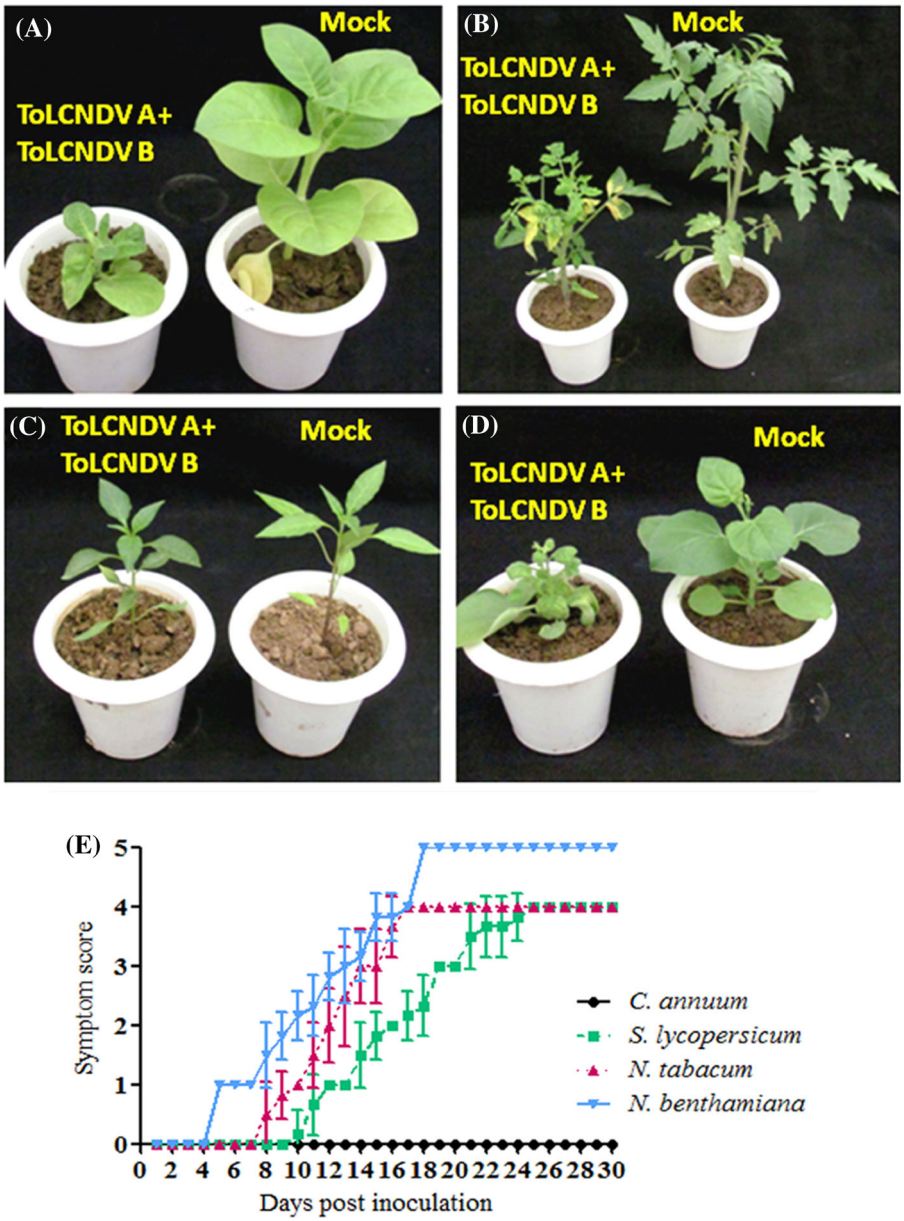

Fig. 1 Disease reaction on ToLCNDV-inoculated solanaceous hosts at 21 days post-inoculation (dpi) (**A**) *N. tabacum*, (**B**) *S. lycopersicum*, (**C**) *C. annuum,* (**D**) *N. benthamiana* (**E**) Time course of symptom severity

Table 1 Infectivity of ToLCNDV in four solanaceous hosts

Host	No. of symptomatic plants/total no. of inoculated plants	Days to first symptom appearance (dpi)	Symptom severity at 30 dpi**	Symptoms	Detection of viral DNA by Southern blotting (21 dpi)#			PCR detection of viral DNA (21 dpi)#			ToLCNDV-infected leaf samples/number of samples analyzed	
					Leaves	Stem	Roots	Leaves	Stem	Roots	By PCR	By Southern hybridization
C. annuum	0/12	None	0	No visible symptoms observed until 30 dpi	No	No	No	Yes*	No	No	8/12	0/12
S. lycopersicum	11/12	10	4	Leaf curling, stunting, yellowing and leaf crinkling	Yes	Yes	No	Yes	Yes	Yes*	11/12	11/12
N. benthamiana	12/12	5	5	Leaf curling, stunting, yellowing of leaf lamina, small leaves, chlorosis and mottling	Yes	Yes	Yes	Yes	Yes	Yes	12/12	12/12
N. tabacum	10/12	8	4	Severe leaf curling, vein banding, stunting, yellowing, chlorosis and crumpling of leaf lamina	Yes	Yes	Yes*	Yes	Yes	Yes*	10/12	10/12

'Yes' indicates amplification/accumulation, * indicates low amplification/accumulation, and 'No' indicates no amplification/accumulation. dpi, days post-inoculation

** Symptom severity (0-5 scale) was measured according to Chakraborty et al. [4]

For DNA isolation, uninoculated uppermost leaves, stem above the inoculation level, and root pieces were used

21 dpi. Results from one representative mock- and virus-inoculated plant per host are shown in Figure 2. The viral titers were similar in the leaves of *N. benthamiana*, *N. tabacum* and *S. lycopersicum*, but we failed to detect viral DNA in the leaves of *C. annuum* (Fig. 2A and D). Furthermore, we detected a higher accumulation of viral DNA in stems of *N. benthamiana* compared to *N. tabacum* and *S. lycopersicum* (Fig. 2B and E). We could not detect viral DNA in *C. annuum* stems (Fig. 2B and E). In roots, a considerably higher level of ToLCNDV DNA was found in *N. benthamiana*, and a lower level in *N. tabacum* (Fig. 2C and F). Significantly less accumulation of viral DNA was found in the roots of inoculated *S. lycopersicum* plants (Fig. 2C and F).

PCR-based detection using virus-specific primers was employed to detect viral DNA in all four solanaceous hosts (Fig. 3). Contrary to the results of Southern analysis, the presence of viral DNA could be ascertained in *C. annuum* leaves, albeit at a low level. However, we could not detect the presence of ToLCNDV in stem and roots of *C. annuum*. In agreement with the Southern hybridization results, we detected viral DNA in leaves, stem and roots of *N. benthamiana*, *N. tabacum* and *S. lycopersicum* (Fig. 3).

ToLCNDV-specific siRNA biogenesis

Although geminiviruses are DNA viruses, they are targets of transcriptional and posttranscriptional gene silencing machineries of the host. To investigate the possible role of the gene silencing pathway in ToLCNDV-mediated symptom induction, ToLCNDV-specific siRNAs were isolated from leaves, stems and roots of all four solanaceous hosts. The maximum level of ToLCNDV siRNA accumulation was observed in leaves. *N. benthamiana* and *S. lycopersicum* leaves accumulated equivalently high levels of ToLCNDV siRNAs, whereas *N. tabacum* accumulated slightly lower amounts (Fig. 4A). *N. benthamiana* plants accumulated significantly higher amounts of ToLCNDV siRNAs in the stem compared to *N. tabacum* and *S. lycopersicum* (Fig. 4B). We could not detect ToLCNDV-specific siRNAs in the leaves and stems of *C. annuum*. Comparative analysis of siRNA accumulation in roots indicated very low levels in *N. benthamiana*. ToLCNDV siRNAs could not be detected in the roots of *C. annuum, N. tabacum* and *S. lycopersicum* (Fig. 4C).

Differential expression of selected defense-related genes

A comparative analysis of expression profiles and patterns from specific (NBS-LRR and RNAi) and nonspecific (LTP) defense-related genes was performed in all four hosts in response to ToLCNDV infection. Activation of PTGS

requires concomitant expression of several host factors. The expression profile of the SGS3 gene indicated no changes in *C. annuum* and *N. benthamiana* but a significantly elevated level in *S. lycopersicum* ($P \geq 0.001$) (Fig. 5A, B and C). *N. tabacum* showed a 7-fold increase of SGS3 transcripts in the inoculated plants compared to the mock-inoculated control ($P \geq 0.001$) (Fig. 5D). RDR6 is another important component of the PTGS pathway and is required for biogenesis of secondary siRNAs. No significant changes in the RDR6 transcript level were observed in *C. annuum, S. lycopersicum* and *N. tabacum*, whereas in *N. benthamiana*, RDR6 expression was reduced ($P = 0.009$) (Fig. 5A-D). In the solanaceous hosts, the expression profile of AGO1 revealed no significant changes in expression in inoculated plants when compared to mock-infected plants, except in *S. lycopersicum* ($P = 0.001$) (Fig. 5A-D).

