dsRNA mediated protection against *Cucumber mosaic virus* and *Tomato leaf curl virus* in *Nicotiana benthamiana* and *Solanum lycopersicum*

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CERTIFICATE

This is to certify that the work embodied in this thesis entitled **"dsRNA mediated protection against Cucumber mosaic virus and Tomato leaf curl virus in *Nicotiana benthamiana* and *Solanum lycopersicum*"** has been carried out at the School of Life Sciences, Jawaharlal Nehru University, New Delhi, India and at the Agricultural University of Athens, Greece. 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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-Tsewang Namgial

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1. INTRODUCTION

Plant-infecting viruses are intracellular parasitic organisms, lacking the molecular machinery essential for virus replication, transcription and translation, so they entirely depend on host plant to perform these biological functions. Although plant viruses are less understood as compared to animal viruses, they comprise major threats to the food production security worldwide. Plant viruses may be DNA or RNA viruses. Among DNA viruses, *Geminiviridae* family is one of the largest plant-infecting viruses which are transmitted by insect vectors. RNA viruses belonging to the family *Bromoviridae* are transmitted either mechanically or by aphids and also known to infect various hosts.

Geminiviruses are known to be responsible for several devastating diseases by infecting various economically important crops such as cassava, cotton, chillies, tomatoes, grains, legumes, okra and maize, causing high yield losses. They are distributed worldwide including the tropical and subtropical regions. Geminiviruses, named for their twinned icosahedral particles, possess circular ssDNA genome (Zhang *et al*., 2001). They multiply through rolling circle replication and recombination-dependent mechanism and their genome encodes for six to eight proteins that govern viral replication, movement, insect transmission and suppression of gene silencing. The *International Committee on Taxonomy of Viruses* (ICTV), based on the insect vector and genome organization, has divided the family *Geminiviridae* into seven genera, namely *Becurtovirus, Begomovirus, Curtovirus, Eragrovirus, Mastrevirus, Topocuvirus* and *Turncurtovirus* (Varsani *et al*., 2014). The genera *Mastrevirus*, *Curtovirus* and *Begomovirus* are well studied. Geminiviruses have small circular DNA genomes encoding viral ORFs needed for replication and systemic spread (Hanley-Bowdoin *et al*., 1999).

Viruses belonging to the family *Bromoviridae* including Cucumber mosaic virus (CMV) are worldwide distributed, in both temperate and tropical climates, affecting many agricultural and horticultural crops such as beans, beets, carrots, celery, lettuce, peppers, melons, squash, tomatoes and spinach. CMV contains tripartite ssRNA genome, with the three strands being encapsidated by a single coat protein forming icosahedral virions. Although the virus name derives from the fact that it causes mosaic on the leaves of cucumber plant, CMV infects plants belonging to various families. The three viral RNAs

encode for different proteins. In particular, RNA1 encodes for 1a protein (necessary for replication of the viral genome), RNA2 encodes for protein 2a (involved in viral genome replication) and 2b (CMV 2b protein suppress RNA silencing mechanism and RNA3 encodes for two proteins including protein 3a (movement protein) and the coat protein (CP).

Tomato (*Solanum lycopersicum* L.) in the family *Solanaceace* is one of the important vegetable crops cultivated worldeide. This crop is highly susceptible to diverse type of viruses; in which leaf curl causing geminiviruses (e.g. ToLCV) and CMV pose major threat to this crop (García-Cano *et al*., 2006). Therefore, it is important to develop antiviral strategies against these viruses in tomato.

RNA interference (RNAi) or RNA silencing is considered to be one of the most effective methods for controlling viral infection in plants. RNAi is initiated by the presence of viral double stranded (ds) RNA molecules that derive either from viral replication intermediates or from RNA hairpins that possess by definition a secondary structure. The dsRNA molecules are recognized in plants by specific RNase type III-like enzymes of the host designated as DICER-like proteins (DCL) which cleave the long dsRNA fragments into smaller dsRNA fragments, called small interfering RNAs (siRNAs), that are 21-27 nucleotides in size (Blevins *et al*., 2006). Subsequently, the siRNAs are incorporated into the RNA inhibition and silencing complex (RISC), with the main part comprised of Argonaute (AGO) protein family. AGO proteins possess slicer activity against target mRNAs. The degradation of target mRNAs is facilitated by sequence complementarity with the one of the siRNA strands, the so-called guide-strand (Hammond *et al*., 2001). The other strand of the siRNA duplex, named passenger strand, is finally degraded. Another possibility is that the siRNA guide strand, after binding to the complementary target mRNAs, could serve as a primer for the action of specific RNA dependent RNA polymerases (RDRs) of the host generating more dsRNAs that could be recognized again by the DCLs (amplification step of RNA silencing). After completion of many similar cycles a huge population of secondary siRNAs will be generated, ultimately leading to an activated plant defence against viruses. It is believed that the activation of plant defence RNAi inhibits the multiplication of the virus via a post transcriptional gene silencing (PTGS) mechanism, one of the means leading to RNA silencing. The final outcome of the

invasion of the virus will depend on the balance between the rate of virus multiplication as well as its silencing suppressor efficacy and the effectiveness of the plant defence mechanisms.

RNA silencing may occur also at transcriptional level by regulating either host or viral gene expression and this is known as transcriptional gene silencing (TGS). In the cytoplasm, small RNAs induce posttranscriptional gene silencing (PTGS) by targeting complementary mRNAs for its degradation or translational repression of mRNA. On the other hand, in the nucleus, small RNAs elicit TGS to the homologous regions through cytosine methylation and/or histone methylation, (Matzke *et al*., 2014). Small-directed DNA methylation also known as RNA-directed DNA methylation (RdDM) is a counter-defense mechanism evolved by plants to combat against invading geminiviruses. In canonical RdDM, ~24 nt siRNAs that are processed by DCL3 are loaded into Argonaute 4 (AGO4) and then targeted to RdDM loci. The AGO4-siRNA complex recruits the DNA methyltransferase which catalyzes the *de novo* DNA methylation at the promoter region of most DNA viruses including geminiviruses (Xie *et al*., 2015; Hanley-Bowdoin *et al*., 2013). Such DNA methylation inhibits the transcription of viral gene and as a result blocks the viral replication.

In many cases, plant protection against viruses has been achieved through transgenic approaches (Waterhouse *et al*., 1998; Smith *et al*., 2000) and other methods including viral cross protection in plants (Zhou and Zhou, 2012). However, direct exogenous application of dsRNA molecules that are derived from viral sequences provides an alternative non-transgenic strategy to induce RNAi for sequence specific degradation of target virus (Tenllado *et al*., 2001; Holeva *et al*., 2007; Yin *et al*., 2009; Gan *et al*., 2010).

1.1 OBJECTIVES

The present study was carried out with the following objectives:

1) Identification of the conserved regions in ToLCGV and *in vitro* synthesis of dsRNA molecules specific to ToLGCV and CMV.
2) Generation of a fusion molecule including both ToLGCV and CMV sequences.
3) To determine the effect of synthetic dsRNA against ToLGCV in *N. benthamiana* and *S. lycopersicum* and CMV in *N. tabacum* plants.

2. REVIEW OF LITERATURE

2.1 Tomato

Tomato (*Solanum lycopersicum* L.) is an important vegetable crops cultivated globally. Because of its life cycle and the availability of substantial genetic information and resources, it represents a model species for all the fleshly fruits. It belongs to the nightshade family *Solanaceae* under the genus *Solanum* which also includes potato. Tomato plant is believed to originate from Southern America in the region of modern day Peru and Ecuador but now is grown throughout the world, in different environments including dry and desert regions. Farmers decided to plant and grow tomatoes rather than picking them from the wild but when or where this happened is not known till to date. People believed that tomatoes were first grown in Peru and Ecuador, where wild tomatoes are still found. Others suggested that tomatoes were cultivated by human in Mexico.

Worldwide, China is listed at the top in tomato production followed by India (at the second position) and other countries including United States of America, Spain, Egypt, Turkey, Iran, Italy, Brazil and Mexico etc. Indian tomato production is around 18.73 million tones, which is about 2.46 % of the total world tomato production. In India the major states producing tomato are: Andhra Pradesh, Arunachal Pradesh, Assam, Bihar Chhattisgarh, Karnataka, Madhya Pradesh, Telangana, Gujarat, Maharashtra, West Bengal, Himachal Pradesh and others. Andhra Pradesh tops the list in tomato production followed by Karnataka, Madhya Pradesh and Telangana.

Tomato is an herbaceous plant, 1-2 meter in height, with a weak woody stem and fruit size varies from cherry tomatoes of 1-2 cm in size in diameter. When ripe, most of the tomato varieties produce red color fruits. Tomato is one of the most popular vegetables grown in India because of its high nutritional value and the wide range of ecological diversity that is suitable to tomato growth. It is rich in vitamin sources and organic acid. Tomato fruits are used for the preparation of variety of foods and food products such as pickle, soup, salad and tomato ketchup which are consumed raw. Tomato also has many medicinal values; it is an excellent source of vitamin C and is also known as poor man's orange (Satyanarayana *et al*., 1992). Its genome is one of the smallest one in comparable to the other *Solanaceae* members. Some varieties of wild type tomato plants possess some

resistance gene like Ty-1, Ty-2 and Ty-3 (Butterbach *et al*., 2014). The Ty-1 resistance gene was recently shown to encode for an enzyme involved in RNA silencing pathway, RNA-dependent RNA polymerase and it has been highlighted that Ty-1 confers geminivirus resistance to viral DNA methylation.

2.2 Leaf curl disease of tomato

2.2.1 The disease

In India, tomato production is severely affected due to pathogen attack including viruses causing huge economic losses and threats to the production of tomato. Tomato leaf curl disease (ToLCD) is considered one of the economically important plant diseases causing serious crop losses in tomatoes. Tomato is the major host plant for the viruses causing leaf curl disease but other potential host plants also exist including cassava, cotton, grain, legumes and other vegetables. The symptoms of ToLCD are characterized by leaf curling, leaf blistering, vein banding and stunting of plant growth (Fig. 2.1). In severe stage, fruit setting gets affected and sometimes, it bear no fruits. Affected plants tend to be distributed randomly or in patches. The occurrence of this disease in India was first reported by Vasudev and Sam Raj (1948).

(a) (b)

Figure 2.1. Symptoms observed on tomato plants infected with *Tomato leaf curl Gujarat virus* (a) shrinking of tomato leaves, (b) leaf curling.

2.2.2 The causal agent

ToLCD is an emerging disease and has been reported from almost all tomato growing areas and is caused by viruses that belong to *Begomovirus* (Family: *Geminiviridae*).

2.2.3 Taxonomy

Geminiviruses are named because of their twinned icosahedra particles that are 18-30 nm in size with a total of 22 pentameric capsomers. They possess circular ssDNA genome, the size of which is ranging from 2.5 to 3 kb in length (Zhang *et al*., 2001). Genome organization of the viruses belonging to these genera is given in Figure 2.2. Among them, *Mastrevirus*, *Curtovirus* and *Begomovirus* are the three well studied genera. Members of geminiviruses can infect both monocot and dicot plants, causing diseases such as yellow mosaic disease and leaf curl disease.

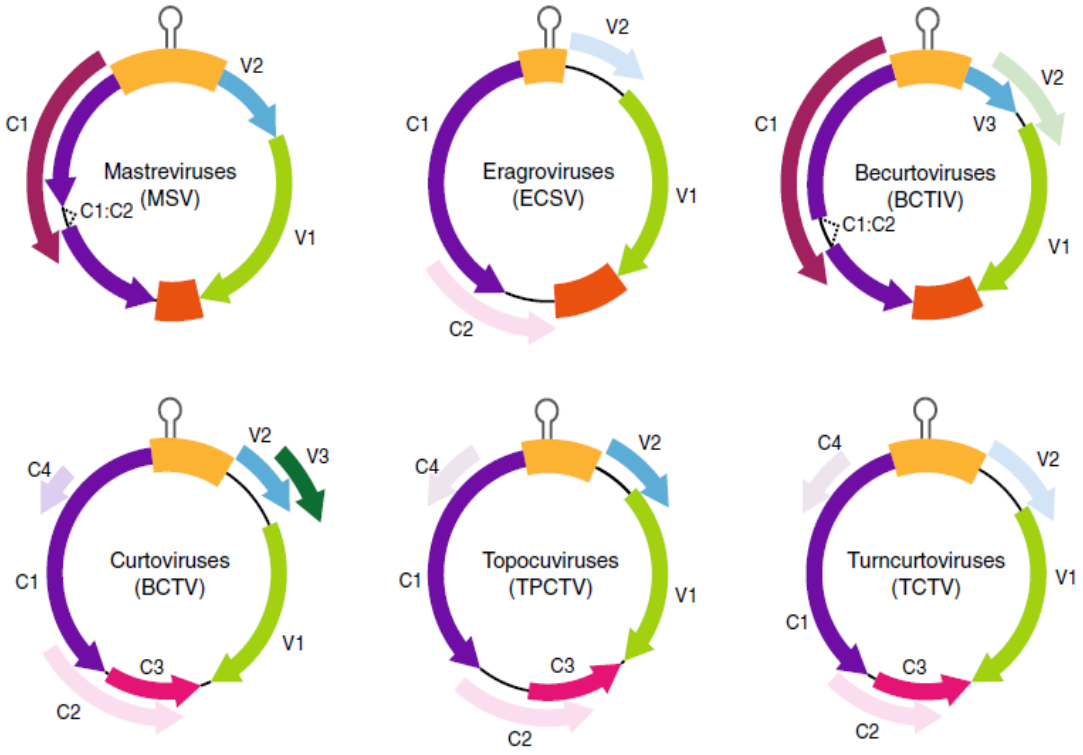

Figure 2.2. Genome organization of various genera of the family, *Geminiviridae* (Adapted from Varsani *et al*., 2014).