Geminiviruses are also known to induce a specific defense response, and earlier studies indicated upregulation of NBS-LRR genes [24, 29]. NBS-LRR-mediated defense mechanisms are involved in specific recognition of pathogen effectors and activate downstream defense signaling pathways to inhibit spread and multiplication of the pathogen. Our analysis indicated upregulation of an NBS-LRR gene in *C. annuum* following ToLCNDV infection. The qRT-PCR analysis revealed >60-fold upregulation of NBS-LRR gene expression in inoculated leaves of *C. annuum* ($P = 0.001$), whereas 5-fold upregulation was

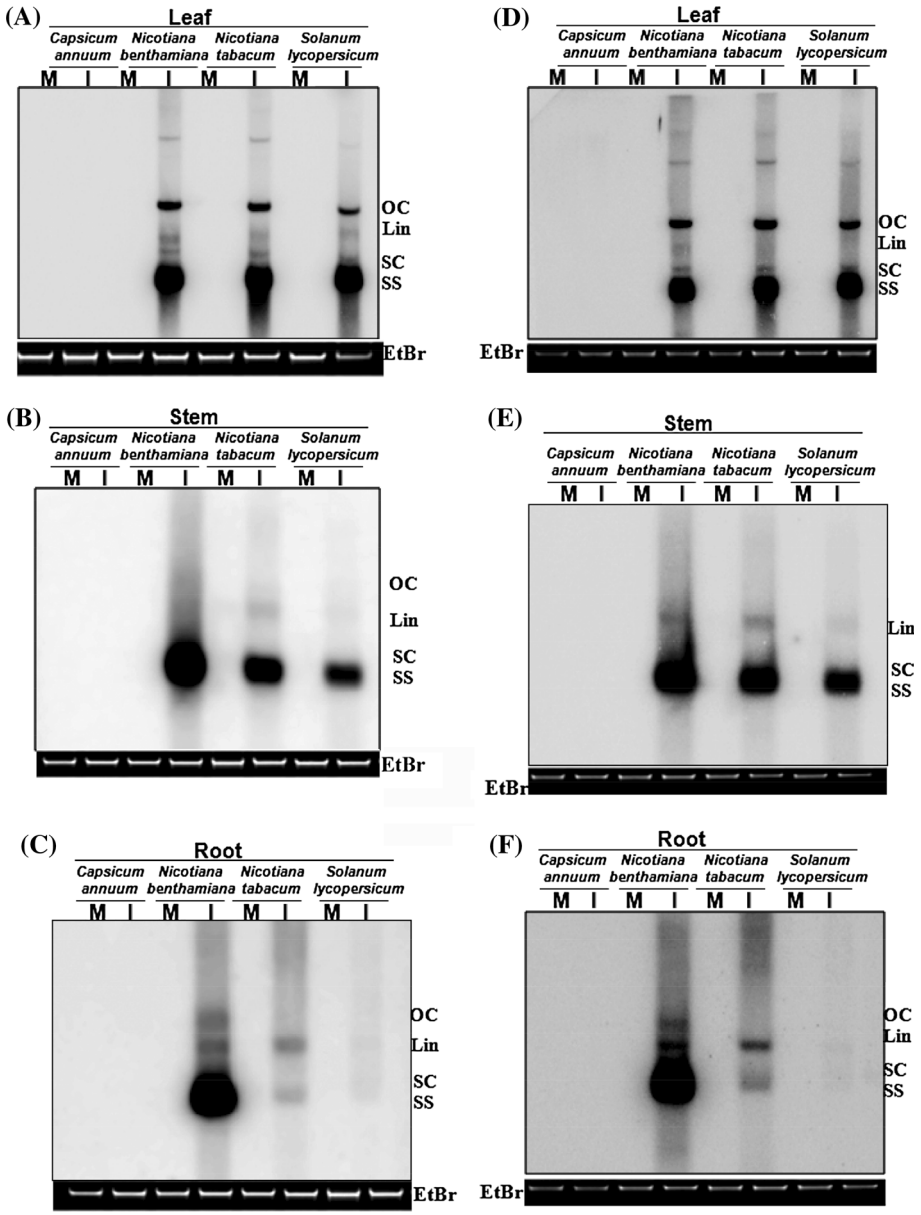

Fig. 2 Comparative levels of ToLCNDV DNA accumulation in solanaceous hosts. Total DNA was isolated from uninoculated uppermost leaves, the stem above the inoculation level and root pieces at 21 dpi. Detection of viral DNA in each host is indicated using an AC1-specific probe in (**A**) leaf, (**B**) stem and (**C**) root and a BC1-specific probe in (**D**) leaves, (**E**) stem, (**F**) root. Ethidium bromide (EtBr)-stained total DNA served as a loading control. Viral replicative forms are indicated as OC, double stranded open circular; Lin, double stranded linear; SC, supercoiled; SS, single stranded. 'M' indicates mock-, and 'I' indicates ToLCNDV-inoculated plants

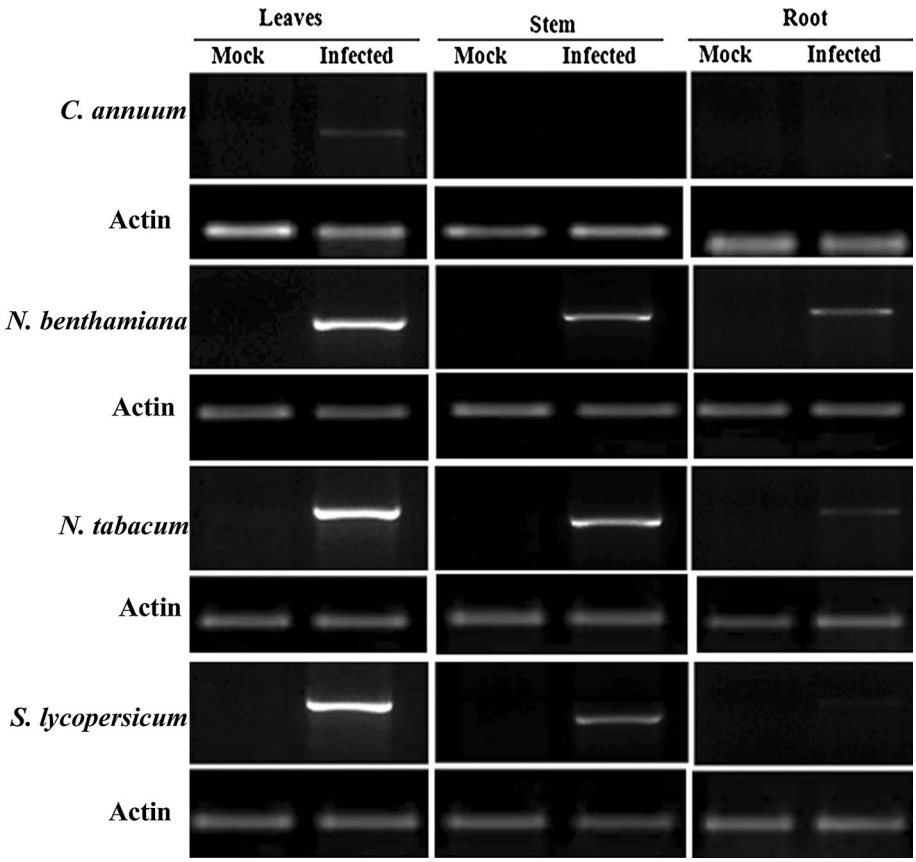

Fig. 3 Detection of viral DNA in different plants parts of solanaceous hosts by PCR at 21 dpi. AC1-specific primers were used to detect the presence of viral DNA in uninoculated uppermost leaves, the stem above the inoculation point, and roots. Actin served as an internal control

observed in inoculated leaves of *S. lycopersicum* ($P \geq 0.001$) (Fig. 5A and B). Contrary to the above, inoculated *N. benthamiana* plants exhibited an approximately 9-fold reduced level of NBS-LRR expression compared to the mock-inoculated plants ($P \geq 0.001$), whereas *N. tabacum* did not show any noticeable change in expression of NBS-LRR (Fig. 5C and D).

Following ToLCNDV infection, LTP expression was significantly elevated in the leaves of *C. annuum* (>15-fold) (Fig. 5A), whereas LTP transcript accumulation was drastically reduced in *S. lycopersicum* ($P \geq 0.001$) (Fig. 5B). Notably, in *N. benthamiana* and *N. tabacum*, a significant reduction of LTP expression was observed ($P = 0.008$ and $P \geq 0.001$, respectively) (Fig. 5C and D).

Discussion

Geminiviruses are ssDNA viruses with a great potential for recombination that are capable of manipulating and efficiently exploiting diverse systems of the host cell for pathogenesis [16, 17, 33, 39]. Nevertheless, the ability of geminiviruses to infect plants of a particular species varies depending on the genetic composition of the host and the capacity of the virus to utilize cellular factors [8, 12, 25]. Tomato leaf curl New Delhi virus is a bipartite begomovirus that has emerged as a severe threat to tomato production in India [4, 34]. Several studies have been carried out to explore resistant mechanisms and to identify host factors to develop efficient antiviral strategies against ToLCNDV and other begomoviruses [27, 40]. However, these studies were based on a single host-virus interaction, whereas most tomato-infecting begomoviruses are known to infect plants of several species [19, 21, 22]. Therefore, plant-virus interactions in a particular host may not provide precise information for developing broad-spectrum resistance to tomato-infecting begomoviruses. The present study on ToLCNDV pathogenesis and interaction in four solanaceous hosts (*C. annuum, N. benthamiana*, *N. tabacum* and *S. lycopersicum*), has generated information that may be exploited to develop antiviral strategies in different hosts.

N. benthamiana has been reported to be a universally susceptible host to most plant viruses [15]. Previous reports and our results have also shown *N. benthamiana* to be a permissive host to geminiviruses, as evidenced by the enhanced level of viral DNA accumulation in all plant parts. Although the precise molecular mechanism of susceptibility of *N. benthamiana* to virus infection is yet to be determined, some efforts have been undertaken to solve

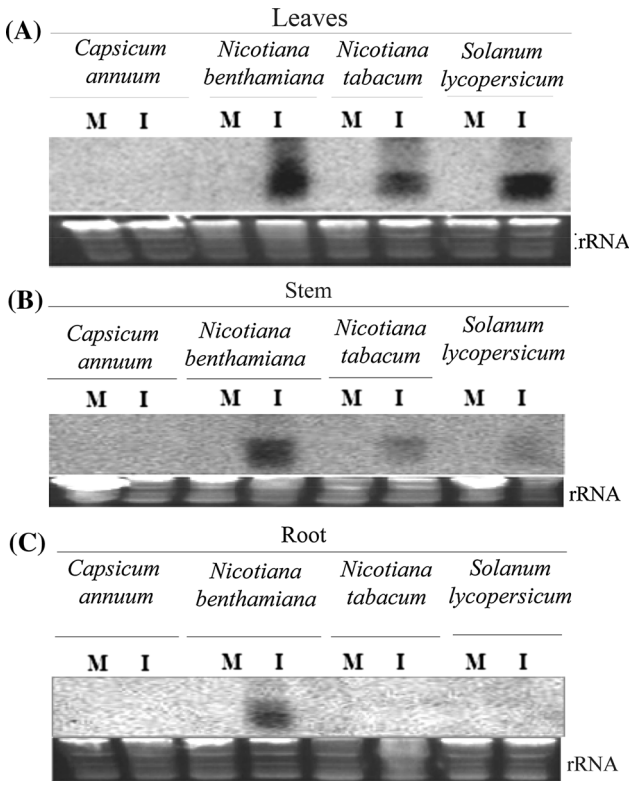

Fig. 4 Detection of ToLCNDV-specific siRNAs in distinct parts of different solanaceous hosts at 21 dpi. The AC2/AC3 overlapping region was used as a probe to detect virus-specific siRNA in (**A**) uninoculated uppermost leaves, (**B**) the stem above the inoculation point and (**C**) roots. 'M' indicates mock- and 'I' indicates ToLCNDV-inoculated plants. Ethidium bromide (EtBr)-stained rRNAs served as a loading control

this enigma. A study by Yang et al. [49] suggested that the enhanced susceptibility to virus infection is at least partly due to a natural mutation in an RNA-dependent RNA polymerase gene (NbRdR1m). RDR1 is one of the crucial players involved in the PTGS-mediated defense mechanism [49, 50]. Tobacco plants expressing the RDR1 gene from *Medicago truncatula* displayed enhanced resistance to virus infection [48]. Unlike *N. benthamiana*, *C. annuum* did not support viral DNA accumulation to a level that could be detected by Southern analysis. *N. tabacum* and *S. lycopersicum* plants displayed intermediate levels of ToLCNDV DNA accumulation.