2.2.3.1 Genus: *Mastrevirus*

The transmission of mastreviruses is mediated by various leafhopper species and they have a nafrrow host range namely to the species of the *Poaceae* family with two exceptions, *Tobacco yellow dwarf virus* (TYDV) and *Bean yellow dwarf virus* (BeYDV) which infect dicots. The monopartite genome of mastreviruses consists of four ORFs: 2 ORFs each in the virion-sense strand (V1 and V2) and in the complementary-sense strand

(C1 and C2). The virion- and complementary-sense strands are separated by two intergenic regions, small intergenic region (SIR) and large intergenic region (LIR) containing the origin of replication (ori) (Kammann *et al*., 1991). The unspliced and spliced transcripts of mastreviruses generate Rep and RepA proteins, respectively.

2.2.3.2 Genus: *Curtovirus*

The transmission of curtoviruses is mediated by various leafhopper species like mastreviruses and it has a wide range of host plants including dicotyledonous plants. *Curtovirus* is monopartite and the genome organization typically consists of seven ORFs and a single intergenic region containing the *ori.* The curtovirus genome also has V3 ORF on the virion-sense strand (Stanley *et al*., 2005).

2.2.3.3 Genus: *Topocuvirus*

The transmission of topocuviruses is mediated either by a leafhopper or by a treehopper, and they infect dicotyledonous plants. This genus is recently separated from curtoviruses. The virus is monopartite with genome organization common to mastreviruses and begomoviruses, revealing that it has evolved as a result of natural recombination event between the two genera (Fauquet and Mayo, 2001).

2.2.3.4 Genus*: Becurtovirus*

It consists of two viral species: *Beet curly top Iran virus* (BCTIV) and *Spinach curly top Arizona virus* (SCTAV) (Varsani *et al.*, 2014). BCTIV has been isolated from the dicot plants exclusively in Iran, while the only SCTAV isolate was reported from Arizona (Hernandez-Zepeda *et al.*, 2013). The nonanucleotide sequence motif in becurtoviruses is found to be unusual (TAAGATTCC) than all other known geminiviruses.

2.2.3.5 Genus: *Eragrovirus*

The type species of *Eragrovirus* is *Eragrostis curvula streak virus* (ECSV) which has been identified from *Eragrostis curvula* in South Africa. Similar to becurtoviruses, all the ECSV isolates have an unusual nonanucleotide sequence (TAAGATTCC).

2.2.3.6 Genus: *Turncurtovirus*

The type species is *Turnip curly top virus* and 20 of these isolates were isolated from *Brassica rapa* and *Raphanus sativus* (Briddon *et al*., 2010; Inoue-Nagata *et al*., 2004). However, this viral species has been also detected by PCR in various other plant species *(*Heydamejad *et al*., 2013). These members have TAATATTAC as the nonanucleotide motif.

2.2.3.7 Genus: *Begomovirus*

Begomovirus is the largest genus in the family *Geminiviridae*. Begomoviruses possess a genome size of approximately 2.7 kb and replicate via rolling circle mode of replication and by recombination-dependent replication. They have been further classified into monopartite begomoviruses (one circular single stranded DNA molecule, DNA-A-like) or bipartite begomoviruses (two circular single stranded DNA molecules, DNA-A and DNA-B) (Fig. 2.3). The proteins encoded by DNA-A molecules are reported to be involved in virus replication while DNA-B encoded proteins are involved in virus movement. Monopartite begomoviruses are often found to be associated with satellite DNA molecules, named, alphasatellites and betasatellites. Betasatellites encode a protein, βC1 which is shown to be a pathogenicity determinant, whereas alphasatellite encode their own replication initiator for their autonomous replication.

2.2.4 Genome organization and function

In geminiviruses, transcription occurs bidirectionally through dsDNA replicative forms (Sunter *et al*., 1989). The intergenic regions of both DNA-A as well as DNA-B contain origin of replication.

2.2.4.1 DNA-A

The DNA-A component encoded 6 ORFs: 2 in virion strand and 5 in complementary-sense. They encode proteins involved in virus replication, transcription, encapsidation and systemic movement of viral DNAs. In begomoviruses, AV2 encodes for pre-coat protein (PCP) is present only in the old world begomoviruses. PCP has been found to be localized at the cell periphery and assist in virus movement (Rothenstein *et al.*, 2007; Priyadarshini *et al*., 2011). The PCP also reported to suppress host's RNA silencing by interacting with SUPPRESSOR OF GENE SILENCING 3 (SGS3) protein (Glick *et al*., 2008).

Table 2.1. Begomovirus- encoded proteins and their functions

Genomic component	Protein encoded	Functions	References
DNA-A	Coat protein	Viral genome packing, Insect transmission, Translocation of viral DNA into nucleus	Mullineaux *et a*l., 1984; Briddon *et al*., 1990; Liu *et al*., 1999
	Pre-coat protein	Suppression of gene silencing	Chowda-Reddy *et al*., 2008; Sharma *et al*., 2010; Glick *et al*., 2008
	Replication initiator protein (Rep)	Replication, Transcription, Suppression of gene silencing	Laufs *et al*., 1995; Hanley-Bowdoin *et al*., 2004; 2013
	Transcriptional activator protein (TrAP)	Activates CP and MP gene expression, Pathogenicity determinant, Suppression of gene silencing	Sunter and Bisaro, 1992 Hong *et al*., 1996; Voinnet *et al*., 1999; Wang *et al*., 2005
	Replication enhancer protein (REn)	Supports viral DNA replication by enhancing Rep mediated ATPase activity	Sunter *et al*., 1990; Pasumarthy *et al*., 2010
	C4/AC4	Silencing suppressor, Cell cycle regulator	Vanitharani *et al*., 2004; Lai *et al*., 2009
DNA-B	Nuclear Shuttle protein (NSP)	Trafficking viral ssDNA between nucleus and cytoplasm, Non-specific DNA binding	Noueiry *et al*., 1994; Rojas *et al*., 1998
	Movement protein (MP)	Intra and intercellular transport of virus, Endosome trafficking	Zhang *et al*., 2001; Lewis and Lazarowitz, 2010

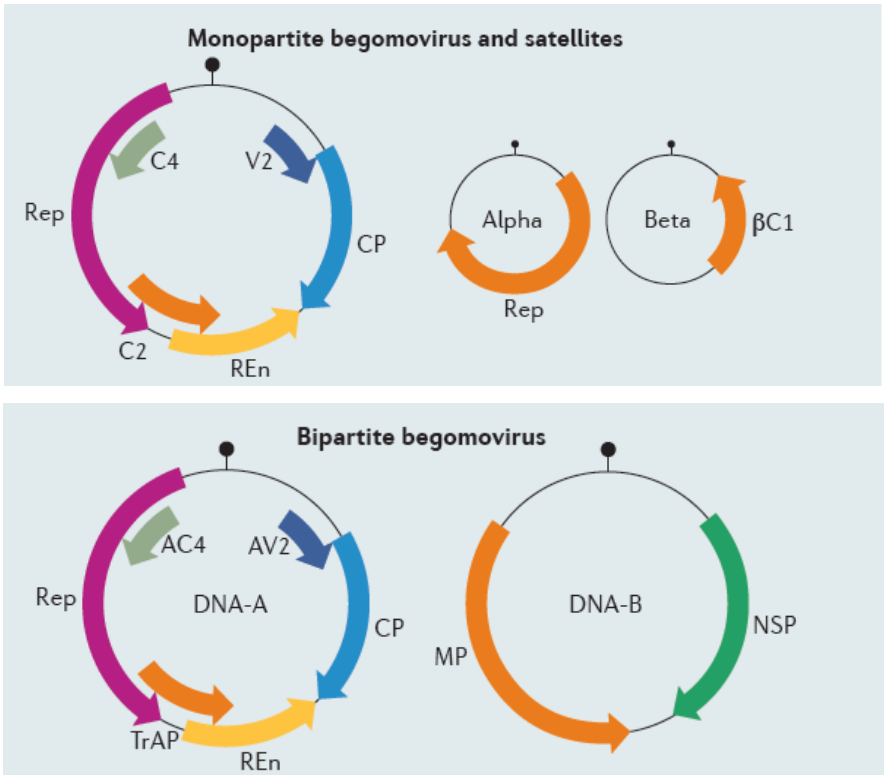

Figure 2.3. Genome organization of begomoviruses and their associated satellite components (Adapted from Hanley-Bowdoin *et al*., 2013).

The function of AV2, in *Mungbean yellow mosaic Indian virus* (MYMIV) had been investigated by mutagenesis and it was found that AV2 affect viral replication in MYMIV (Rouhibakhsh *et al*., 2011).

AV1 encodes the viral coat protein and helps in insect transmission. The exchange of *African cassava mosaic virus* (ACMV)-CP with CP of BCTV in order to investigate the relation between virus and vector transmission suggested that viral CP may be the determinant of vector specificity (Briddon et *al*., 1990). After transmission through whitefly, geminivirus genome needs to be transported to the plant nucleus for replication. N-terminal region of CP of monopartite and bipartite viruses has a nuclear localizing signal (NLS). The presence of YFP-CP fusion protein in the nucleus and the fact that deletion of the N terminal NLS of CP restricted viral distribution to the cytoplasm, suggested that CP is also involved in intracellular movement (Poornima *et al*., 2011). In monopartite viruses, CP also have a nuclear export signal (NES) located in its C-terminal half (Rhee *et al*., 2000), which suggests its role as nuclear shuttle protein (NSP).

Replication initiator protein (Rep) is encoded by the ORF AC1 in the complimentary-sense strand and is the first protein to be synthesized after viral uncoating. It is the highly conserved virus-encoded multifunctional protein which is very essential for virus replication. The three main regions of Rep protein are: N-terminal region, central region and C-terminal region. The N-terminal region is responsible for DNA binding and cleavage function, the central region for oligomerization, which overlaps with the DNA binding domain, and the C-terminal region includes an ATPase domain, encompassing Walker A and Walker B motifs. The presence of motif I, II and III in the N-terminal of Rep suggests a strong evolutionary link between this protein and eubacterial plasmids (Koonin *et a*l., 1992). Replication initiator protein (Rep) binds to the Rep complex for successful completion of viral replication. Rep, a multifunctional protein, possesses structural regions which are universally present in rolling-circle replication initiator proteins and, as mentioned above, these are the N-terminal region, central oligomerization region and C-terminal region.

Generation of different deletion mutants of Rep confirms that the N-terminal region has three functional motifs. Motif I is involved in DNA binding. The Rep complex of Motif I, II and III cleaves the phosphodiester bond between the last T and A in the invariant loop sequence. Motif II possesses a metal binding site and the catalytic site needed for DNA cleavage and ligation is present in motif III (Arguello-Astorga *et al.*, 2004; Laufs *et al*., 1995; Orozco *et al*., 1998). The nicking activity of N terminus motif II does not require ATP (Hanley-Bowdoin *et al*., 1999) but the cleavage activity is enhanced in presence of ATP (Pant *et al*., 2001).

The central region possesses the oligomerization domain (Settlage *et al*., 1996). The oligomerization domain was mapped by generating a series of truncated Rep and by assaying their ability to interact with full-length Rep. It was found that oligomerization domain lies between amino acids 120 and 181 (Beverly *et al.,* 1999). The oligomerization step is required for their replication (Orozco *et al*., 2000). The oligomerization domain allows interaction of Rep with several cellular factors. Finally, both the Rep and RepA oligomerization was shown to be pH sensitive, with pH $\leq$ 7.0 favoring oligomerization and pH $\geq$ 7.4 resulting in monomers (Missich *et al.,* 2000).

C-terminal region, encompassing the AAA+ domain, imparts Rep with ATPase and helicase activity and the fragment (120-182 amino acid) behave as an efficient helicase and ATPase. The ATPase activity of Rep is essential for the viral DNA replication in host plant cells, and the Rep having mutation in ATP binding motif is unable to support DNA replication *in vivo* (Desbiez *et al*., 1995). The oligomerization domain of the Rep has been demonstrated to be involved in the helicase acitivity of this protein. The oligomerization domain is required for the unwinding activity and it was shown that the MYMIV Rep forms higher-order oligomers (24mers) (Choudhury *et al*., 2006). Rep depends on oligomerization for unwinding activity, besides this they also depend on ATP binding and helicase activity. It has also been found that Rep was unable to utilize ADP as the energy source, and in presence of ADP, Rep activity is completely inhibited.

AC2 encodes for transcription activator protein (TrAP) which is involved in transcriptional control and gene silencing. It activates the transcription of virus-encoded late genes (Sunter and Bisaro, 1991, 1992). AC2 of TGMV and C2 of BCTV have been demonstrated to affect SNF1 kinase and adenosine kinase (ADK) activities (Sunter *et al*., 2001; Hao *et al*., 2003; Raja *et al*., 2008; Wang *et al*., 2003). TrAP also been shown to be a suppressor of RNA silencing. The NLS or Zn finger has been shown to be needed for suppression of silencing (Trinks *et al*., 2005). Silencing maintenance mechanisms can change during development or in response to stress (Jamie *et al*., 2015).

AC3 encodes for replication enhancer (REn) protein which is involved in enhancing the replication of geminiviruses and it is conserved across all the begomoviral species. Sunter *et al.* (1990) have shown that mutation in the AC3 created a large reduction in the levels of ssDNA and dsDNA. They proposed that replication depends on the interaction between Rep and the REn. REn interacts with retinoblastoma-related protein (pRb) and it was found that Rep and pRB bind to the same region on the REn (Settlage *et al*., 2001). In addition to this, by using yeast two-hybrid and pull-down assays, it was demonstrated that REn interacts with proliferating cell nuclear antigen of tomato (LePCNA) and the interaction between PCNA and REn/Rep takes place during virus infection (Castillo *et al*., 2003). Furthermore, it was found that SlNAC1 expression and upregulation requires REn. Pasumarthy *et al.* (2010) has demonstrated that *Tomato leaf curl Kerela virus*

(ToLCKeV) REn protein has been demonstrated to enhance the *in vitro* ATPase activity of Rep, thereby leading to increased replication of viral DNA.