Geminiviruses are known to encode multiple viral suppressors of RNA silencing [2, 44]. Tomato golden mosaic virus (TGMV) AC2 suppresses TGS by reduction of cytosine methylation [3]. AC4 encoded by cassava-infecting bipartite begomoviruses suppresses PTGS by binding directly with either siRNA or miRNA [6]. Moreover, AV2 interacts with and outcompetes SGS3 for substrate dsRNA recognition [11, 14]. Interaction between viral suppressors and host proteins is largely responsible for determining viral pathogenesis in the host. Nevertheless, the strength of geminivirus-encoded suppressors of RNA silencing may vary from one viral strain/species to another [2, 45]. ToLCNDV might have exploited the transcription machinery of *N. benthamiana* most efficiently, generating viral transcripts that were eventually degraded by the host PTGS machinery. Both *N. tabacum* and *S. lycopersicum* produced a considerable amount of ToLCNDV-specific

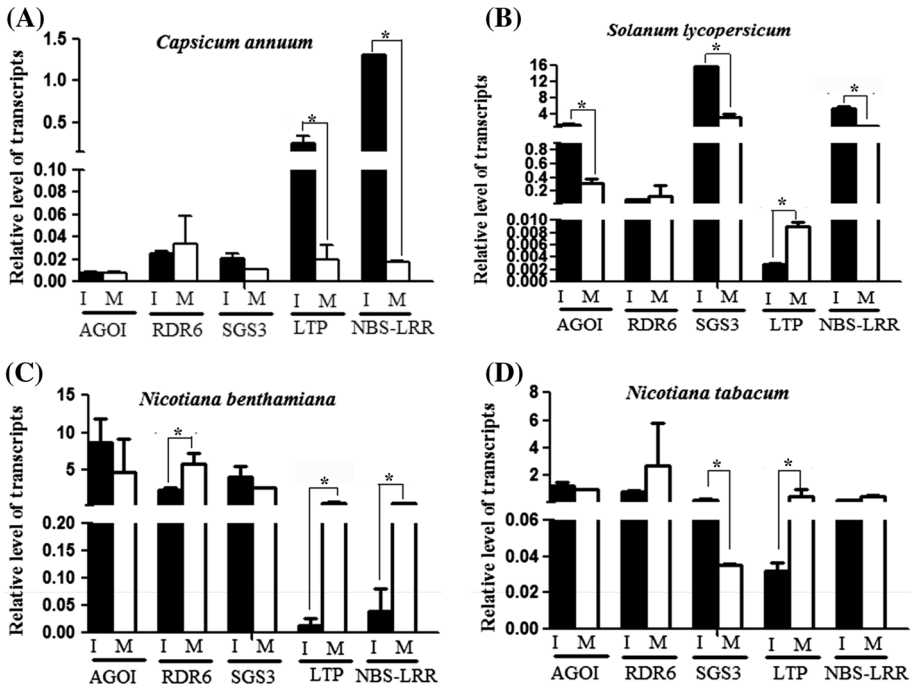

Fig. 5 Comparative analysis of host gene expression in different ToLCNDV-inoculated solanaceous hosts at 21 dpi. Relative level of transcript accumulation of AGOI, RDR6, SGS3, LTP and NBS-LRR in the leaves of (**A**) *C. annuum*, (**B**) *S. lycopersicum*, (**C**) *N. benthamiana* and (**D**) *N. tabacum*. 'I' denotes ToLCNDV-inoculated plants, and 'M' denotes mock-inoculated plants. '*' indicates a significant difference in the expression level of the respective transcript, as determined using Student's t-test

siRNA. On the other hand, *C. annuum* generated very little ToLCNDV-specific siRNA, which could not be detected in Northern blot analysis. This is likely related to the low level of viral DNA accumulation in this non-permissive host. Therefore, reduced accumulation of viral DNA and ToLCNDV-derived siRNAs in *C. annuum*, *S. lycopersicum* and *N. tabacum* compared to *N. benthamiana* indicates inefficient interactions with host factors from those three hosts.

Differential expression levels of selected defense-related genes highlights the variable response of solanaceous hosts following ToLCNDV infection. In *C. annuum*, different components of the PTGS pathway were found to be expressed at low levels in both mock-infected and virus-inoculated plants in comparison with the other host species tested. It can be argued that *C. annuum* does not provide a congenial situation for viral replication. As a consequence, both viral DNA and transcripts remain below the threshold detection level of the PTGS machinery, which therefore remained at a basal or low level. ToLCNDV infection resulted in elevated levels of SGS3 transcripts in tomato and tobacco, while they stayed unchanged in *N. benthamiana*. SGS3 is a key component of the PTGS machinery responsible for biogenesis of secondary virus-derived siRNAs [14, 47]. RDR6 is another important component of the PTGS machinery. In *N. benthamiana*, RDR6 is known to be involved in the systemic spread of RNA silencing [36]. The expression of RDR6 was significantly reduced only in *N. benthamiana*. It is relevant that siRNAs could be detected in all plants parts of *N. benthamiana*. However, the expression profile of AGO1 did not indicate significant variation, highlighting its conserved role in the PTGS-mediated defense response in these hosts.

Specific defense-related host genes could also be involved in resistance against ToLCNDV. Several genes that are involved in defense against various pathogens have been characterized from *Arabidopsis* [43], tomato [40], pepper [51] and potato [1]. One of the recent reports indicated upregulation of an NBS-LRR gene in tomato resistance to the begomovirus tomato yellow leaf curl virus [7]. The present study suggests a specific role of an R gene of chilli in conferring resistance against ToLCNDV. NBS-LRR expression was significantly enhanced in ToLCNDV-inoculated *C. annuum* plants, which also showed a high degree of resistance to this pathogen. Expression of the NBS-LRR gene was moderately upregulated ($\sim$1.5-fold) in *S. lycopersicum*, which supported ToLCNDV multiplication to a lesser degree compared to *N. benthamiana*. NBS-LRR expression was drastically reduced in *N. benthamiana*, the most susceptible host species, and this can be correlated with elevated levels of viral DNA. Further study is required to investigate the specifics of the R-gene-mediated resistance response against geminiviruses.

Lipid transfer proteins (LTPs) are basic 9-kDa pathogenesis-related (PR) proteins known to be induced following pathogen attack [13]. However, LTP expression is not pathogen-specific and is involved in imparting basal defense against several pathogens [42]. The role of the LTP-mediated defense mechanism is largely unknown, but the differential expression of LTP suggests that is has a role in defense against geminiviruses. LTP expression was elevated in *C. annuum*, was slightly reduced in *N. tabacum* and *S. lycopersicum*, and was drastically reduced in *N. benthamiana*. LTP expression was inversely proportional to viral DNA accumulation in these hosts.

Viruses often reprogram host cells to make the cellular environment more permissive to viral replication and survival. On the other hand, plants have evolved a coordinated action of several layers of defense, which are activated following pathogen attack. ToLCNDV infection induces expression of genes that are involved in an RNA-silencing-mediated acquired immune response against plant viruses. Host-specific induction and expression of an R gene and an LTP gene upon inoculation suggest the involvement of other defense machineries in *C. annuum* against a ToLCNDV isolate obtained from tomato. Conversely, downregulation of these genes may result in enhanced susceptibility of *N. benthamiana* to ToLCNDV, a situation that is further aggravated by the lack of a functional RDR1 protein in *N. benthamiana*. Taken together, our data suggest a relevant role of basal defense responses in chilli pepper to limit spread of infection and to maintain the viral load below the detection limit of the host RNA silencing surveillance system. NBS-LRR and LTP probably act as the first line of defense against the pathogen, which, in coordination with RNA-silencing-based surveillance, contributes to resistance in the host plant. However, other incompatible virus-host interactions involved in several stages of viral pathogenesis might also lead to resistance in a non-permissive host.

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RESEARCH ARTICLE

Identification of siRNA generating hot spots in multiple viral suppressors to generate broad-spectrum antiviral resistance in plants

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Abstract Viruses are one of the most devastating plant pathogens causing severe economic losses worldwide. RNA silencing is a robust technology to knock down the expression of specific genes. This mechanism can be exploited to generate virus resistant plants through expression of the viral derived sequences. Viruses in turn have evolved to encode suppressors of RNA silencing to combat host defense. Mixed infection of plants is of common occurrence in nature and simultaneous targeting of suppressor(s) of multiple viruses offers an effective strategy. In this study, we have in silico designed siRNAs against suppressors of the two most devastating viruses of tomato, leaf curl causing tomato begomoviruses and *Cucumber mosaic virus*. Three different siRNA prediction programs were used to evaluate siRNAs generating capability of each sequence and common putative candidate siRNAs were selected fulfilling the stringent parameters. Our results indicated that in the case of each suppressor a particular region of 100–150 base pairs could be source of potent siRNAs referred as hotspots. Expression of these viral hot spots as a single construct in the plants would facilitate development of transgenic plants with a high degree of broad spectrum resistance against multiple viruses.

Keywords RNA silencing · siRNA · Viral suppressors · Mixed infection · Geminivirus · CMV

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Introduction

Viruses are obligate intracellular parasites and are responsible for several devastating diseases of food, fiber and medicinal crops causing significant quantitative and qualitative losses of several crops. Tomato (*Solanum lycopersicum* L.) belongs to the *Solanaceae* family which also constitutes of several other agriculturally valuable crops. Tomato is one of the most economically important vegetable crops in the world and is being used as vegetable, sauce etc.. Several studies have revealed that this crop is highly susceptible to diverse type of viruses in natural condition (García-Cano et al. 2006). Among the different viruses infecting tomato, leaf curl causing geminiviruses and Cucumber mosaic virus (CMV) are the two major viruses imposing significant threat to successful cultivation of this crop worldwide. During mixed infections, the synergism might result in the exacerbation of disease symptoms, the increase of virus titer, and the complementation of movement defects due to host restrictions, so that either of them can spread systemically and accumulate at higher level (García-Cano et al. 2006; Chakraborty et al. 2008; Rentería-Canett et al. 2011). Geminiviruses are ssDNA viruses causing leaf curl disease in several crop plants including chilli, okra, cotton, cassava, radish, cabbage etc. (Moffat 1999; Boulton 2003; Mansoor et al. 2006; Chakraborty et al. 2008; Singh et al. 2012). During the last decade, geminiviruses have emerged as the most notorious plant pathogens causing severe crop loss worldwide. CMV is another devastating virus which infects about 1,200 plant species, causing significant economic losses in many vegetables and horticultural crops. CMV is a positive sense tripartite RNA virus which belongs to the family *Bromoviridae* (Palukaitis et al. 1992). CMV infected tomato plants exhibit stunted growth, yellowing and bushy phenotype with leaves showing shoestring phenotype. Under field conditions, plants

are infected with multiple viruses and mixed infection is a common phenomenon. The disease reaction hence generated is a consequence of the interaction between viruses belonging to same or different groups. Co-infection of a plant with two viruses has been demonstrated to produce more severe disease reaction as compared to single virus infected plants (Carr and Kim 1983; Wege and Siegmund 2007; Wege 2009; Syller 2012). Infections of crop plants with geminiviruses have been reported to be accompanied with the other devastating virus; CMV (Carr and Kim 1983).

RNA interference or post transcriptional gene silencing (PTGS) is a natural antiviral defense mechanism in plants. PTGS is a natural sequence specific mRNA degradation mechanism that is triggered by double stranded RNA (dsRNA) involving coordinate action of several proteins. Long dsRNA is cleaved by an endonuclease dicer into small interfering RNAs (siRNAs) (Bernstein et al. 2001). These nascent siRNAs are then incorporated into the RNA–induced silencing complex (RISC) and act as a guide to recognize a complementary RNA for its degradation (Hammond et al. 2000). Plant viruses with either DNA or RNA genomes are subjected to small RNA directed degradation of viral transcripts resulting in resistance against viruses. This has led to the outcome that RNA silencing mechanism can be exploited to generate resistance against viral pathogens. Pathogen derived resistance (PDR) (Sanford and Johnston 1985) strategies based on RNA silencing mechanism have been utilized to develop resistance against RNA or DNA viruses (Chen et al. 2004; Di Nicola-Negri et al. 2005, Bonfim et al. 2007; Ramesh et al. 2007; Zrachya et al. 2007; Vanderschuren et al. 2009; Patil et al. 2011). Recently, RNAi-based resistance to mixed infection of three different viruses has been reported in soybean plants expressing separate short hairpins from a single transgene (Zhang et al. 2011).

On the other hand, viral encoded proteins, called silencing suppressors (VSRs) have been evolved as one of the most potent arms against the host surveillance system. VSRs are found in almost all the plant viruses though reports of few viral suppressors from insects and mammalian viruses are also available at present. CMV encoded 2b protein is a potent suppressor of RNA silencing and inhibits RNA silencing through binding to dsRNA (Brigneti et al. 1998) and interfering with Ago mediated cleavage (Zhang et al. 2006). Three proteins namely AC2, AC4 and AV2 encoded by geminiviruses have been reported to suppress host RNA silencing with varied efficacy. AC2 protein inhibits transcriptional gene silencing through inactivation of adenosine kinase and Sucrose non fermenting 1 (SNF1) (Hao et al. 2003; Bisaro 2006), AC4 interferes with RNA silencing through binding with single stranded siRNAs (Chellappan et al. 2005) and AV2 interferes with RNA silencing by inhibiting RDR6-SGS3-siRNA mediated signal amplification (Glick et al. 2008).

Targeting these viral suppressors of RNA silencing using RNA silencing mechanism offers a promising tool to generate a high degree of resistance in plants and may provide a solution to this problem of major significance. In field condition, plants are not infected by single virus alone and targeting suppressor(s) of one particular virus will render the suppressor(s) of other viruses to inhibit RNA silencing hence making the approach non feasible. Earlier reports of in silico designing of putative siRNAs against viral suppressors (Saxena et al. 2011, 2013) do not provide a solution to the problem caused by mixed infection. In order to address this, we have adopted an approach of designing of highly efficient and off target filtered siRNAs using consensus sequences of suppressors of CMV and tomato infecting begomoviruses. We have used three different siRNA prediction programs (http://siRNA.wi.mit.edu/siRNA_search.cgi, http://biodev.extra.cea.fr/DSIR/selectOT.php and http://plantgrn.noble.org/pssRNAit/) to predict highly efficient siRNAs. We assume that targeting multiple suppressors of different viruses using single ihpRNAi construct may provide effective and broad spectrum resistance against tomato infecting viruses.

Materials and methods

Retrieval (Collection) of viral target sequences

Sequences of 18 different isolates of CMV, and 27 different tomato infecting begomoviruses were retrieved from NCBI (www.ncbi.nlm.nih.gov) database. The sequences of suppressors of CMV (2b protein), and begomoviruses (AC2, AC4, and AV2) were deduced from full length genomes.

Multiple sequence alignment of viral suppressors to generate consensus sequences

Different viral suppressor sequences were aligned and a consensus sequence for each suppressor was identified for each alignment using Genamics expression program (http://www.genamics.com/expression/). Multiple sequence alignment was performed to identify the most conserved regions of different suppressors among different viral isolates. The consensus sequence generated for each suppressor was further used as a query for designing highly effective putative siRNA sequences.

Designing of highly effective and virus specific siRNAs against target sequences

For designing highly effective siRNAs, we used three different siRNA designing softwares namely DSIR (http://biodev.extra.cea.fr/DSIR/selectOT.php) (Vert et al. 2006), siRNA at White head (http://sirna.wi.mit.edu/siRNA_search.cgi?tasto=

1012545632) (Yuan et al. 2004), and pssRNAit (http://plantgrn.noble.org/pssRNAit/). The consensus sequence derived from alignment of different suppressors was used as input query in above mentioned programs. Initially, siRNAs were designed using DSIR and siRNA at whitehead programs. Subsequently, the consensus sequences were used to generate siRNA using pssRNAit program. This program designs effective and specific siRNAs with genome-wide off-target gene assessment and have the feature for filtering off target siRNAs using the BLAST search against various plant species. We used this program to filter off target siRNA through a BLAST search against available tomato (*Solanum lycopersicum*) genome database.

Target secondary structure prediction and siRNA binding

Secondary structures for target sequences and siRNA binding sites were generated using Sfold (http://sfold.wadsworth.org/cgi-bin/index.pl) program. Consensus sequence for each suppressor was used as input query in Sfold and secondary structure with minimum free energy was selected.

Target accessibility prediction

Target accessibility of selected siRNAs was computed using RNAup program. siRNAs for each sequence were used in RNAup program to predict the favourable interaction of siRNA antisense strand with target mRNA.

Parameters for designing siRNAs

For designing highly efficient siRNAs various parameters were considered. These parameters included GC content range between 30 and 55 %, RISC binding score for sense and antisense strand, target accessibility, avoidance of four or more A, U, G and C, and filtering of off target and immunostimulatory motifs containing siRNAs.

Filtering of off target siRNAs

Initially, consensus sequence for each of viral suppressor was used to select common siRNAs that were generated by using two different programs namely DSIR, and siRNA at white head. Later, these consensus sequences were again used in pssRNAit program and siRNAs were generated using this program with a BLAST search with tomato genome to filter any off target siRNA.

Selection of effective siRNAs

Finally, the best siRNAs were selected which were common among at least two programs (but must be predicted by pssRNAit program) and fulfilling all the parameters such as GC content, high score, target accessibility, RISC loading, lack of immunostimulatory motifs and no off targets.

Results

Retrieval of sequences and generation of consensus sequences

Nucleotide sequences of AC2, AC4 and AV2 genes were deduced from 27 different tomato infecting begomoviruses reported worldwide and CMV 2b gene sequences were deduced from 17 different CMV isolates (Supplementary Table 1 and 2). Multiple sequence alignment (MSA) of each suppressor sequence was performed using the CLC workbench program and the results of MSA were used to generate phylogenetic trees for each sequence (Supplementary fig. 1–4). Result of MSA did not provide a high degree of conservation among selected suppressor sequences due to the large number of diverse viral sequences used in the study. So we generated consensus sequences for each suppressor using Genamics expression software (http://www.genamics.com/expression/). Use of consensus sequence for prediction of siRNA offers advantage of broad spectrum resistance against multiple viruses.

Designing of highly efficient siRNA with filtered off targets

The consensus sequences generated for each suppressor was used as the input for the prediction of all the possible siRNAs. To obtain highly efficient siRNAs we used three different siRNA predicting softwares (DSIR, siRNA at Whitehead and pssRNAit). Out of these three programs pssRNAit has a novel feature of BLAST with different plant genomes to avoid any off target siRNA. Initially we designed siRNAs using DSIR and siRNA at Whitehead programs. All these programs have option of BLAST with the human genome to avoid any off target siRNA against human genome. To filter siRNAs targeting any tomato gene, BLAST was performed with *Solanum lycopersicum* cDNA/transcript libraries. In addition to removing off target siRNAs we further filtered siRNAs which may have immunostimulatory motifs. Next, we selected siRNAs which were assigned high rank and common to at least two programs but must be predicted by pssRNAit.