AC4 encodes for a small protein, with the gene completely nested within the Rep gene, and is involved in the virus movement. Rojas *et al.* (2001) suggested that AC4 protein contains an N-terminal putative myristoylation domain which may be responsible to deliver viral DNA to the plasmodesmata and mediate cell-to-cell transport. A few studies have shown that this protein is also involved in pathogenesis and suppression of RNA silencing (Fondong, 2007). It has demonstrated that AC4 transformed *N. benthamiana* plants exhibit stunted growth, leaf curling and enation on the surface of the leaves as compared to the untransformed plant. In bipartite begomoviruses, it has been found that AC4 protein is involved in binding of miRNA (Chellappan *et al*., 2005). The hairpin RNA targeting AC4 gene has been shown to reduce MYMV DNA accumulation (Sunita *et al*., 2013).

2.2.4.2 DNA-B

DNA-B is present only in bipartite begomoviruses. It encodes for two proteins one each on the viral- and complementary-sense strands designated as BV1 (nuclear shuttle protein, NSP) and BC1 (movement protein, MP), respectively. The products of both genes facilitate viral movement. BV1 encodes for NSP that forms a complex with ssDNA by interacting with positively charged domain and this complex of viral DNA movement has the ability to bind with ss-DNA or dsDNA and can transport the viral DNA to the neighboring cells (Gilbertson *et al*., 2003; Rojas *et al*., 2001). BC1 (MP) takes over and transfers the viral DNA to the adjacent cells through plasmodesmata. BC1 functions as MP by two manners: 1) by increasing the size exclusion limit of plasmodesmata (Rojas *et al*., 1998), and 2) by inducing the formation of a tubular structure from endoplasmic reticulum which facilitates movement of viruses between adjacent cells (Ward *et al*., 1997). BC1 is involved in pathogenicity as transgenic tomato and tobacco plants expressing BC1 showed characteristic symptoms of viral infection (Duan *et al*., 1997; Hou *et al*., 2000; Saunders, 2001). BV1-BC1 interaction is vital for virus movement, for some bipartite viruses such as Squash leaf curl virus (SLCV). In the presence of defective or mutated BV1 gene, the CP can provide an alternative function to BV1 gene of DNA-B

components (Qin *et al*., 1998). Thus for monopartite begomoviruses CP and C4 may act similar to BV1-BC1 interaction for virus movement.

2.2.4.3 Betasatellite DNA (DNA-β)

The majority of the begomoviral diseases are caused by bipartite begomovirus infections. Nevertheless a number of diseases of the Old World are associated with the monopartite begomoviruses, with the exception of several diseases of tomato, most of the diseases are caused by a monopartite begomovirus which are association with essential satellite component discovered as betasatellite DNA (DNA-β) (Briddon *et al*., 2006). These molecules are single-stranded DNA, symptom-modulating, which are dependent on a helper virus for replication, encapsidation and transmission by whitefly vectors. The replication of DNA-β can also be supported by other geminiviruses other than ToLCV. In many viruses, the replication of DNA satellites and viral genome suppresses the replication of the helper virus genome and this result in disease reduction. However, some of the betasatellites enhance disease symptoms that are manifested due to the helper virus or produce a novel symptom which is usually not related to helper virus infection (Roossinck *et al*., 1992).

In the case of ToLCV, the presence of satellite(s) has no considerable effect on viral replication and symptom expression. In contrast to that, some other begomoviruses need DNA β satellites associated with them for infection and induction of disease symptoms in some host plants (Briddon *et al*., 2001; Jose and Usha, 2003; Saunders *et al*., 2000; Zhou *et al*., 2003). In their composition, betasatellites, encode a single gene BetaC1 (βC1, 13 kDa protein), in the complementary-sense strand of their genome, contain an adenine-rich region as well as a satellite-conserved region (SCR), which is highly conserved among all the betasatellites known today (Briddon *et al*., 2003). Betasatellites have a nonanucleotide (TAATATT/AC) sequence which shares homology with the helper virus. Strikingly, βC1 has a similar function as ASYMMETRIC LEAVES 2 (AS2) and directly binds with ASYMMETRIC LEAVES 1 (AS1), thus altering leaf development by suppressing the expression of selective jasmonic acid (JA)-responsive genes (Yang *et al*., 2008). Yang *et al.* (2011) reported that βC1 inhibits the methylation and transcriptional gene silencing (TGS) by interacting with SAHH protein required for TGS. The intracellular distribution of βC1 suggested that it has a role in transporting the DNA A from the nuclear site of

replication to the plasmodesmatal exit sites of the infected cell (Saeed *et al*., 2007). It was also reported that the βC1 increases the performance of transmission by whitefly that carries the geminivirus by the suppression of plant terpene biosynthesis (Li *et al*., 2014).

2.2.4.4 Alphasatellites (DNA-1)

Alphasatellites (previously called DNA-1) are single-stranded DNA molecules identified in begomovirus-infected plants. Xie *et al*. (2010) have sequenced thirty-three alphasatellite molecules ranging from 1360 to 1376 nucleotides. All alphasatellites contain three conserved features: a single open reading frame (Rep), a conserved hairpin structure and an adenine-rich (A-rich) region. These satellite-like molecules depend on the helper virus for movement, encapsidation and vector transmission but they replicate independently of their helper virus. Like nanovirus, single ORF of the alphasatellite encodes a replication initiator protein (Rep) that catalyses initiation of ssDNA replication. Nanoviruses are single-stranded DNA (ssDNA) plant viruses but they possess six to eight genomic DNA components of approximately 1 kb each and encapsidated in icosahedral particles. It has been assumed that some of the genomic components have adapted to geminiviruses during the course of evolution, and the resulting helper virus-dependent individual molecules are termed as DNA 1 satellites, or α-satellites (Paprotka *et al*., 2010). Alphasatellites (including a conserved nonanucleotide 'TAGTATT/AC'), may have adapted to whitefly transmission after begomoviruses and nanoviruses had mix infected common host plants (Mansoor *et al*., 1999; Saunders *et al*., 2002; Saunders and Stanley, 1999).

2.3 *Cucumber mosaic virus* (CMV)

CMV has been listed as one of the top 10 most devastating plant viruses (Scholthof *et al*., 2011; Rybicki, 2015) and it belongs to the genera *Cucumovirus* of the family *Bromoviridae*. CMV is sub-divided into two major subgroups (I and II). The mosaic disease caused by CMV was first documented in 1916. CMV strains have very broad host range and infect several monocot and eudicot plants including fruit crops, vegetables and ornamentals (Scholthof *et al*., 2011). CMV is transmitted by aphids and although with low frequencies, seed transmission of CMV occurs in several weeds (Palukaitis *et al*., 1992). CMV genome contains five genes which are involved in virus

replication, RNA silencing suppression and systemic spread. CMV 2b protein and coat protein are expressed from subgenomic RNAs as RNA 4A and RNA 4 (Palukaitis and García-Arenal, 2003; Palukaitis and García-Arenal, 2003. Scholthof *et al*., 2011). Tomato losses due to CMV infection have been reported 25 to 50% in China (Tien and Wu, 1991). In the absence of any effective control measures, CMV incidence has increased in tropical and subtropical regions (Scholthof *et al*., 2011). Management of CMV through vector control does not yield a positive response in disease eradication (Scholthof *et al*., 2011; Tien and Wu, 1991).

2.4 Control of virus diseases

Plant pathogens, continuously posing stress on plants for their survival, manifest their presence through abnormal morphology, physiology and low productivity of the infected plants. As per the report of the International Committee on Taxonomy of Viruses (2013), 2828 virus species are known to exist. The classical methods used to combat virus diseases have not been proven sufficient and no strategy is known to counter viruses through use of chemicals under field condition. The resistance against viruses has been achieved through protein- as well as nucleic acid-derived methods.

2.4.1 Protein mediated resistance

2.4.1.1 Coat protein mediated resistance (CPMR)

The first demonstration of viral sequences mediated resistance in transgenic plants was shown by Beachy and colleagues (Abel *et al*., 1986). Earlier studies conducted with *Tobacco mosaic virus* (TMV), *Alfalfa mosaic virus* (AlMV), and *Tobacco streak virus* (TSV) had shown that the resistance conferred by endogenous CP could be overcome by inoculation with viral RNA (Loesch Fries *et al*., 1987; Nelson *et al*., 1987). Replication of PVX was reduced in transgenic plants and transgenic protoplasts expressing wild type or mutant (CP^{T28W}) TMV CP. Similarly transgenic protoplasts expressing *Potato virus X* (PVX) CP conferred transient protection against infection with TMV and TMV RNA. PVX CP conferred heterologous interference to PVX and TMV infection (Bazzini *et al*., 2006). Plants transformed with truncated, antisense or non-expressing CP genes of *Cucumber mosaic virus* (CMV), *Potato leaf roll virus* (PLRV), PVX, and *Potato virus Y*

(PVY) provided measurable protection or even complete immunity against the corresponding viruses (Lindbo *et al*., 1992a and 1992b).

2.4.1.2 Movement protein mediated resistance (MPMR)

Movement proteins (MP) encoded by the plant viruses are responsible for their local (cell to cell) and systemic movement in the hosts enabling infection to reach distant sites. Transgenic tobacco plants expressing defective TMV movement protein lacking three N-terminal amino acids (Cooper *et al*., 1995) or a temperature sensitive mutant (Malyshenko *et al*., 1993) of it showed delayed and attenuated symptoms. Chimeric MP constructs made with N- terminal sequences of *Tomato golden mosaic virus* (TGMV) BLl and carboxy-terminal sequences of *African cassava mosaic virus* (ACMV) homologue BCl, inhibit the replication and symptom development by ACMV (Von Arnim and Stanley, 1992). Transgenic plants expressing the 13 kDa gene bearing mutation in a region conserved among all viruses were shown to be resistant to systemic infection by *White clover mosaic virus* (WClMV).

2.4.1.3 Replicase protein mediated resistance (RPMR)

RPMR involves the expression of virus genes encoding full-length, truncated or mutant form of virus replicase protein. Expression of 54 kDa full-length replicase gene of *Pea early browning virus* (PEBV) conferred high degree of resistance to *Broad bean yellow band virus* and an uncharacterized isolate of PEBV but not to other tobraviruses (MacFarlane and Davies, 1992). Wintermantel and Zaitlin (2000) showed that transgene translatability is required for effective resistance against CMV. Expression of full-length, mutated or truncated rep gene derived from ssDNA viruses (geminiviruses) and dsDNA virus, *Rice tungro bacilliform virus* (RTBV) had been shown to provide resistance in transgenic plants. *N. benthamiana* transgenic plants expressing truncated or full-length rep (AC1) gene from *Tomato yellow leaf curl virus* (TYLCV) and *African cassava mosaic virus* (ACMV) remained symptom free till four weeks post inoculation and accumulated 70% less viral DNA in inoculated leaf discs and protoplasts as compared to infected control plants (Hong and Stanley, 1996; Noris *et al*., 1996). Transient expression of the oligomerization domain of Rep of *Tomato leaf curl New Delhi virus* interfered with DNA accumulation of *African Cassava Mosaic Virus, Potato yellow mosaic virus and Pepper huasteco virus* (Chatterji *et al*., 2001).

2.4.2 Nucleic acid mediated resistance

In 1990, two different groups attempted to generate transgenic petunias that were expected to be purpler due to introduction of a transgene designed to overexpress the chalcone synthase enzyme. Surprisingly, over-expression of chalcone synthase transgenes did not increase coloration. Instead, some flowers were completely colorless (white), and others showed patterns such as loss of pigmentation along the veins. This phenomenon was then termed as cosuppression (Napoli *et al*., 1990). Later it was found that transgene derived small RNAs (21-24nts) are mediators of this sequence specific recognition and degradation of homologous RNA (Hamilton and Baulcombe, 1999).

RNA interference (RNAi) is an evolutionarily conserved gene regulatory mechanism triggered by double-stranded RNA (dsRNA) in which expression of a gene is inhibited through specific degradation of mRNA (post transcriptional gene silencing), inhibition of transcription of transposons and repetitive DNA elements (transcriptional gene silencing) and suppression of gene expression at the translational level (Hamilton and Baulcombe, 1999). Several attempts have been made to generate virus resistant transgenic plants exploiting RNAi mechanism against both RNA and DNA viruses. Based on the siRNAs-mediated RNA silencing mechanism, constructs were designed against viral genes that play important roles in their life cycle. The success of generating virus resistant transgenic plants depends upon following three criteria (1) approach used to induce RNAi; (2) selection of RNAi targets; and (3) pathogens targeted by RNAi.

2.4.2.1 Transgene-mediated resistance through sense or antisense viral sequences

In several cases of protein mediated resistance, the mechanism of resistance could not be explained as transgene encoded protein could not be detected and correlation between the amount of protein and the level of resistance was not observed. Therefore, it was postulated that transgene transcripts might be responsible for resistance. Further investigations into the role of RNA revealed that transgene transcripts degraded the homologous RNA in a sequence specific manner. Kawchuk (1991) developed resistance in potato against *Potato leaf roll virus* (PLRV) using CP derived sense and antisense constructs. Van Der Vlugt *et al*., (1992) generated transgenic tobacco plants resistant to *Potato virus Y* (PVYN) infection using CP cistron.