Properties of selected siRNAs

Several parameters like GC content, lack of self structure, high A/U content at 5′ end of antisense strand or high G/C at the 5′ end of the sense strand were considered while predicting putative siRNAs . Stretches of 4 or more A or T were avoided as it can lead to transcription termination. In this study, we have selected 30-55 % GC content range for siRNA as it has been shown that functional siRNAs tend to have a moderate to

Table 1 Potential siRNA sequences identified within the viral genomes

Gene	Position	Target sequence	Putative siRNA sequences	RISC binding Antisense score	RISC binding sense score
CMV 2b					
	179	UAGAUGGUUCGUGAACUGAUA	Sense strand: GAUGGUUCGUGAACUGAUAGA Antisense strand: UAUCAGUUCACGAACCAUCUA	1.16	−0.58
	85	CACAAGCAGAAUCGACGGGAA	Sense strand: CAAGCAGAAUCGACGGGAACG Antisense strand: UUCCCGUCGAUUCUGCUUGUG	1.15	−0.97
	187	UCGUGAACUGAUAGAGAUGUA	Sense strand: GUGAACUGAUAGAGAUGUACC Antisense strand: UACAUCUCUAUCAGUUCACGA	−0.34	−1.57
	75	ACGAAGGUCUCACAAGCAGAA	Sense strand: GAAGGUCUCACAAGCAGAAUC Antisense strand: UUCUGCUUGUGAGACCUUCGU	0.59	−2.16
	74	GACGAAGGUCUCACAAGCAGA	Sense strand: GAAGGUCUCACAAGCAGAAUC Antisense strand: UUCUGCUUGUGAGACCUUCGU	−0.11	−0.68
	173	AUCAAGUAGAUGGUUCGUGAA	Sense strand: CAAGUAGAUGGUUCGUGAACU Antisense strand: UUCACGAACCAUCUACUUGAU	1.37	−1.91
AC2					
	292	CCGUCCAAAUACAGUUCAACC	Sense strand: CCGUCCAAAUACAGUUCAACC Antisense strand: UUGAACUGUAUUUGGACGGUG	1.81	−2.83
	281	CGACAUCAUCACCGUCCAAAU	Sense strand: CGACAUCAUCACCGUCCAAAU Antisense strand: UUGGACGGUGAUGAUGUCGUG	1.86	−1.85
	368	GGACGAGCUUACAGCCUCAGA	Sense strand: GGACGAGCUUACAGCCUCAGA Antisense strand: UGAGGCUGUAAGCUCGUCCAG	2.03	−1.46
	391	GGUCGUUUCUUAAGAGUAUUU	Sense strand: GGUCGUUUCUUAAGAGUAUUU Antisense strand: AUACUCUUAAGAAACGACCAG	0.58	−1.20
	385	CAGACUGGUCGUUUCUUAAGA	Sense strand: CAGACUGGUCGUUUCUUAAGA Antisense strand: UUAAGAAACGACCAGUCUGAG	1.63	−2.10
AV2					
	48	GGUGUAUGCUAGCAAUUAAAU	Sense strand: GGUGUAUGCUAGCAAUUAAAU Antisense strand: UUAAUUGCUAGCAUACACCUA	0.48	−2.08
	148	GGCUAGAAAUUAUGUCGAAGC	Sense strand: GGCUAGAAAUUAUGUCGAAGC Antisense strand: UUCGACAUAAUUUCUAGCCCG	1.56	−1.5
	164	GAAGCGACCAGCAGAUAUAAU	Sense strand: GAAGCGACCAGCAGAUAUAAU Antisense strand: UAUAUCUGCUGGUCGCUUCGA	0.69	−1.11
	49	GUGUAUGCUAGCAAUUAAAUA	Sense strand: GUGUAUGCUAGCAAUUAAAUA Antisense strand: UUUAAUUGCUAGCAUACACCU	0.2	−2.24
	47	AGGUGUAUGCUAGCAAUUAAA	Sense strand: AGGUGUAUGCUAGCAAUUAAA Antisense strand: UAAUUGCUAGCAUACACCUAA	0.96	−1.06
	125	CGUGAUUUAAUUUCAGUUAUU	Sense strand: CGUGAUUUAAUUUCAGUUAUU Antisense strand: UAACUGAAAUUAAAUCACGAA	1.84	−1.3
	88	GACGUAUUCUCCCGAUACAUU	Sense strand: GACGUAUUCUCCCGAUACAUU Antisense strand: UGUAUCGGGAGAAUACGUCUU	0.25	−1.99
AC4					
	137	CCUCCGAUGUCAAGUCCUACA	Sense strand: CCUCCGAUGUCAAGUCCUACA Antisense strand: UAGGACUUGACAUCGGAGGUG	2.66	−2.22
	201	CGAUGGAAGAUCUGCUAGAGG	Sense strand: CGAUGGAAGAUCUGCUAGAGG Antisense strand: UCUAGCAGAUCUUCCAUCGAU	1.02	−0.28

Table 1 (continued)

Gene	Position	Target sequence	Putative siRNA sequences	RISC binding Antisense score	RISC binding sense score
	149	AGUCCUACAUGGACAAAGACG	Sense strand: AGUCCUACAUGGACAAAGACG	1.43	−1.43
			Antisense strand: UAAUUGCUAGCAUACACCUAA		
	24	CGUGCUCAUCCAAUUCGAAGG	Sense strand: CGUGCUCAUCCAAUUCGAAGG	2.44	−1.66
			Antisense strand: UUCGAAUUGGAUGAGCACGUG		
	113	CGAACAUUCAGGGAGCUAAAU	Sense strand: CGAACAUUCAGGGAGCUAAAU	1.40	−1.67
			Antisense strand: UUAGCUCCCUGAAUGUUCGGA		

low GC content. Another important parameter of determining siRNA efficacy is efficiency score which is based upon several rules. To get highly efficient siRNAs we further eliminated siRNAs which were assigned an efficiency score below eight to ensure that selected siRNAs are efficient in functioning (Table 1).

Target accessibility

Designing of siRNAs based on only thermodynamic parameters does not always guarantee successful silencing of the target; the main cause of this positional effect is the presence of local mRNA structure/ secondary structure at the target site (Fig. 1). In this study we addressed this significant problem by using pssRNAit program. In pssRNAit program, RNAup program is employed to calculate target accessibility of each selected siRNA, which is represented by the energy, required to open secondary structure around the target site. The results showed that the energy required for specific interaction (ΔGi) of the antisense strand of selected siRNAs ranges from −15 kcal/mol to-33 kcal/mol. This energy requirement is quite lower than the energy of secondary structures formed around the siRNA binding region on mRNA (Fig. 2).

Influence of guide strand on siRNA efficiency

The guide strand of siRNA plays crucial role in efficient gene silencing and thermodynamic asymmetry is fundamentally important for siRNA functions and loading into the RISC complex. The most effective siRNAs tend to have a relatively low Tm and duplex stability at 5′-end of the guide strand. Our results also show that 5′-end of the antisense strand in the case of each siRNA is A/U rich and relatively less stable compared to 5′-end of sense strand which is G/C rich. This antisense strand of each selected siRNA has a high probability to load into RISC complex. In addition, the RISC binding score assigned by the pssRNAit program to each siRNA also indicates the higher probability of antisense strand to load into the RISC complex (Table 1).

Off-target filtering

One important problem associated with in silico designed siRNAs is off target silencing of unintended host transcript. To address this problem we performed a BLAST search against human genome (DSIR, pssRNAit) and tomato genome sequence (pssRNAit) to filter off target siRNAs.

Hotspots of siRNA generation

For each suppressor sequence used in this study, we have identified a stretch of 100–200 nt (nucleotides) which we referred as hot spots for highly efficient siRNA generation.

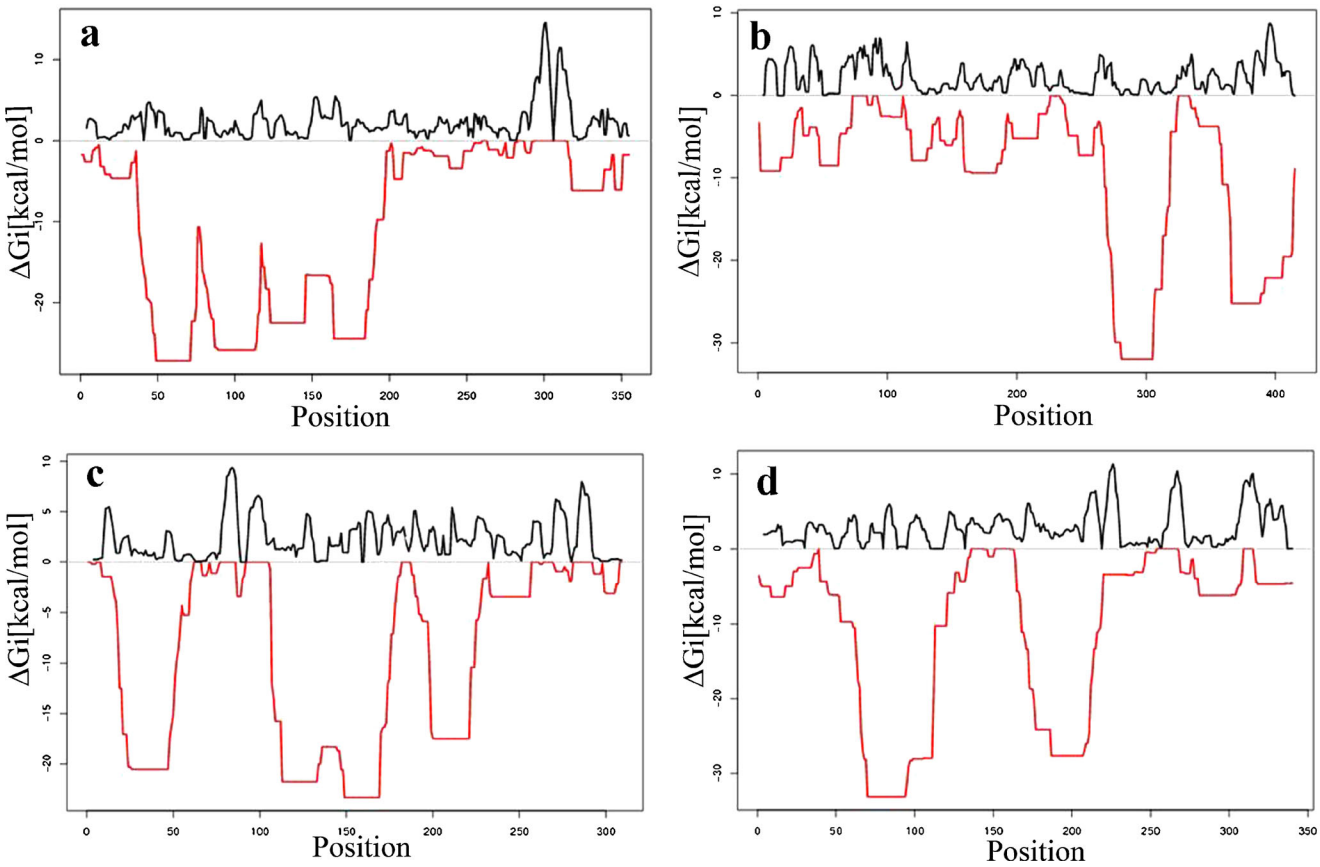

Fig 1 Computational prediction of siRNA and corresponding target mRNA hybridization plot. **a** AV2, **b** AC2, **c** AC4 and **d** CMV2b. The plot shows that the interaction energy (ΔGi) required for siRNA-mRNA interaction is very low compared to the energy required for mRNA secondary structure formation

This region contains sequences, which give rise to siRNAs fulfilling all the parameters. This region extends from 75 to 190 bp for CMV2b, 100 to 220 bp for AC4, 280–415 bp for AC2 and from 40 to 200 bp for AV2.