2.4.2.2 Virus-derived inverted repeats (IRs)/hairpin RNAi transgene-mediated resistance

One of the major drawbacks of sense/antisense transgene constructs mediated resistance is instability and delayed resistance or resistance with low efficacy. IRs can generate effective RNA-mediated resistance against a wide range of RNA viruses where sense-RNA-mediated silencing was not effective (Chen *et al*., 2004). Interestingly, plant infecting DNA viruses like caulimoviruses and geminiviruses are also known to be targets of host endogenous RNA silencing pathway (Al-Khaff *et al*., 1998; Lucioli *et al*., 2003). Transgenic tomato expressing IR of CP plants showed resistance to TYLCV by targeting (Zrachya *et al*., 2007).

2.4.2.3 Artificial miRNA mediated resistance

Plant miRNA are important regulators of diverse developmental processes, metabolism and stress. This technology was used to modify endogenous miRNA precursors to generate amiRNAs for specific gene silencing in different plants species (Molnar *et al*., 2009; Ossowski *et al*., 2008; Qu *et al*., 2007). Qu et al. (2007) compared siRNA and miRNA mediated approach against CMV. They designed ihpRNAi construct and miRNA construct targeting the same conserved site on 2b protein. They observed that miRNA construct was superior to ihpRNAi construct in anti-CMV mechanism in both transient and transgenic systems. The resistance provided by amiRNA construct was correlated with miRNA expression. Another group developed high degree of resistance against PVX and PVY using amiRNA against the suppressors of PVX and PVY (Ai *et al*., 2011).

3. MATERIALS AND METHODS

3.1 Experimental plants used and growth conditions

Nicotiana benthamiana, Nicotiana tabacum cv. Xanthi *and Solanum lycopersicum* cv. Clodin plants were grown and maintained in a greenhouse under the constant conditions of 25±2 °C and 14 hrs/10 hrs light-dark photoperiod. Test plants were grown under insect-proof conditions (Fig. 3.1).

Figure 3.1. *Nicotiana benthamiana* plants were grown and maintained in a greenhouse under the constant conditions of 25±2 °C and 14 hrs/10 hrs light-dark photoperiod.

3.2 Identification of target sequences for double stranded (ds) RNA production and DNA construction

To identify conserved regions among *Tomato leaf curl virus Gujarat Virus* (ToLCGV) different DNA-A genomes belonging to 3 distinct species of begomoviruses (ToLCGV; *Tomato leaf curl New Delhi virus*, ToLCNDV and *Tomato leaf curl Patna virus*, ToLCPav) were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Based on the alignment result, 449 base pair (bp) conserved overlapping region targeting AC1 and AC2 genes and another conserved region of 432 bp targeting AV1 and AV2 in case of TOLCGV were selected. In case of *Cucumber Mosaic Virus* (CMV), a 336 bp conserved region targeting the CMV-2b gene was selected for generating dsRNA (Fig. 3.2).

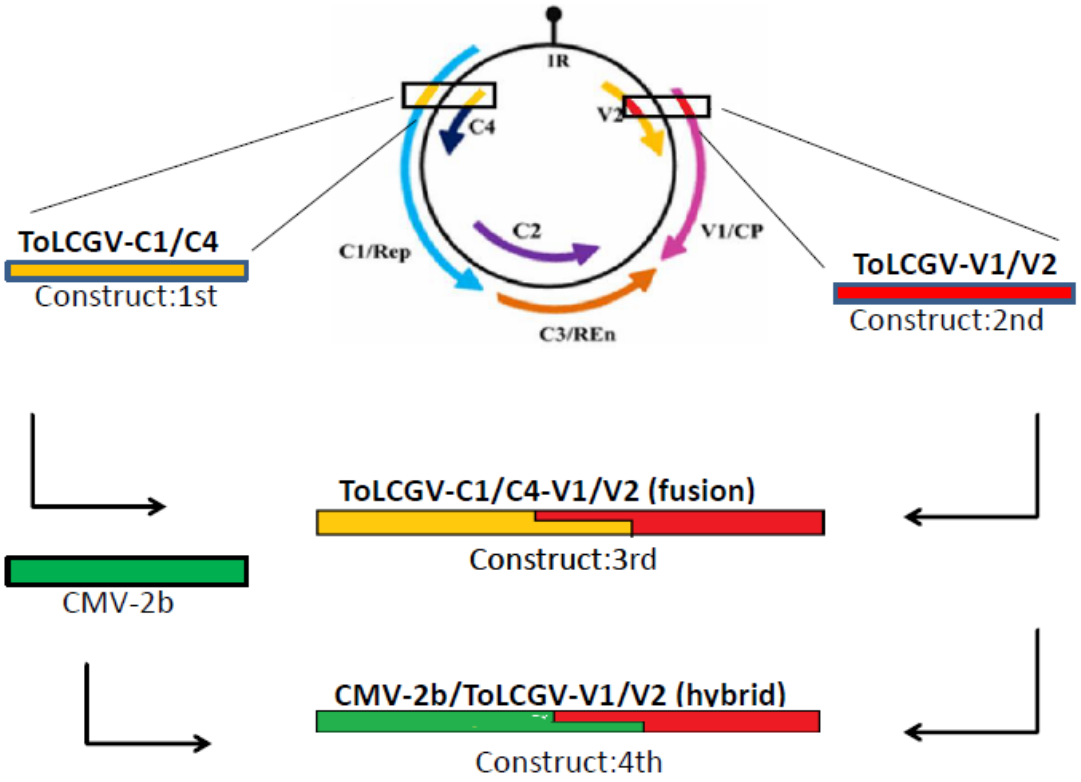

Figure 3.2. Construction of different constructs named as ToLCGV-C1/C4, ToLCGV-V1/V2, ToLCGV-C1/C4-V1/V2 and CMV-2b-ToLCGV-V1/V2.

3.3 Amplification of targeted fragments, incorporation of T7 promoter and *in vitro* transcription

For the production of the dsRNA molecules, the *in vitro* method that is based on a two-step PCR approach and the *in vitro* transcription, was followed as described previously (Voloudakis *et al*., 2015). In brief, the DNA-A genome of ToLCGV was already cloned in pUC18, used as a template for PCR. The targeted fragments of ToLCGV-C1/C4 (449 bp), ToLCGV-1/V2 (432 bp) and CMV-2b (336 bp) gene parts were amplified using Eppendorff Master Cycler (Eppendorf, UK) using primers that possessed a linker sequence at their 5'ends.

3.4 Polymerase chain reaction conditions for all constructs and their primer sequences

3.4.1 ToLCGV_C1/C4 (449 bp) – Construct 1

The PCR conditions were 94˚C-5 min, 94˚C-30 sec, 60˚C-30 sec, 72˚C-1 min and 72˚C-1 min and PCR reaction consisted of: 1 µl of DNA sample, 0.4 µl dNTPs (10 mM), 2 µl

10X KAPA Taq Polymerase buffer, 0.08 µl of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa), and each 0.8 µl of primers ToLCGV_C1/C4_151-F3 and ToLCGV_C1/C4_559-R4; all primers were adjusted to 10 pm/µl and PCR was run for 35 cycles.

Table 3.1. Primers used in this study.

PRIMER NAME	PRIMER SEQUENCE (5' to 3')
Primers for 1st construct	
1) ToLCGV_V1/V2_20-F	GGGGATCCACGAGTTTCCTGAAACCG
2) ToLCGV_V1/V2_451-R	GGGGATCCCCTCGTCACATCGGACAAAC
Primers for 2nd construct	
3) ToLCGV_C1/C4_151-F	GGGGATCCCACGATGATGGGACTCCTCA
4) ToLCGV_C1/C4_599_R	GGGGATCCGCCCACGCTTCAAGTTCTTC
Primers for 3rd construct	
5) ToLCGV_C1/C4-V1/V2-F	AAGCGTGGGCACGAGTTTCC
6) ToLCGV_C1/C4-V1/V2-R	GGAAACTCGTGCCCACGCTT
Primers for 4th construct	
7) 2b_F_LINKER-cor	GGGGATCCATGGAATCGAACGAAG
8) CMV_2b/ToLCGV_V1-V2_F	GGTGCGTTTTGAACGAGTTTCCTG
9) CMV_2b/ToLCGV_V1-V2_R	CAGGAAACTCGTTCAAAACGCACC
10) ToLCGV_V1/V2_451-R	GGGGATCCCCTCGTCACATCGGACAAAC

Sequences highlighted represent "Linker sequences"

IN CASE OF SEQUENCE OF PRIMERS 5 AND 6

Green letters indicate sequence within V1/V2

Black letters indicate sequence within C1/C4

IN CASE OF SEQUENCE OF PRIMERS 8 AND 9

Red letters indicate sequence within CMV-2b

Black letters indicate sequence within V1/V2

3.4.2 ToLCGV_V1/V2 (432 bp) – Construct 2

The PCR conditions were 94˚C-3 min, 94˚C-30 sec, 55˚C-30 sec, 72˚C-30 sec and 72˚C-7 min and PCR reaction consisted of: 1 µl of DNA sample, 0.4 µl dNTPs (10 mM), 2 µl 10X KAPA Taq Polymerase buffer, 0.08 µl of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa), and each 0.8 µl of primers ToLCGV_V1/V2_20-F and ToLCGV_V1/V2_451-R; all primers were adjusted to 10 pm/µl and PCR was run for 35 cycles.

3.4.3 ToLCGV_C1/C4-V1/V2 (449 bp + 432 bp = 881 bp) – Construct 3

Third construct is the fusion construct of two regions of ToLCGV, namely ToLCGV_C1/C4 and ToLCGV_V1/V2. The fusion was done via PCR following the procedure described below:

Step 1) Amplification of ToLCGV_C1/C4 region

The PCR conditions were 94˚C-3 min, 94˚C-30 sec, 55˚C-30 sec, 72˚C-30 sec and 72˚C-7 min and PCR reaction consisted of: 1 µl of DNA sample, 0.4 µl dNTPs (10 mM), 2 µl 10X KAPA Taq Polymerase buffer, 0.08 µl of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa), and each 0.8 µl of primers of ToLCGV_C1/C4_151-F3 and ToLCGV_C1/C4-V1/V2-R6; all primers were adjusted to 10 pm/µl and PCR was run for 35 cycles.

Step 2) Amplification of ToLCGV_V1/V2 region

The conditions were 94˚C-3 min, 94˚C-30 sec, 55˚C-30 sec, 72˚C-30 sec and 72˚C-7 min and PCR reaction consisted of: 1 µl of DNA sample, 0.4 µl dNTPs (10 mM), 2 µl 10X KAPA Taq Polymerase buffer, 0.08 µl of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa), and each 0.8 µl of primers **ToLCGV_C1/C4_151-F** and ToLCGV_V1/V2_451-R; all primers were adjusted to 10 pm/µl and PCR was run for 35 cycles.

Step 3) Joining of ToLCGV_C1/C4 region and ToLCGV_V1/V2 region

Mixed the PCR products of step 1(1:10,000X) and step 2(1:1000X) and used this PCR products as template for 3rd construct. The conditions were 94˚C-3 min, 94˚C-30 sec, 60˚C-30 sec, 72˚C-30 sec and 72˚C-7 min and PCR reaction consisted of: 1 µl of DNA

sample (PCR products of step 1 and 2), 0.4 µl dNTPs (10 mM), 2 µl 10X KAPA Taq Polymerase buffer, 0.08 µl of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa), and each 0.8 µl of primers ToLCGV_C1/C4_151-F and ToLCGV_V1/V2_451-R; all primers were adjusted to 10 pm/µl and PCR was run for 32 cycles.

4) CMV_2b-ToLCGV_V1/V2 (768bp) – Construct 4

Fourth construct is the hybrid construct containing part of CMV and part of ToLCGV, namely part of CMV_2b gene and part of ToLCGV_V1/V2 gene. This single construct is hypothesized that could provide resistance against two serious viral pathogens of tomato (CMV and ToLCGV).

Step 1) Amplification of CMV_2b region (336 bp)

Total RNA extracted from the CMV-infected *N. tabacum* cv Xanthi leaves employing TRIzol (Life Technologies, USA) was used as a template. cDNA was synthesized using 100 ng total RNA in a 10 µl reaction volume employing Superscript II reverse transcriptase (Invitrogen) and a CMV 2b gene specific reverse primer, using the conditions of 25˚C-10 min, 42˚C-50 min and 70˚C-15 min. The targeted fragment of CMV-2b gene (336 bp) was amplified in an Eppendorff Master Cycler using the forward primers that possessed a linker sequence at their 5'ends. The PCR conditions were 94˚C-3 min, 94˚C-30 sec, 55˚C-30 sec, 72˚C-30 sec and 72˚C-7 min and PCR reaction consisted of: 1 µl of DNA sample, 0.4 µl dNTPs (10 mM), 2 µl 10X KAPA Taq Polymerase buffer, 0.08 µl of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa), and each 0.8 µl of primers CMV_2b_F_LINKER-cor and CMV_2b/ToLCGV_V1-V2_R; all primers were adjusted to 10 pm/µl and PCR was run for 35 cycles.

Step 2) Amplification of ToLCGV_V1/V2 region (432 bp)

The PCR conditions were 94˚C-5 min, 94˚C-30 sec, 55˚C-30 sec, 72˚C-30 sec and 72˚C-7 min and PCR reaction consisted of: 1 µl of DNA sample, 0.4 µl dNTPs (10 mM), 2 µl 10X KAPA Taq Polymerase buffer, 0.08 µl of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa), and each 0.8 µl of primers CMV_2b/ToLCGV_V1-V2_F and ToLCGV_V1/V2_451-R; all primers were adjusted to 10 pm/µl and PCR was run for 35 cycles.