Discussion

Viruses are obligate intracellular parasites and cause severe losses of food, fiber and medicinal crops. They invade plants and hijack host cellular machinery for successful replication and life cycle in host cells. In order to neutralize plant defense machinery of RNA silencing viruses have evolved suppressors which inhibit different steps of RNAi machinery. Targeting of viral suppressors using the RNAi mechanism offers a plausible solution to develop a high degree of broad spectrum resistance against multiple viruses.

Tomato is one of the most economically important vegetable crops worldwide and is susceptible to viruses from different taxa. Two most devastating tomato infecting viruses are tomato leaf curl viruses belonging to the family *Geminiviridae* and CMV. In natural conditions, mixed viral infection of plants is a common phenomenon and this synergistic interaction among viruses helps both partners to avoid host defense mechanism and successfully propagate in the host. Previous reports suggest the strategy of in silico designing of siRNA against geminivirus suppressors as a tool to develop generic and broad spectrum resistance against tomato leaf curl disease (Saxena et al. 2011, 2013). The above approaches seem non feasible and impractical based on two important facts firstly, how to continuously feed these siRNAs into the field without generating transgenic plants as RNA molecules are highly labile and secondly, targeting suppressors of one virus does not offer an effective strategy as in fields single plant is infected by different viruses and the suppressor of other virus will inhibit RNA silencing. In order to answer these questions, we adopted the in silico approach of siRNA designing to target suppressors of multiple viruses which naturally occurred as mixed infection and we suggest that transgenic expression of these hot spots may provide broad spectrum transgenic resistance against multiple viruses.

We have employed consensus sequences instead of conserved sequences for prediction of siRNAs as siRNA designed using consensus sequence is likely to target broad ranges of

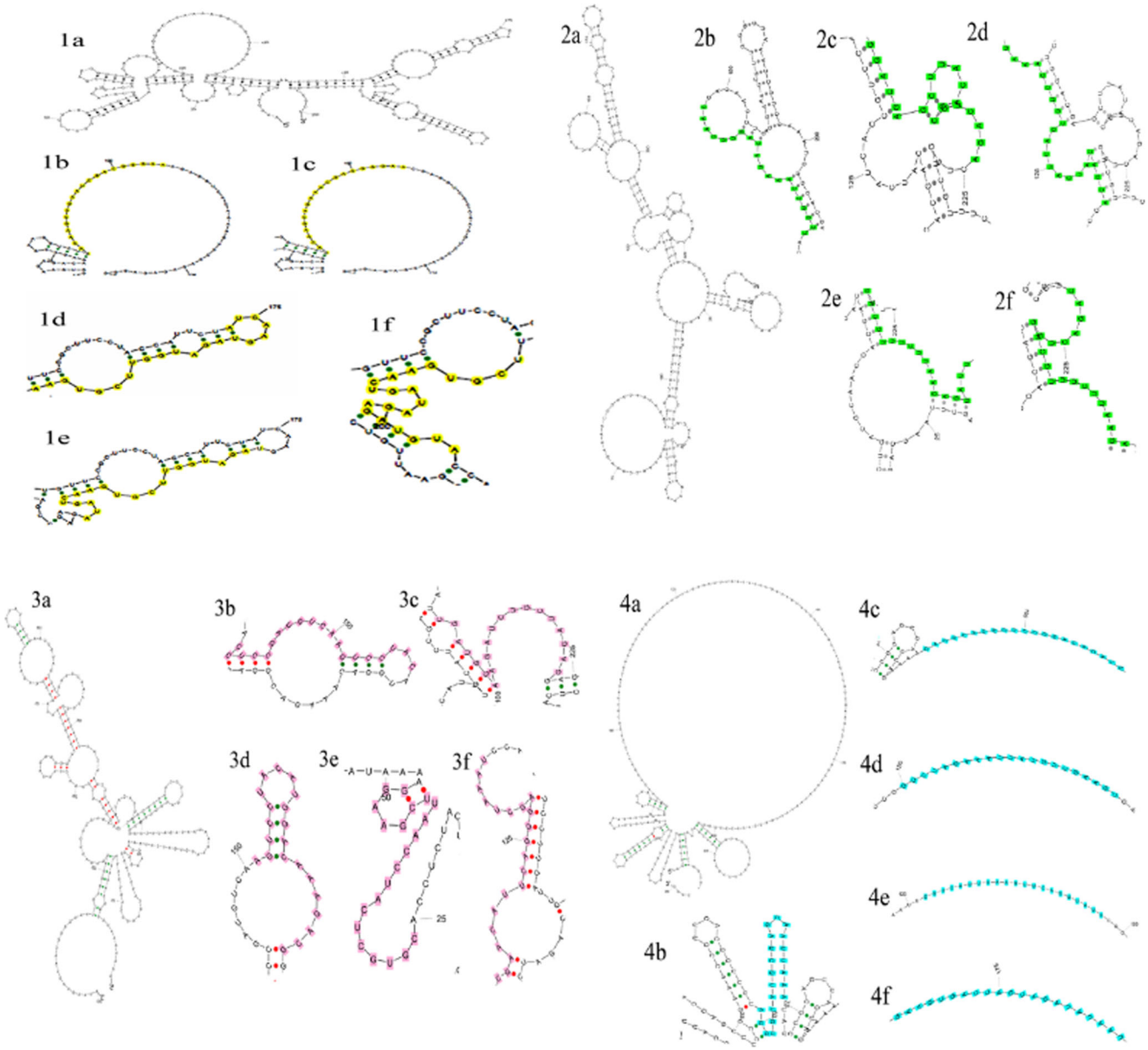

Fig. 2 Computational prediction of local secondary structures of target sequence and siRNA binding sites. This picture shows the predicted secondary structures of target sites sequences. Local secondary structures with the lowest free energy were generated using the fold program. The colored region shows the binding site for siRNA guide strand on target sequences. *1a–1f* for CMV2b, *2a–2f* for AC2, *3a–3f* for AC4 and *4a–4f* for AV2

viruses compared to siRNAs designed using conserved sequences. The consensus sequences for each suppressor were used as an input query in three different programs to find out highly efficient siRNAs. We obtained several putative siRNA for each consensus sequence but in order to assure that designed siRNA are highly efficient and ensure effective silencing of target gene, we filtered siRNA with a low efficiency score. Next to find out best siRNA, we picked siRNAs which were common to at least two programs but must be predicted by pssRNAit fulfilling all the parameters as mentioned in methods section and assigned a high rank by each program.

Thermodynamic features of siRNAs are important determinants of their potency, predominantly by factors relating to RISC loading. Effective siRNAs tend to have a relatively lower duplex stability (Tm; less stable, more A/U rich) toward the 5′-end of the strand that remains in RISC (the ‘guide strand’) and a relatively higher Tm (more stable, more G/C rich) toward the 5′-end of the degraded strand (the ‘passenger strand’) (Aza-Blanc et al. 2003). Thermodynamic asymmetry is a fundamental property of functional siRNAs and microRNAs (miRNAs) and directly correlates with strand loading during RISC assembly (Khvorova et al. 2003;

Schwarz et al. 2003; Tomari 2004). GC content of siRNAs has been shown to be another important parameter for siRNA efficacy. In general, functional siRNAs have a moderate to low GC content (30 to 55 %) and regions of high local GC content may be prone to problems with secondary structure formation which leads to stalled RISC (Holen 2005). Target site accessibility is another parameter that impacts the effectiveness of silencing (Muckstein et al. 2006; Tafer et al. 2008). Due to secondary structure formation in mRNA, some target sites are inaccessible to interact with siRNAs resulting in inhibition of RNAi (Bohula et al. 2003; Vickers et al. 2003). Our results of siRNA:mRNA hybridization analysis indicate that interaction energy required for binding of selected siRNA to the corresponding target mRNA is very low as compared to mRNA secondary structure (Fig. 1). These results explain that antisense strands of selected siRNAs are accessible to target sites lying in highly folded regions. It has been shown that siRNA efficacy predictions based upon a combination of the most probable mRNA secondary structure and siRNA duplex-end stabilities improved the number of correct predictions from 60 to 70 % (Heale 2005).

Unintended off target silencing is a major concern while designing siRNA against any sequences as off target silencing of host genes may lead to an abnormal phenotype (Persengiev et al. 2004; Saxena et al. 2003; Scacheri 2004; Lin et al. 2005,) and is triggered by a 7 nt complementation between siRNA and off target (Xu et al. 2006). One possible way to eliminate off target effect is to use the lesser concentration of siRNAs (Semizarov et al. 2003; Persengiev et al. 2004), chemical modification of siRNA (Jackson 2006) or use of siRNAs with no homology to host genome. It is important to filter any off target siRNA suspected to target genes other than specific target. PssRNAit program (earlier siRNA scan) designs better siRNA for PTGS by minimizing off-target gene silencing in plants. In addition this program also filters siRNAs which may have an immunostimulatory motif.

We have identified the hot spots of siRNA generation in each viral sequence. These hot spots are 100 bp to 200 bp in

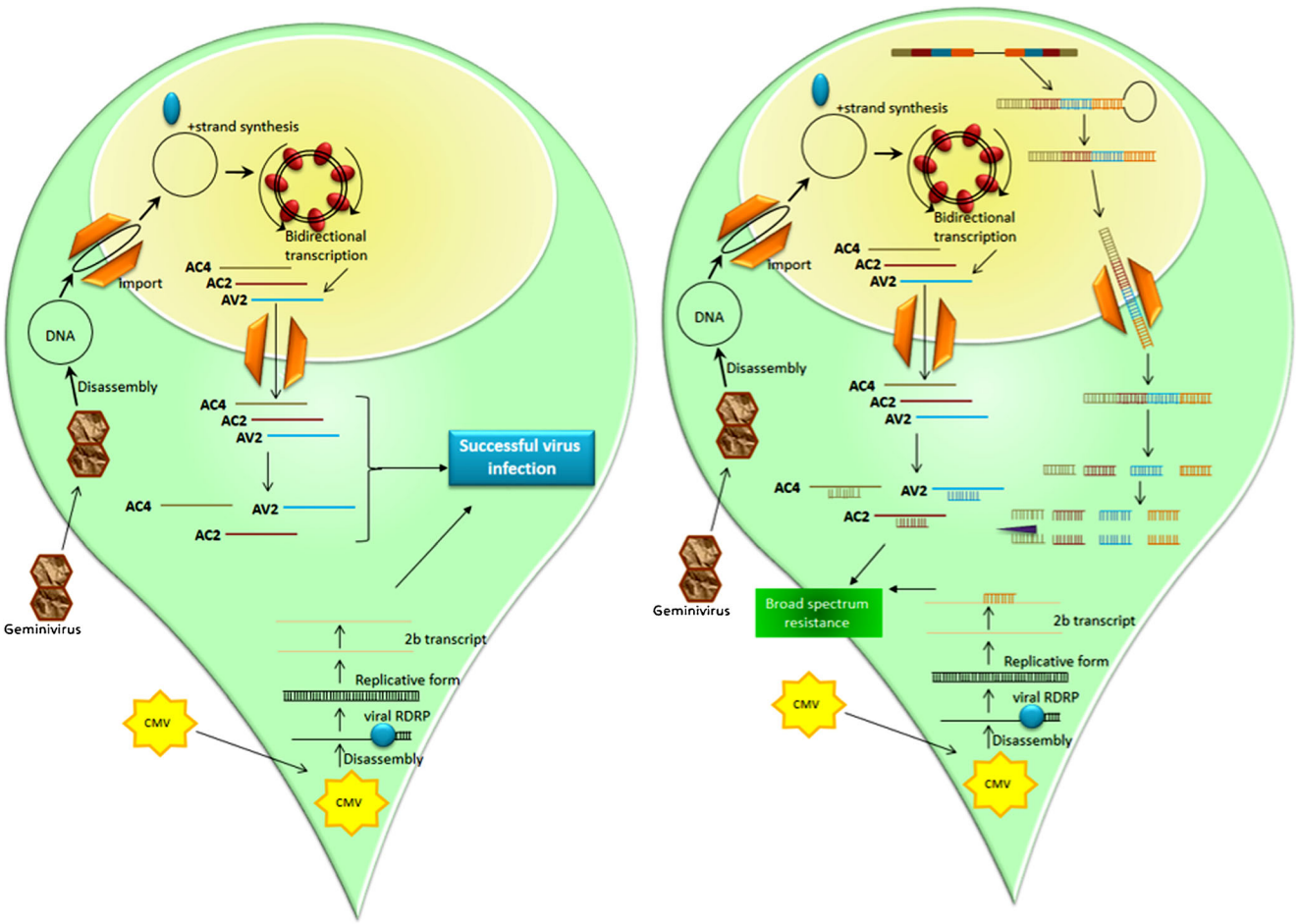

Fig. 3 Schematic diagram depicting the consequences of mixed infection in non transgenic and transgenic plant based on our proposed strategy. **a** In natural condition, mixed infection result in the severe symptom production in the infected plants due to cumulative effect of viral suppressors. **b** Transgenic plant harboring intron hairpin construct containing suppressor sequences of multiple viruses. Intron hairpin construct will generate siRNA against suppressor of multiple viruses which would facilitate the sequence specific degradation of viral transcripts and inhibition of the viral multiplication resulting in broad spectrum resistance

length. The siRNAs predicted to be generated by these hot spots were the most effective siRNA in terms of fulfilling all the stringent parameters. These hot spots regions could be the prime targets of host PTGS machinery. These regions could be identified in other viral sequences and may be targeted by RNAi mechanism to generate effective broad spectrum resistance.

In case of mixed infections, different viruses code for proteins which are responsible for viral replication, transcription and movement to allow successful virus infection (Fig. 3a). Different viruses code for suppressor proteins which act on and block different steps of RNA silencing machinery of host. Thus targeting suppressor(s) of one virus is not competent enough to block infection by another virus, as the suppressor(s) encoded by the later can also block host RNAi mediated antiviral response (Fig 3a). We, therefore, propose that transgenic expression of hot spot regions identified in viral suppressors of tomato infecting begomoviruses and CMV, as single chimeric intron hairpin RNAi construct would generate highly effective siRNAs against multiples suppressors in tomato. These siRNAs would target the suppressors of both these destructive viruses through PTGS and facilitate development of broad-spectrum transgenic resistance (Fig. 3b).

This is the first report of in silico designing of highly efficient and specific siRNA against suppressors of multiple viruses to develop a high degree of broad spectrum transgenic resistance in tomato against devastating tomato leaf curl viruses and CMV using three different programs. We have firstly identified the presence of hot spots in geminivirus and CMV suppressor sequences through in silico search. This approach can be further extended to other economically important crops susceptible to multiple virus infections.

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Author Disclosure The authors declare that they have no competing financial interests.

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To.
The Chairperson
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Re: Complete patent application no. 2619/DEL/2015 dated 24.08.2015 for "Chilli Leaf curl virus DNA-based chimeric construct for efficient plant inoculation" in the name of Jawahar Lal Nehru University.

Dear Sir,

This is in reference to the above said invention, complete specifications has been filed at the Indian Patent office, New Delhi. A copy of the same is enclosed herewith along with official filling receipt for your record.

Further, we enclose herewith **Form-1 (in triplicate)** and **Power of Attorney (In Original)** which may kindly be executed by the inventor and applicant wherever indicated therein and returned to us.

Kindly note that, executed Power of Attorney is required to be filed at the Patent Office **Within one Month** from the date of filling of the application according to the circular issued b the C.G., Further, executed Form-1, is required to be Filed **within 6 Months** from the date of filling of the application failing which a petition will be required to file along with the prescribed govt.fee for late submission of Application form at the Patent Office

Also please find enclosed our invoice for the same.

Thanking You,

Yours sincerely,

Shaleen Raizada

Dr. (Mrs.) Shaleen Raizada
MD & CEO

for filing & follow up
R Bamzai
07/10/15

Enclosures-

1. Copy of the as filed as papers.
2. Our Invoice
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4. Form-1 (In triplicate)
5. Power Of Attorney (In Original)

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I/We, the above named applicant/s hereby authorize G.S. DAVAR, INDIRA BANERJEE, SUDIPTA BANERJEE, SOMA RANI MISHRA, and all representatives Of L.S.DAVAR & CO., having their offices at 32, Radha Madhav Datta Garden Lane, Kolkata-700 010 and 5/1, Kalkaji Extn., New Delhi-110019, India, all of Indian nationality, jointly and severally to act my/our

behalf in connection with letter patent from Govt. of India in respect of an invention for "Development of Chilli leaf curl virus DNA-based chimeric construct for efficient plant inoculation"

including substituting and/or authorizing any other person/s their behalf and request that all notices, requisitions and communications relating thereto may be sent to such agent at the above address.

I/We hereby revoke all previous authorisation, if any in respect of the same matter or proceeding.

I/We also confirm all action already taken by them in this matter.

Dated this 24th day of August, 2015

JAWAHARLAL NEHRU UNIVERSITY

Chairperson
Intellectual Property Management
JNU, New Delhi

FORM – 1

THE PATENTS ACT, 1970
(39 of 1970)

THE PATENTS RULES, 2003

APPLICATION FOR GRANT OF A PATENT

[See section 7, 54 & 135 and rule 20(1)]

1. Applicant (S)

WE,

JAWAHARLAL NEHRU UNIVERSITY, NEW DELHI 110067, INDIA. AN INDIAN UNIVERSITY.

2. Inventor (s)

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All are Indian Nationals.

3. Title of the Invention: "Development of Chilli leaf curl virus DNA-based chimeric construct for efficient plant inoculation"

4. Address for Correspondence of Applicant/Authorised Patent agent in India:

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5. Priority Particulars of the Application (s) filed in convention country.

.....Nil.....

6. Particulars for filing patent cooperation treaty (PCT) National phase application

.....Nil.....

7. Particulars for filing divisional application

.....Nil.....

8. Particulars for filing patent of addition

.....Nil.....

9. Declarations:

(i) Declaration by the inventor(s)

SUPRIYA CHAKRABORTY

NIRBHAY KUSHWAHA

ASHISH KUMAR SINGH

I/We, the above named inventor(s) is/are the true & first inventor(s) for this invention and declare that the applicant(s) herein is/are my/our assignee or legal representative.

(ii) Declaration by the applicant(s) in the convention country

I/We, the applicant(s) in the convention country declare that the applicant(s) herein is/are my/our assignee or legal representative.

Date:

JAWAHARLAL NEHRU UNIVERSITY, NEW DELHI

Chairperson
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JNU New Delhi

(iii) Declaration by the applicant (s):

I/We, the applicant(s) hereby declare(s) that:-

- **I am/We are in possession of the above-mentioned invention.**
- **The provisional/complete specification relating to the invention is filed with this application.**
- **The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by me/us before the grant of patent to me/us.**

- **There is not lawful ground of objection to the grant of the Patent to me/us.**
- **I am/We are the assignee or legal representative of true & first inventors.**
- **The application or each of the application, particulars of which are given in para 5 was the first application in convention country/countries in respect of my/our invention.**

- **I/We claim the priority from the above mentioned application(s) filed in convention country/countries and state that no application for protection in date by me,/us or by any person from which I/We derive the title.**
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10. Following are the attachments with the application:

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I/we, hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/We request that a Patent may be granted to me/us for the said invention.