Step 3) Joining of CMV_2b region and ToLCGV_V1/V2 region

The PCR products of step 1 and step 2 were used as templates for construct 4th construct. The PCR conditions were 94˚C-5 min, 94˚C-30 sec, 60˚C-30 sec, 72˚C-1 min and 72˚C-7 min and PCR reaction consisted of: 1 µl of DNA sample(PCR products of step 1 and 2), 0.4 µl dNTPs (10 mM), 2 µl 10X KAPA Taq Polymerase buffer, 0.08 µl of KAPA Taq DNA polymerase (KAPA Biosystems, South Africa), and each 0.8 µl of primers CMV_2b_F_LINKER-cor and ToLCGV_V1/V2_451-R ; all primers were adjusted to 10 pm/µl and PCR was run for 35 cycles.

Step 4) Cloning the hybrid construct of CMV_2b and ToLCGV_V1/V2

Because of the existence of many non-specific bands in the first PCR product (Fig. 3.3) of this fourth construct, we isolated DNA by gel extraction of the particular amplicon based on the expected size and cloned this product in the pGEM-T vector. Elution of PCR product was performed by using a PCR Clean up gel extraction kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The elusion product was cloned using the pGEM-T cloning kit (Promega, USA).

3.5 Incorporation of T7 promoter in the four DNA constructs

For all four constructs, the T7 RNA polymerase promoter sequence was introduced at both 5' and 3' ends in the second PCR reaction using a specific T7 linker primer.The PCR conditions were 95˚C-5min, 98˚C-20sec, 50˚C-15 sec, 72˚C-45 sec and 72˚C-7 min for ToLCGV- C1/C4; 95˚C -5 min, 98˚C-20 sec, 50˚C-15 sec, 72˚C-45 sec and 72˚C-7 min for ToLCGV- V1/V2; 94˚C-5 min, 95˚C-20 sec, 55˚C-15 sec, 72˚C-45 sec and 72˚C-7 min for ToLCGV- C1/C4-V1/V2; and 94˚C -5 min, 95˚C-20 sec, 60˚C-30 sec, 72˚C-45 sec and 72˚C-7 min for CMV-2b-ToLCGV-V1/V2; each reaction mixture comprised of 5 µl 5X KAPA polymerase buffer, 1 µl of the 1st PCR products, 0.5 µl of KAPA HiFi DNA polymerase, 0.75 µl dNTPs (10 mM), 1.5 µl volume of T7 linker primer (10 pm/µl) and the final volume was adjusted to 25 µl with water. The 2nd PCR product was used as a template for *in vitro* transcription and production of the respective dsRNA.

3.6 Sequencing and annotation

Full-length sequencing of the clones was carried out commercially at Genewiz (formerly Beckman Coulter Genomics). Vector sequence were recognized and removed by

Vecscreen software available within NCBI (www.ncbi.nlm.nih.gov). Sequences were analysed using BLAST (www.ncbi.nlm.nih.gov).

3.7 *In vitro* transcription for dsRNA production

In vitro transcription was executed by using T7 Ribomax™ Express large scale RNA production system (Promega, USA). The reaction mixture comprised of 10 µl Ribomax™ Express T7 2X buffer, 3 µl of 2nd PCR for each construct, 2 µl enzyme mix and the final volume was adjusted to 20 µl with water. The reaction mixture was maintained at 37˚C for 4 hrs, 85˚C for 10 min and 25˚C for 20 min. Concentration of total nucleic acids was estimated spectrophotometrically by Fisher Scientific Multiskan FC Reader (Thermo Fisher Scientifics, UK), ranging between 8-15 µg/µl.

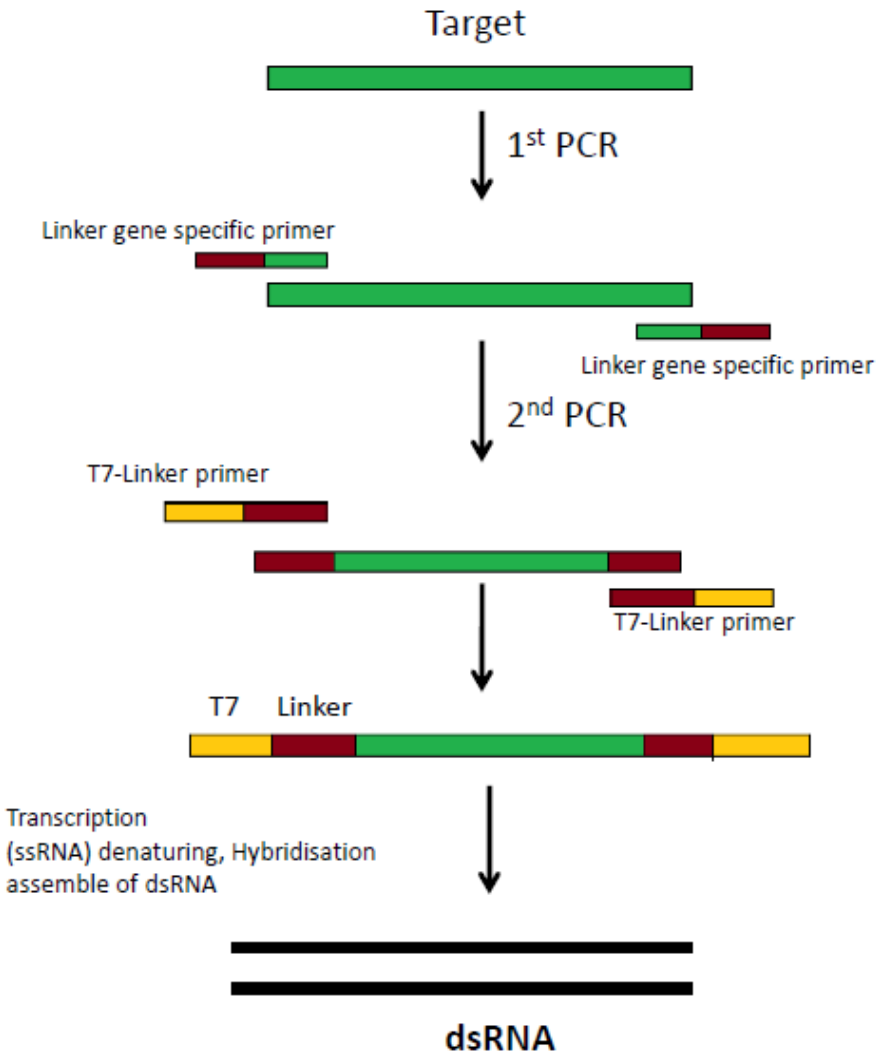

Figure 3.3. Schematic representation of *in vitro* transcription of dsRNA production.

3.8 Exogenous application of dsRNA molecules onto tomato plants, *N. tabacum* cv. Xanthi and *N. benthamiana*

For ToLCGV, *in* vitro produced dsRNAs (construct 1st, 2nd ,3rd and 4th) were manually applied by rubbing the solution onto the upper surface of carborundum dusted leaves of 35 days old tomato and *N. benthamiana* plants ToLCGV was introduced, the same day as dsRNA, in plants via agroinfiltration (Chakraborty *et al.* 2008) in the lower surface of the same leaf where dsRNA was applied onto. dsRNA inoculum consisted of 2 µl of *in vitro* produced dsRNA with 18 µl of sterile double distilled water. The negative control consisted of water-treated tomato / *N. benthamiana* plants. Twelve plants per treatment were inoculated by gently rubbing onto single fully expanded carborundum-dusted leaves with 20 µl inoculation mixture per leaf (Fig. 3.4). Agroinoculated plants were kept under insect-free greenhouse conditions as mentioned above (section 3.1). Symptom observation and disease progress assessment was done at regular time intervals till 40 dpi for tomato and 14 dpi for *N. benthamiana* plants.

3.9 Preparation of ToLCGV inoculum

Test plants were infected using *Agrobacterium tumefaciens* mediated inoculation (Chakraborty et *al.* 2008). For this, *A. tumefaciens* strain EHA105 harboring DNA A and DNA B viral constructs of ToLCGV were inoculated in 50 ml LB media supplemented with Kanamycin (50 µg/ml) and Rifampicin (30 µg/ml) and grown at 28°C for 24 hrs at 190 rpm. Bacterial cells were pelletized at 3500 rpm for 15 min and pellet was resuspended in sterile double distilled water. The optical density (OD_{600}) of each inoculum was brought to 1.0 and equimolar concentration of each construct (DNA A & DNA B) was mixed and the mixture was used for inoculation. Inoculation of plants was carried out by agro-infiltration.

For *Cucumber mosaic virus* (CMV), the host plant was N. tabacum *cv.* Xanthi and the inoculation consisted of solution containing dsRNAs (construct 4(2b_V1/V2) and CMV-b) and CMV-infected plant sap; was prepared by adding 4 µl of *in vitro* produced dsRNA (CMV-2b) and 16 µl of sap (at 1:25 and 1:50 final dilution) that was extracted from 14 days post infection (dpi) CMV-infected tobacco plants. The positive control group comprised of inoculating single leaves of experimental tobacco plants with CMV sap. Twelve tobacco plants per treatment were inoculated by gently rubbing the inoculation

mixtures onto single fully expanded carborundum-dusted leaves with 20 µl inoculation mixture per leaf (Fig. 3.4a). The inoculated plants were grown in the conditions mentioned above and disease symptom development was monitored daily until 14 dpi.

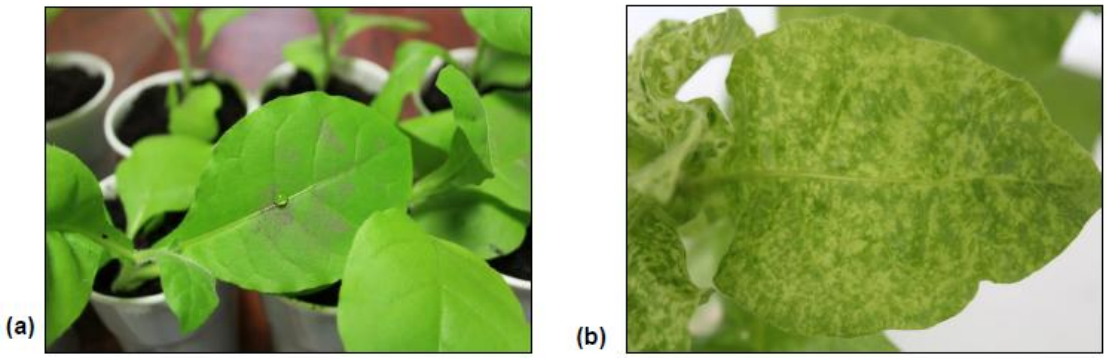

Figure 3.4. Agro-infiltration of CMV on *Nicotiana tabacum* cv. Xanthi plants. (a) *Nicotiana tabacum* cv. Xanthi leaf that has been carborundum-dusted with a drop of dsRNA solution ready for rubbing. (b) CMV infected leaf (14dpi) used for preparing sap to be inoculated in the challenge experiments.

3.10 Plant DNA Isolation

For plant DNA isolation we used the Quick DNA Prep for PCR, adapted from Weigel and Glazebrook (2002). Briefly, a small amount of systemic tissue (a leaflet from second leaf from top) from each plant was grinded by micropestle in a 1.5 ml microcentrifuge tube, in the presence of 400 µl extraction buffer (200 mM Tris-Cl pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS). After centrifugation at maximum speed, 300 µl of the supernatant from each sample were transferred to new tubes. Then an equal volume of isopropanol was added, the two phases were mixed well by shaking and a second centrifugation at maximum speed was done, to precipitate the DNA. After discarding the supernatant, the pellet was washed once by 70% ethanol, air dried to remove residual ethanol and resuspended in 100 µl of TE buffer (10 mM Tris-Cl pH 8; 1 mM EDTA). One µl of the total nucleic acid extract from each sample was used for PCR with gene specific primers in order to detect the replication levels of the tested virus (ToLCGV or CMV).

4. RESULTS

4.1 Identification of the target regions for the production of dsRNA

The genomic sequences of *Tomato leaf curl Gujarat virus* (GenBank accession no. AY190290) is mentioned below. The same was considered for identification of the target region. The viral genome is of 2757 nt in length.