Dated this 24th day of August , 2015

(SOMA RANI MISHRA)
OF L.S. DAVAR & CO.,
Patent Agent No. IN/PA(1159)
APPLICANT'S ATTORNEY

To,
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The Patent Office Branch,
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OF VIROLOGY & IMMUNOLOGY

Indian Virological Society

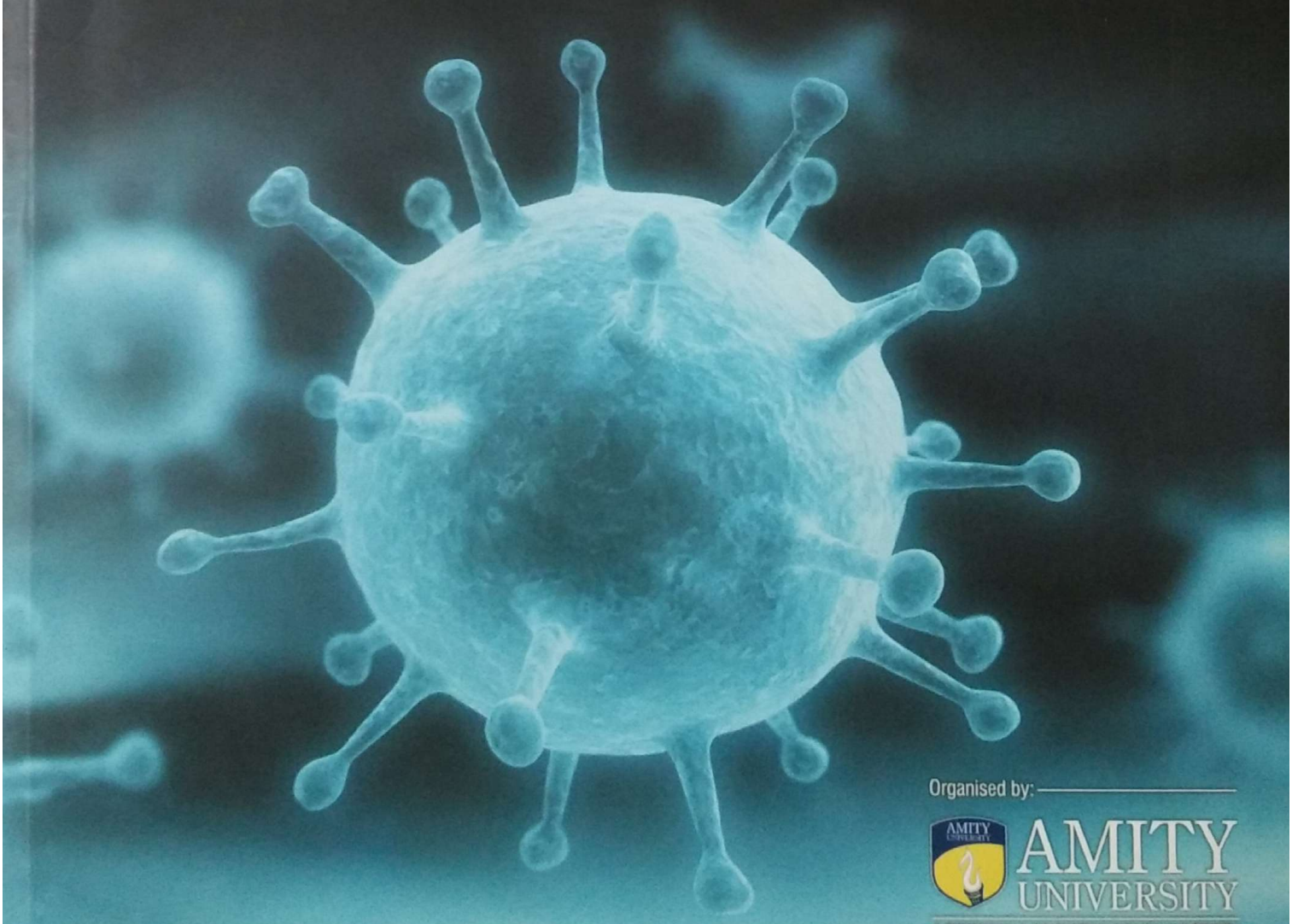
ABSTRACT BOOK

Indian Virological Society (IVS)

VIROCON 2013

ASIA-PACIFIC CONGRESS OF VIROLOGY

17th-20th Dec., 2013 • Amity University, Sec.-125, Noida (New Delhi NCR), INDIA



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Synergistic interaction of begomoviruses results in breakdown of natural resistance in chilli

Abstract

Multiple infection of viruses leads to the synergistic or antagonistic interaction with each other. During synergistic interaction, at the cellular level viruses exert positive influence on the replication, movement of each other which eventually results in the manifold virus accumulation and severe symptoms. We observed the breakdown of resistance in resistant chilli variety (Capsicum annuum cv. BS35 and C. annuum cv. Kalyanpur Chanchal) due to multiple infections of four genomic components of begomoviruses which were isolated and identified as DNA-Aof Tomato leaf curl New Delhi virus, DNA- B of Tomato leaf curl Gujarat virus, DNA A of Chilli leaf curl Multan virus and DNA β of Tomato leaf curl Bangladesh betasatellite. The nature of molecular interactions among these four molecules of begomovirus was analyzed in model plants N. benthamiana. Symptom severity could be correlated with higher viral DNA accumulation in mixed infected plants. Quantitative assessment of viral DNA accumulation in chilli plants revealed presence of higher level of viral titer in dual infected resistant plants. Results of qRT PCR indicated suppression of defense machinery in mixed infected plants which finally leads to the breakdown of resistance and establishment of pathogenesis in hitherto known resistant chilli varieties.

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Genetic transformation of an elite Indian Genotype of cotton (Gossypiumhirsutum L.) for CLCuD resistance

Cotton, an economically important crop of India, valued for textile production with about 9.7 million hectares under cultivation, is plagued by devastating Cotton leaf curl disease CLCuD is caused by Cotton leaf curl virus (CLCuV) in association with DNA β. CLCuV,a begomovirus, is transmitted by whitefly (Bemisiatabaci) vector. There is an urgent need to employ advanced and more potent strategies, such as the one driven by an RNAi-based approach, to safeguard cotton crop against CLCuD led devastation. Genetic engineering of cotton plant is a viable alternative for protection against CLCuD. However, Low transformation efficiency and genotype dependence are the two most limiting factors in the development of genetically modified cotton. Most of them are recalcitrant and not amenable to genetic manipulation. The main aim of this study was to develop a rapid and efficient Agrobacterium-mediated genetic transformation protocol of an elite Indian genotype of cotton cv. Narsimha for resistance against CLCuD employing RNAi approach. Shoot tips, hypocotyls and nodal explants were used for transforming with the RNAi cassettes, and planted on selection medium. The regenerated explants were further transferred to rooting medium, hardened and transferred to glass house. The putative cotton transgenic plants were screened by PCR and the integration of the gene in genome was confirmed by Southern blot analysis. The transgenicplants were raised in the glasshouse and screened for virus resistance by inoculating with viruliferous whiteflies for biological testing. Level of resistance in transformed cotton against CLCuD will be discussed.

AMITY INSTITUTE OF VIROLOGY & IMMUNOLOGY

Indian Virological Society

BEST POSTER AWARD IN PLANT VIROLOGY

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ASHISH KUMAR SINGH

This award was conferred during **ASIA PACIFIC CONGRESS OF VIROLOGY** "VIROCON-2013" held at Amity University Uttar Pradesh, Noida.

Prof. Narayan Rishi	Prof. A.K. Prasad	Prof. Neeraj Aggarwal	Dr. Govind P. Rao
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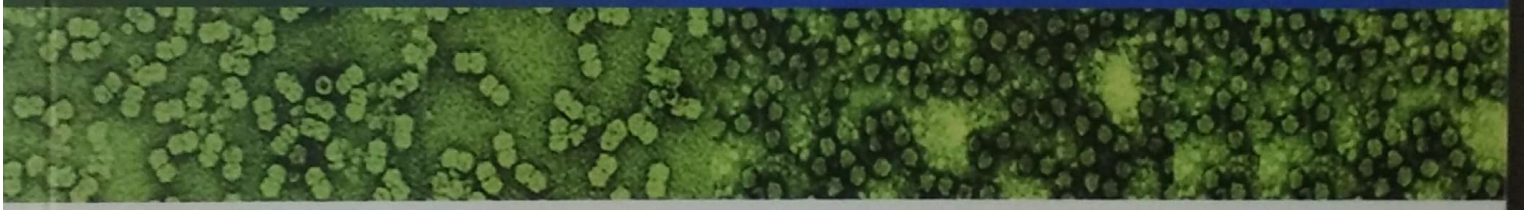
December 20, 2013

The Abstract Book

8th International Geminivirus Symposium
&
6th International ssDNA Comparative Virology Workshop



November 7-10, 2016
New Delhi, India

PA55

Study of radish leaf curl viruses and their host range

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Radish and okra plants showing leaf curl symptoms were studied for identification of the casual organism. Whitefly transmission of virus from symptomatic samples to healthy radish and okra produced typical leaf curling symptoms 12-15 days post inoculation of plants. Total DNA isolated from infected plants was used for amplification of virus genome with degenerate primers. The amplicons were cloned and plasmid DNA isolated from two individual colonies was sequenced. A comparative analysis of these sequences with corresponding gene sequences of other Geminiviruses in the GenBank revealed highest sequence identity with Radish leaf curl virus (family: *Geminiviridae*, genus: *Begomovirus*). Transmission of RaLCV to commercial vegetable crops, symptoms, putative recombination of virus with other Geminiviruses and host range will be discussed.

PA56

Consequences of synergistic interaction among chilli-infecting begomoviruses

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Plants are continuously exposed to several pathogens. Multiple virus infections may lead to either synergistic or antagonistic interaction among the invading pathogens. During synergistic interaction, at the cellular level viruses exert positive influence on the replication, movement and provide suitable environment for each other which eventually favours virus accumulation and pathogenesis. We observed breakdown of natural resistance in the chilli cultivar (*Capsicum annuum* cv. Kalyanpur Chanchal) due to the association of four genomic components of begomoviruses such as DNA-A of Tomato leaf curl New Delhi virus, DNA- B of Tomato leaf curl Gujarat virus, DNA-A of Chilli leaf curl virus and DNA β of Tomato leaf curl Bangladesh betasatellite. Further, frequent association of these four genomic components was also observed in the symptomatic plants of other resistant and susceptible chilli cultivars (*Capsicum chinense* cv. Bhut Jolokia and *Capsicum annuum* cv. Kashi Anmol) grown in the experimental field. The nature of molecular interactions among these four molecules of begomoviruses was analyzed in laboratory host *N. benthamiana* as well as in natural host chilli. Symptom severity was proportional with the higher viral DNA accumulation in plants infected with more than one begomovirus. Semi-quantitative assessment revealed presence of high viral titer in mixed infected resistant chilli plants. Analysis of the host basal (ascorbate peroxidase, thionin, polyphenol oxidase) and specific defense-related gene (NBS-LRR) expression revealed that mixed infection of chilli-infecting begomoviruses suppress the expression of defense genes that finally leads to the breakdown of resistance and establishment of pathogenesis in hitherto known resistant chilli cultivars.

8th International Geminivirus Symposium
&
6th International ssDNA Comparative Virology Workshop

November 07-10, 2016

*This is to certify that **Ashish Kumar Singh, India** has actively participated / made a poster / ~~oral~~ presentation in the 8th International Geminivirus Symposium & 6th International ssDNA Comparative Virology Workshop held during November 07-10, 2016 at New Delhi, India.*

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