TAATATTACCGGATGGCCGCGATTTTTTTTTTTGGACCCCACAACGCACTAACTGACAAAGACATGCGGACC
AATCACCTGACGCGCTCAAAGCTTAATTGTTTTGTGTTTTCTATTTAAACTTGGCCCCAAGTATTGTAGTTC
GTACTATGTGGGATCCTTTGTTAAACGAGTTTCCTGAAACCGTTCACGGTTTTAGATGTATGTTAGCAGTTA
AATATCTTCAATTAGTAGAAAAGACTTATTCTCCTGACACAGTCGGTTACGATTTAATCAGGGATTTAATTT
CAGTTATTAGGGCTAGAAATTATGTCGAAGCGACCAGCAGATATGCTCATTTTCACGCCCGCTTCGAAGGTA
CGTCGCCGTCTCAACTTCGACAGCCCATCGGTGAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACCAAAGCA
AAGGCATGGGCCAACCGGCCCATGTATCGGAAGCCCAGAATCTACAGAATGTACAGGAGCCCTGATGTGCCT
AAGGGCTGTGAGGGTCCGTGTAAGGTCCAATCGTTTGATGCTAAGAACGATATTGGTCACATGGGTAAGGTG
ATCTGTTTGTCCGATGTGACGAGGGGAATTGGGCTGACCCATCGAGTGGGTAAACGTTTTTGCGTTAAGTCT
TTGTATTTCGTCGGCAAGATCTGGATGGACGAGAATATTAAGGTGAAGAACCACACCAACACCGTTATGTTT
TGGATAGTACGTGATAGGCGTCCTAGTGGGACTCCCAATGATTTTCAGCAGGTTTTTAACGTATATGATAAT
GAGCCCTCTACTGCGACTGTTAAGAATGACCAGCGTGATCGCTTTCAAGTCTTACGGAGGTTTCAAGCGACT
GTTACTGGAGGACAATATGCAGCCAAGGAGCAGGCGATAATTAGAAGATTTTTTCGTGTTAATAATTACGTA
GTTTATAATCACCAGGAAGCTGGGAAGTACGAAAATCATACTGAGAATGCTTTGTTGTTGTATATGGCATGT
ACTCATGCCTCTAATCCTGTGTATGCGACTCTCAAAGTGAGGAGTTATTTCTATGACTCAGTCACAAACTAA
TAAATATTAAATTTTATTATATTTGAACTGTCTACAAATGTTGTTTGCGTCAATGCATCCCATAGTACATAA
TTCACTGCTCTAATTACATTATTCAAACTAATTACACCCAAATTATTAAGAAATTTCATCACTTGTGTTTTA
AATACCCTTAAGAAACGACCAGTCGGAGGCTGTGAGGTCATCCAGCATCGGTAGGTTAGGAAACATTTGTGT
ATCCCCAACACTTTCCTCAGGTTGTGATTGAACTGTATCTGGACGGTGATGATGTCTTCGTTCATCAGGAAG
GGCCGGTTTTGGTGCTCTGTTATCTTGAAATATAGGGGATTTTGAATCTCCCAGATAAACACGCCATTCTCT
GCTTGAGCTGCAGTGATGAATTCCCCTGTGCGTGAATCCATGGTCGTTGCAGGTTAATGCTATGAAGTAAGA
ACAGCCACACGGTAGATCAACTCGTCGACGTCTGGTCCCCTTCTTGGCTAGCCTGTGCTGTACTTTGATTGG
TACTTGAGTAGAGTGGGCCTTCGAGGGTGACGAAGGTTGCATTTTTTAAAGCCCAGTTTTTTAGTGCAGAAT
TCTTTTCCTCATCCAAGTATTCTTTATAGCTGGAGTTGGGTCCTGGATTGCAGAGGAAGGTAGTGGGAATTC
CGCCTTTAATTTGAACTGGCTTCCCGTACTTTGTATTTGATTGCCAGTCCCTTTGGGCCCCCATGAATTCTT
TAAAGTGCTTTAGGTAGTGGGGGTCGACGTCATCAATGACGTTGTACCAGGCATCATTACTGTAAACCTTTG
GGCTAAGGTCTAGATGACCACACAAATAATTATGTGGTCCCAATGATCTGGCCCACATAGTCTTCCCCGTCC
TACTGTCACCCTCAATTACTATACTTATGGGTCTCAGAGGCCGCGCAGCGGGACTGCAGACGTTCTCGGCAG
CCCACGCTTCAAGTTCTTCTGGAACTTGGTCGAAAGAAGAAGATATAAAAGGTGAAACATAAACATCCATTG
GAGGTGTAAAAATCCTATCTAAATTAGAATTTAAATTGTGAAATTGTAAAACAAAATCTTTGGGAGCTTTCT
CCCTTAATATATTGAGGGCCTGAGCTTTGGACCCTGAATTGATTGCCTCGGCATATGCGTCGTTGGCAGATT
GGCAACCTCCTCTAGCTGATCGTCCATCGACTTGGAAAACTCCATGATCAATGACGTCTCCGTCTTTTTCCA
TGTAGGATTTGACATCGCTTGAGCTCTTAGCTCCCTGAATGTTCGGATGGAAATGTGCTGACCTGGTTGGGG
ATGTGAGGTCGAAGAATCTATTGTTTTTGCATTGGAACTTTCCTTCGAACTGGATGAGCACATGCAAGTGAG
GAGTCCCATCATCGTGAAGCTCTCTGCAAATTCTAATAAATTTTTTGAAAGTGGGTGTTTGGAGATTTAATA
ATTGGGAAAGTGCCTCTTCTTTAGTTAGAGAGCACTTGGGATAAGTGAGAAAATAATTTTTGGCATTTATTT
TAAAGCGATTGGGGGCTGCCATGTTGACTTGGTCAATCGGTGTCTTTCAACTCTCTCTATGTATCGGTGTAT
TGGAGTCCTATATATATGGAGACTCCAATGGCATAAATGTAAATATTGAACTTTAAATCAAAACCCTAACGC
TCCAAAAAGCGGCCATCCGTA

Yellow color-V2, Yellow Red color (overlapping region) -V1_V2, Purple color-V1, Blue color-C1, Yellow color-C4.

UNDERLINE regions represent the sequences of primers

The following sequences of the constructs were generated and confirmed.

4.1.1 Construct ToLCGV (V1/V2) 432 nt

ACGAGTTTCCTGAAACCGTTCACGGTTTTAGATGTATGTTAGCAGTTAAATATCTTCAATTAGTAGAAAAGA
CTTATTCTCCTGACACAGTCGGTTACGATTTAATCAGGGATTTAATTTCAGTTATTAGGGCTAGAAATTATG
TCGAAGCGACCAGCAGATATGCTCATTTTCACGCCCGCTTCGAAGGTACGTCGCCGTCTCAACTTCGACAGC
CCATCGGTGAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACCAAAGCAAAGGCATGGGCCAACCGGCCCATG
TATCGGAAGCCCAGAATCTACAGAATGTACAGGAGCCCTGATGTGCCTAAGGGCTGTGAGGGTCCGTGTAAG
GTCCAATCGTTTGATGCTAAGAACGATATTGGTCACATGGGTAAGGTGATCTGTTTGTCCGATGTGACGAGG

4.1.2 Construct ToLCGV (C1/C4) Reverse complement (449 nt)

CACGATGATGGGACTCCTCACTTGCATGTGCTCATCCAGTTCGAAGGAAAGTTCCAATGCAAAAACAATAGA
TTCTTCGACCTCACATCCCCAACCAGGTCAGCACATTTCCATCCGAACATTCAGGGAGCTAAGAGCTCAAGC
GATGTCAAATCCTACATGGAAAAAGACGGAGACGTCATTGATCATGGAGTTTTCCAAGTCGATGGACGATCA
GCTAGAGGAGGTTGCCAATCTGCCAACGACGCATATGCCGAGGCAATCAATTCAGGGTCCAAAGCTCAGGCC
CTCAATATATTAAGGGAGAAAGCTCCCAAAGATTTTGTTTTACAATTTCACAATTTAAATTCTAATTTAGAT
AGGATTTTTACACCTCCAATGGATGTTTATGTTTCACCTTTTATATCTTCTTCTTTCGACCAAGTTCCAGAA
GAACTTGAAGCGTGGGC

4.1.3 Construct ToLCGV (C1/C4-V1/V2): Fusion construct of C1/C4 followed by V1/V2 (881nt)

CACGATGATGGGACTCCTCACTTGCATGTGCTCATCCAGTTCGAAGGAAAGTTCCAATGCAAAAACAATAGA
TTCTTCGACCTCACATCCCCAACCAGGTCAGCACATTTCCATCCGAACATTCAGGGAGCTAAGAGCTCAAGC
GATGTCAAATCCTACATGGAAAAAGACGGAGACGTCATTGATCATGGAGTTTTCCAAGTCGATGGACGATCA
GCTAGAGGAGGTTGCCAATCTGCCAACGACGCATATGCCGAGGCAATCAATTCAGGGTCCAAAGCTCAGGCC
CTCAATATATTAAGGGAGAAAGCTCCCAAAGATTTTGTTTTACAATTTCACAATTTAAATTCTAATTTAGAT
AGGATTTTTACACCTCCAATGGATGTTTATGTTTCACCTTTTATATCTTCTTCTTTCGACCAAGTTCCAGAA
GAACTTGAAGCGTGGGCACGAGTTTCCTGAAACCGTTCACGGTTTTAGATGTATGTTAGCAGTTAAATATCT
TCAATTAGTAGAAAAGACTTATTCTCCTGACACAGTCGGTTACGATTTAATCAGGGATTTAATTTCAGTTAT
TAGGGCTAGAAATTATGTCGAAGCGACCAGCAGATATGCTCATTTTCACGCCCGCTTCGAAGGTACGTCGCC
GTCTCAACTTCGACAGCCCATCGGTGAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACCAAAGCAAAGGCAT
GGGCCAACCGGCCCATGTATCGGAAGCCCAGAATCTACAGAATGTACAGGAGCCCTGATGTGCCTAAGGGCT
GTGAGGGTCCGTGTAAGGTCCAATCGTTTGATGCTAAGAACGATATTGGTCACATGGGTAAGGTGATCTGTT
TGTCCGATGTGACGAGG

4.1.4 Construct CMV-2b/ToLCGV -V1/V2): Hybrid construct of CMV-2b followed by V1/V2 (768nt)

ATGGAATCGAACGAAGGCGCAATGACAAACGTCGAACTCCAACTGGCCCATATGCTAGAGGTGAAGAGACAG
AGACGAAAGTCTCACAAGAAGAATCGACGGGAACGAGGTCACAAAAGTCCCAGCGAGAGGGCGCGTTCAAAT
CTCAGACTATTCCGCTTCCTACCATTTTATCAGGTAGATGGTTCGGAACTAATAGAGATGCACCACCATGCG
AGCATGGTGGAACTATCCGAGTCTGAGGCCCCTTGTTTCACGTTACCAGCGGAAGAAGACCATGACTTTGAC
GATACAGATTGGTTCGCTGGTAACGAATGGGCG**GAAGGTGCGTTTTGA****ACGAGTTTCCTGAAACCG**TTCACG
GTTTTAGATGTATGTTAGCAGTTAAATTCTTCAATTAGTAGAAAAGACTTATTCTCCTGACACAGTCGGTTA

CGATTTAATCAGGGATTTAATTTCAGTTATTAGGGCTAGAAATTATGTCGAAGCGACCAGCAGATATGCTCA
TTTTCACGCCCGCTTCGAAGGTACGTCGCCGTCTCAACTTCGACAGCCCATCGGTGAGCCGTGCTGCTGCCC
CCATTGTCCGCGTCACCAAAGCAAAGGCATGGGCCAACCGGCCCATGTATCGGAAGCCCAGAATCTACAGAA
TGTACAGGAGCCCTGATGTGCCTAAGGGCTGTGAGGGTCCGTGTAAGGTCCAATCGTTTGATGCTAAGAACG
ATATTGGTCACATGGGTAAGGTGATCT**GTTTGTCCGATGTGACGAG**

4.2 Sequencing result of construct 4th hybrid, cloned in the pGEMT vector

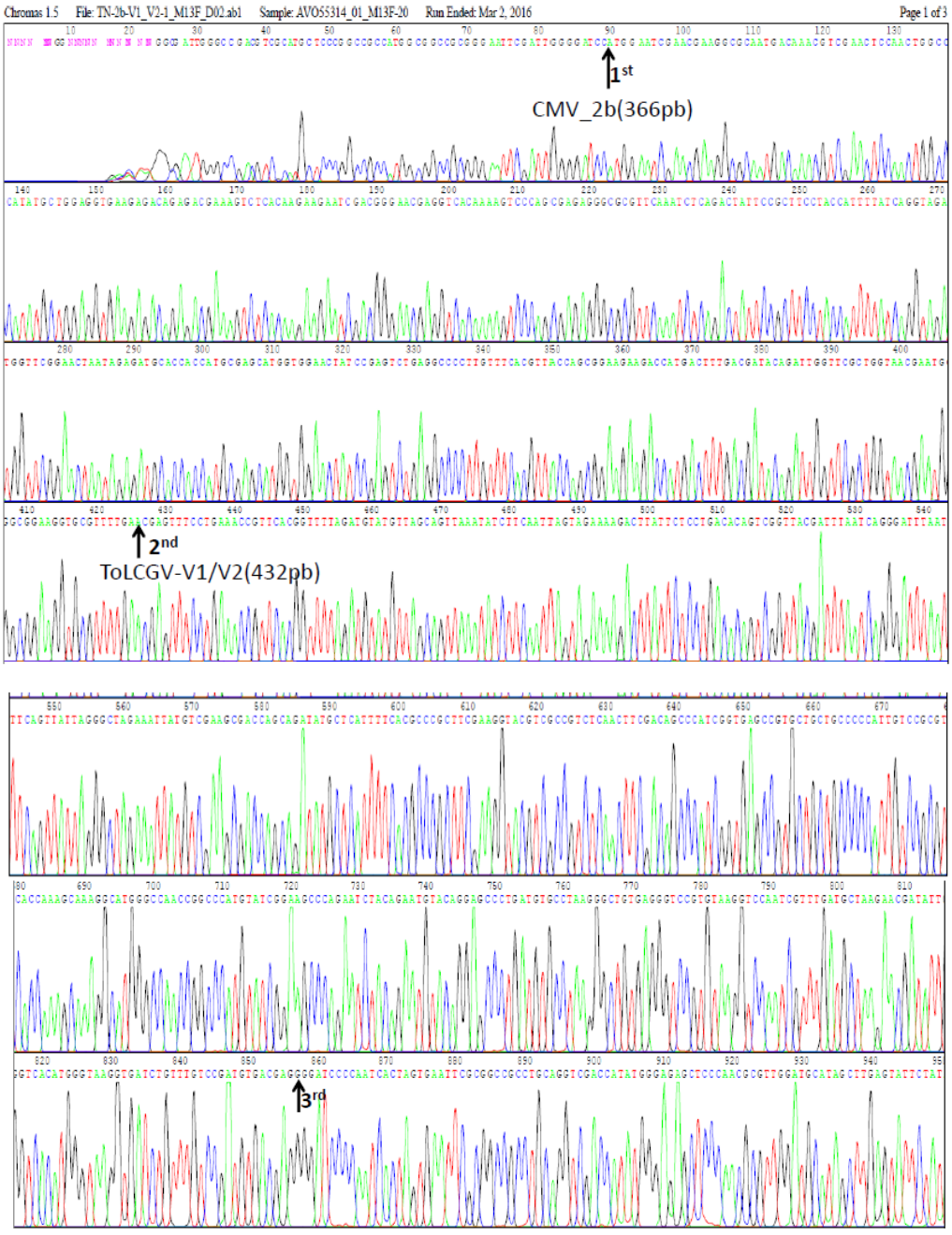

Figure 4.1. DNA sequencing of cloned viral constructs. Chromatograms showing the sequencing result of construct 4 (CMV_2-ToLCGV-V1/V2) (786 bp) that had been cloned in pGEMT-vector.1st arrow shows the beginning of CMV_2b (366 bp) whereas 2nd arrow shows the beginning of ToLCGV-V1/V2 (432bp) and 3rd arrow shows the ending of ToLCGV-V1/V2.

4.3. dsRNA production for different regions of ToLCGV and CMV

In the current study, a method based on two sequential PCR reactions coupled with *in vitro* transcription was employed to produce dsRNA molecules for different regions of TYLCGV and CMV genomes, as described in the materials & methods section. More precisely, in case of *Tomato leaf curl virus* dsRNA molecules were produced for: 1) ToLCGV-C1/C4 (overlapping region of 449bp targeting C1 and C4 genes), 2) ToLCGV-V1/V2 (overlapping region of 432bp targeting V1 and V2 genes), 3) ToLCGV-C1/C4-V1/V2 (fusion construct of 881 bp composed of constructs 1 and 2, 4) CMV-2b_ToLCGV-V1/V2 (hybrid construct of 768 bp composed of a CMV-2b region and construct 2). In case of *Cucumber mosaic virus*, apart from construct 4, dsRNA molecules were also produced for CMV-2b gene alone (a conserved region of 336 bp). The above procedure has the advantage that it is not time consuming compared to transgenic development and at the same time produces significant quantities of dsRNA, that typically range between 10-15 μg/μl. At the first PCR reaction, the target fragments were amplified using primers with a linker sequence at their 5' end. At the second PCR reaction, the use of a T7-linker primer sequentially introduced a T7 promoter sequence at the 5' ends of both sense and anti-sense strands of the targeted DNA fragments. The second PCR product was used as a template for *in vitro* transcription by employing the T7 Ribomax express large scale RNA production system. The products of first PCR, second PCR and the *in vitro* transcription reaction for constructs 1, 2, 3 (that target regions of ToLCGV), for construct 5 (that target a region of CMV) and for hybrid construct 4 (that target regions of both CMV and ToLCGV) are shown in Fig. 4.2.

The following constructs: were generated in the present study :-

- Construct 1 : ToLCGV-C1/C4 (overlapping region)
- Construct 2: ToLCGV-V1/V2 (overlapping region)
- Construct 3: ToLCGV-C1/C4-V1/V2 (fusion)
- Construct 4: CMV-2b_ToLCGV-V1/V2 (hybrid)
- Construct 5: CMV-2b

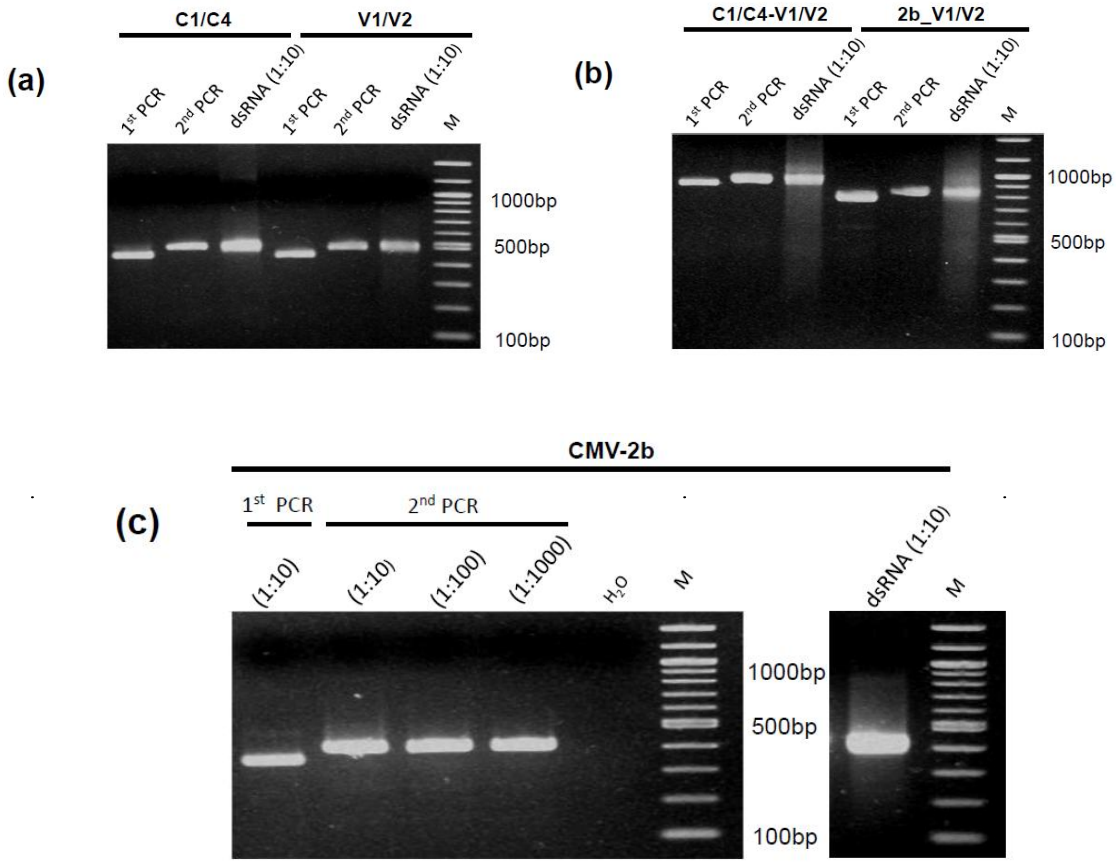

Figure 4.2. PCR amplification for generation of target constructs. First PCR, second PCR product and *in vitro* produced dsRNA of target sequences of all five constructs of *Tomato leaf curl virus* and *Cucumber mosaic virus* as evidenced by 1.5% agarose gel electrophoresis. **(a)** ToLCGV-C1/C4, ToLCGV-V1/V2. **(b)** ToLCGV-C1/C4-V1/V2, CMV-2b_ToLCGV-V1/V2. **(c)** CMV-2b. M is a 100 bp DNA ladder (New England Biolabs, USA**).**

4.4. dsRNA mediated resistance against ToLCGV in *N. benthamiana and S. lycopersicum* and against CMV in *N. tabacum*

4.4.1 *N. benthamiana*

Five experiments were performed using *N. benthamiana* as experimental plant. At the first two experiments, the plants were infected the plants with ToLCGV by applying *A. tumefaciens* cells (carrying DNA A and DNA B) at an optical density (OD_{600}) brought to 1.0, except for the second experiment in which 200 times diluted *A. tumefaciens* cells were applied together with dsRNA for construct CMV-2b_ToLCGV-V1/V2. From these initial experiments it became evident that *N. benthamiana* were extremely sensitive to ToLCGV, since all plants (with or without application of dsRNA) developed disease symptoms within 12 days post inoculation (dpi). Thus, a third experiment was performed

using serial dilutions of ToLCGV agro-innoculum to find out the highest dilution at which all plants from the positive control group (ToLCGV only) become infected. Finally, two more experiments were conducted using ToLCGV agro-inoculum diluted 2×10^3 and 10^4 times, respectively. Interestingly, it was observed that in case of *N. benthamiana*, the plant which received dsRNA having larger leaves and their height was taller as compared to control plants which were only treated with virus (Fig. 4.3).

Figure 4.3. *N. benthamiana* plants inoculated with ToLCGV showing the leaf curl disease symptoms. **(a)** ToLCGV inoculated plants having small size leaves, **(b)** ToLCGV plus dsRNA inoculated plants having larger leaves as compare to control plants (ToLCGV treated plants).

4.1.1 Experiment I

In order to examine if the exogenously applied dsRNA molecules have the capacity to confer resistance against ToLCGV infection, 12 *N. benthamiana* plants were used for each of the following treatments:

a) ToLCGV+ds1 (C1/C4)
b) ToLCGV+ds2 (V1/V2)
c) ToLCGV+ds3 (C1/C4-V1/V2)
d) ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2)
e) ToLCGV (positive control)
f) Water (negative control)

For the infection of plants with ToLCGV, *A. tumefaciens* cells carrying DNA A and DNA B were diluted in water at an optical density (OD_{600}) brought to 1.0, and the inoculum was agroinfiltrated into *N. benthamiana* plants. The main symptoms of ToLCGV-

inoculated *N. benthamiana* plants were the characteristic curling of the systemic, newly emerging leaves and the shorter size as compared to the negative control plants, treated with water only (Fig. 4.4).

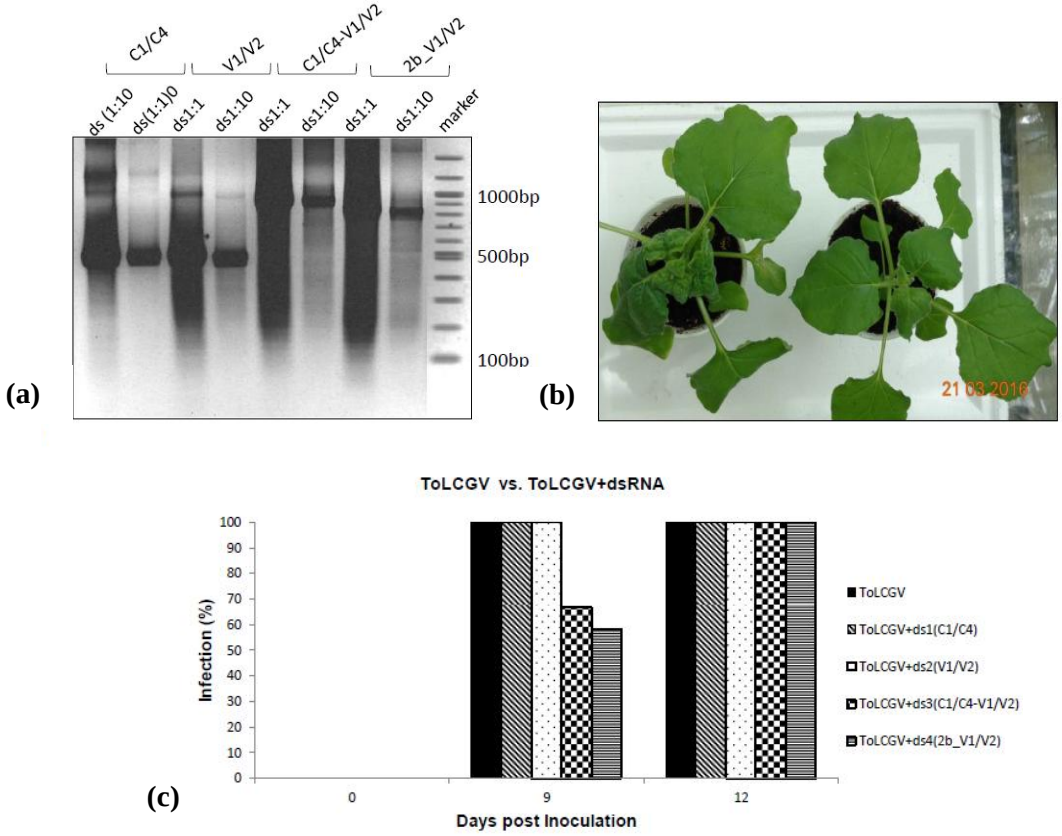

Figure 4.4. Effect of dsRNA construct on symptom induction of ToLCGV on *N. benthamiana* experiment I. **(a)** Gel electrophoresis image of dsRNAs for constructs 1, 2, 3 and 4, that were exogenously applied on *N. benthamiana* plants. **(b)** Phenotype of a *N. benthamiana* plant inoculated with ToLCGV (left) as compared to a healthy non-inoculated plant (right). **(c)** The treatments namely ToLCGV; ToLCGV+ds1 (C1/C4); ToLCGV+ds2 (V1/V2); ToLCGV+ds3 (C1/C4-V1/V2); ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) were applied. Disease incidence was calculated based on the number of plants infected out of 12 plants used in each treatment.

The disease symptom development was monitored in all treatments daily until 12 dpi. From the experiment performed, the disease incidence data indicated that at 9 dpi, 100% of ToLCGV (positive control), ToLCGV+ds1(C1/C4) and ToLCGV+ds2(V1/V2) treated plants were infected, suggesting that the respective dsRNAs did not induce any resistance against ToLCGV. Interestingly at 9 dpi, *N. benthamiana* plants that received dsRNAs for ds3 (C1/C4-V1/V2) and ds4 (CMV-2b_ToLCGV-V1/V2) showed 33% and 41% resistance against ToLCGV, respectively. However at 12 dpi, all plants from

ToLCGV+ds3(C1/C4-V1/V2) and ToLCGV+ds4(CMV-2b_ToLCGV-V1/V2) treatments were finally infected, suggesting that these two constructs (3 & 4) conferred only a three-day delay in ToLCGV symptom development.

4.1.2 Experiment II

In the experiment I, all *N. benthamiana* plants for all treatments finally developed curling of the systemic leaves that is the characteristic symptom of the disease caused by ToLCGV indicating no protection induced by exogenous dsRNA application. A possible explanation for the incapacity of dsRNAs to protect against ToLCGV could be that the concentration of the virus that was agro-inoculated into *N. benthamiana* plants was high. The experiment was repeated using the same treatments:

a) ToLCGV+ds1(C1/C4)
b) ToLCGV+ds2(V1/V2)
c) ToLCGV+ds3(C1/C4-V1/V2)
d) ToLCGV+ds4(CMV-2b_ToLCGV-V1/V2)
e) ToLCGV (positive control)
f) Water (negative control)

For the above-mentioned reason, it was felt necessary to perform one more test for construct 4 (that gave the more satisfactory results in experiment I, using lower concentration of the inoculated *A. tumefaciens* cells. Thus after bringing the optical density (OD_{600}) to 1.0, the bacterial cells were further diluted 200 times (OD ~ 0.005), injected the agro-inoculum into *N. benthamiana* plants by agro-infiltration, and finally applied ds4 at the same leaves. The disease symptom development was monitored in all treatments daily until 12 dpi. From the experiments performed, the disease incidence data indicated that at 7 dpi, 91% of ToLCGV treated plants (positive control) were infected. At the same time, 83% of the plants from treatments ToLCGV+ds1 (C1/C4), ToLCGV+ds2 (V1/V2), ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) and 75% of the plants from treatment ToLCGV+ds3 (C1/C4-V1/V2) (fusion construct) were infected. At 9 dpi, ToLCGV (positive control), ToLCGV+ds3 (C1/C4-V1/V2) and ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) groups showed 100 % infection. In case of ToLCGV+ds1 (C1/C4) and ToLCGV+ds2 (V1/V2) groups 91% of the plants were infected at 9 dpi. Finally at 12

dpi, all the plants treated with the different dsRNAs and ToLCGV showed 100% infection (Fig. 4.4a).

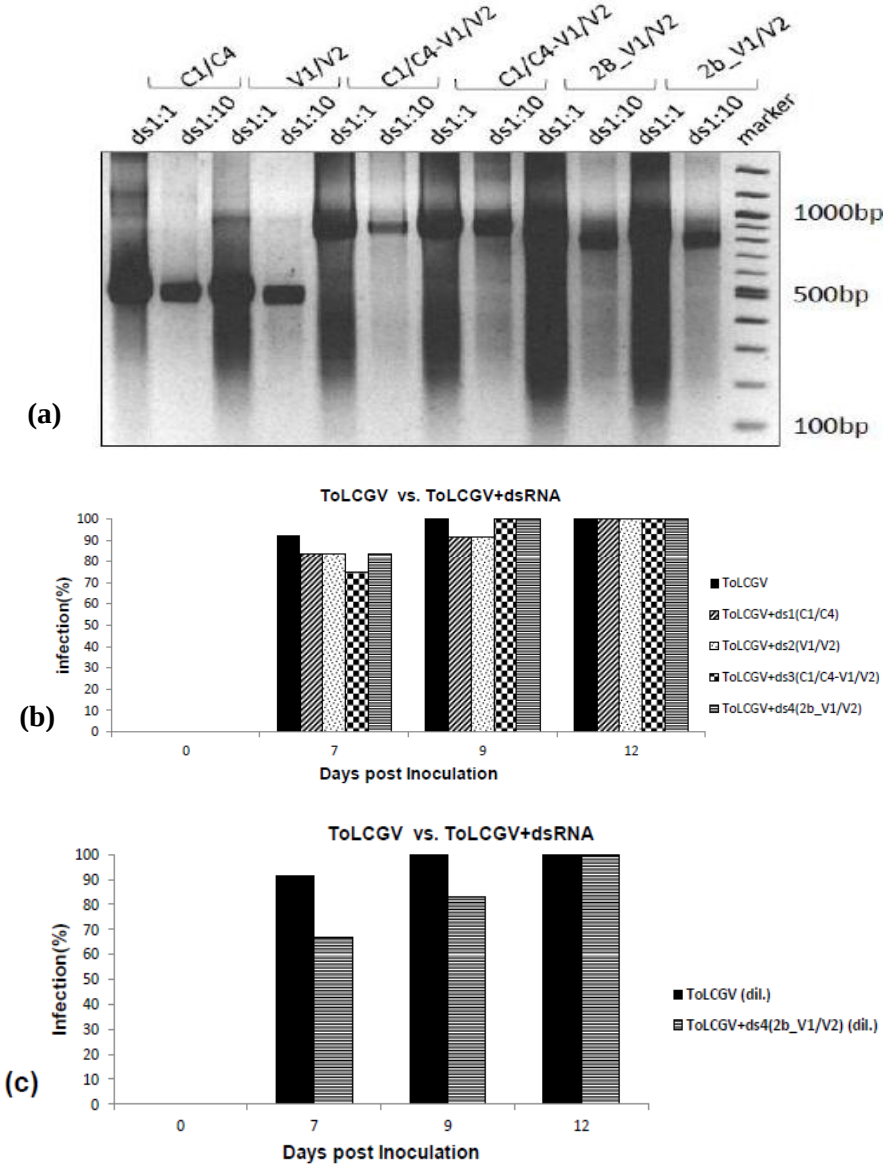

Figure 4.5. Effect of dsRNA construct on symptom induction of ToLCGV on *N. benthamiana* (2nd experiment) **(a)** Gel electrophoresis image of dsRNAs for constructs 1, 2, 3 and 4 that were exogenously applied on *N. benthamiana* plants. **(b)** The treatments namely ToLCGV; ToLCGV+ds1(C1/C4); ToLCGV+ds2(V1/V2; ToLCGV+ds3(C1/C4-V1/V2); ToLCGV+ds4(CMV-2b_ToLCGV-V1/V2) were applied. The optical density (OD) of the Agro-inoculum was 1.0. Disease incidence was calculated based on the number of plants infected out of 12 plants used in each treatment. **(c)** Showing the delay in ToLCGV symptom development at plants that received dsRNA for CMV-2b_ToLCGV-V1/V2 (Hybrid), when the Agro-inoculum was diluted 200 times (at an optical density of 0.005).

On the other hand, plants treated with the 200 times diluted Agro-inoculum of ToLCGV together with ds4 (CMV-2b_ToLCGV-V1/V2) at 7 dpi and 9 dpi exhibited 27% and 16% resistance against ToLCGV symptoms. However, at 12 dpi, again all plants showed symptoms of infection (Fig. 4.5b). The percentages of the protection conferred by the application of ds4 were higher when the virus was diluted (e.g. compare Figs 4.5a and 4.5b). These results suggest that *N. benthamiana* plants are extremely sensitive to ToLCGV infection and if a further dilution of the virus would be used, then the protection conferred by the exogenously applied dsRNAs could reach satisfactory levels.

4.1.3 Experiment III

From the above two experiments it was found that all the *N. benthamiana* plants became infected by ToLCGV at 12 dpi. So, in this experiment different dilutions of virus concentration were performed in order to find out the lowest concentration of the applied *A. tumefaciens* cells at which all plants get infected. Thus after bringing the optical density (OD_{600}) to 1.0, cells were further diluted i) 2 x 10^1 (OD ~ 0.05), ii) 2 x 10^2, iii) 2×10^3, iv) 2×10^4, v) 2×10^5, vi) 4×10^6, vii) 8×10^7 and viii) 16×10^8 . Ten *N. benthamiana* plants were used for each of the above dilutions. The disease symptom development was monitored in all treatments daily until 14 dpi.

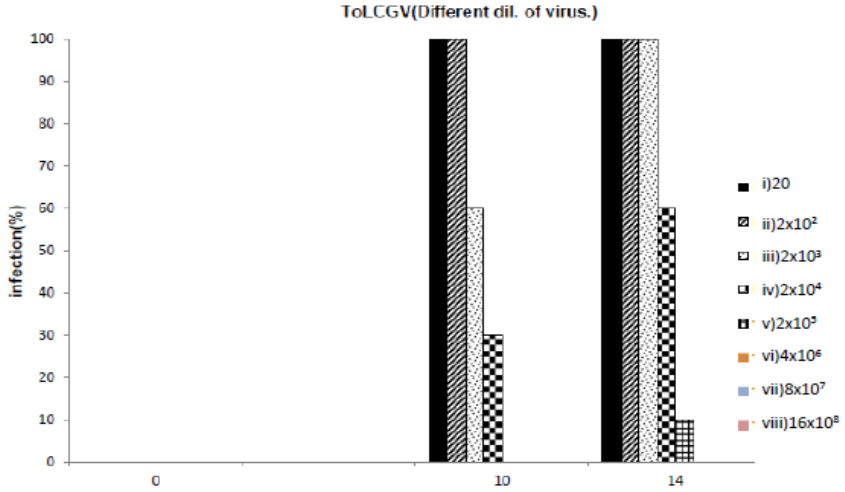

Figure 4.6. Disease incidence at different dilutions of ToLCGV. Agro-inoculum at 7, 10 and 14 days post inoculation: i) 20 (OD ~ 0,050), ii) 2×10^2, iii) 2×10^3, iv) 2×10^4, v) 2×10^5, vi) 4×10^6, vii) 8×10^7 and viii) 16×10^8.

At 7 dpi, 100% of the plants were infected in case of dilutions (i) and (ii), whereas in case of dilution (iii) only 30% of plants were infected. In case of remaining dilutions (iv), (v), (vi), (vii) and (viii) all plants were healthy at 7 dpi. At 10 dpi, 60% of plants in case of dilution (iii) and 30 % of plants in case of dilution (iv) were infected. In case of remaining dilutions (v), (vi), (vii) and (viii) all plants were healthy at 10 dpi. At 14 dpi, 100% of the plants were infected in case of dilution (iii), where as in case of dilution (vi) and dilution (v) 60% and 10% of plants were infected. In case of remaining dilutions (vi), (vii) and (viii) all plants were healthy. So for further experimentation, we considered as the most convenient the dilution (iii) i.e. 2×10^3, in which there is a delay in the development of ToLCGV disease symptoms but finally 100% of the plants become infected at 14 dpi (Fig. 4.6).

4.1.4 Experiment IV

In this experiment the dilution of 2×10^3 (dilution iii) of 3rd experiment and infiltrated the agro-inoculum into *N. benthamiana* plants. Twelve *N. benthamiana* plants were used for each of the following treatments:

a) ToLCGV+ds1 (C1/C4)
b) ToLCGV+ds2 (V1/V2)
c) ToLCGV+ds3 (C1/C4-V1/V2)
d) ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2)
e) ToLCGV (positive control)
f) Water (negative control)

The disease symptom development was monitored in all treatments daily until 14 dpi. At 7 dpi, 50% of ToLCGV (positive control) and ToLCGV+ds3 (C1/C4-V1/V2), 54% of ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) and 33% of ToLCGV+ds1 (C1/C4) treated plants were infected. Interestingly, at 7 dpi plants that received ds2 (V1/V2) together with ToLCGV exhibited the lowest percentage of infection (16%). At 10 dpi, ToLCGV (positive control) inoculated plants showed 91% of infection, whereas ToLCGV+ds1 (C1/C4), ToLCGV+ds3 (C1/C4-V1/V2) and ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) inoculated plants showed 83% of infection. At 10 dpi, again ToLCGV+ ds 2(V1/V2) inoculated plants showed the lowest percentage of infection, namely 66%. At 14 dpi, all

plants showed 100% infection except ToLCGV+ds2 (V1/V2) inoculated plants which showed 91% of ToLCGV infection (Fig. 4.7).

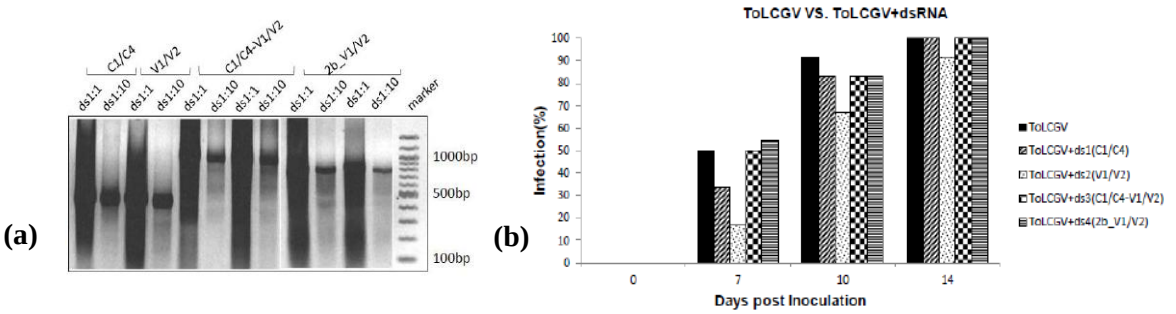

Figure 4.7. Effect of dsRNA constructs on disease incidence of a $2x10^3$ dilution of ToLCGV on *N. benthamiana* (4^{th} experiment). **(a)** Gel electrophoresis image of dsRNAs for constructs 1, 2, 3 and 4, that were exogenously applied on *N. benthamiana* plants. (**b**) The treatments namely ToLCGV; ToLCGV+ds1 (C1/C4); ToLCGV+ds2 (V1/V2); ToLCGV+ds3 (C1/C4-V1/V2); ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) were applied. Disease incidence was calculated based on the number of plants infected out of 12 plants used in each treatment.

As mentioned above, the main symptom of ToLCGV-inoculated *N. benthamiana* plants is the characteristic curling of the systemic, newly emerging leaves, while at the same time the older leaves remain healthy. An interesting observation that was done at the end of the 4^{th} experiment in *N. benthamiana* was that the older healthy leaves from plants treated with ToLCGV and dsRNA (ds1 or ds2 or ds3 or ds4) were bigger in size than the respective leaves from plants treated with ToLCGV alone (Fig. 4.3).

4.1.5 Experiment V

Because in 4^{th} experiment - in which 2×10^3 dilution of ToLCGV agro-inoculum was applied - the application of dsRNAs again proved to be largely ineffective against ToLCGV infection, the possible reason is that *A. tumefaciens* cells (carrying DNA A and DNA B) need to be diluted even more. Thus, in experiment V a 10^4 dilution was made to infiltrate the agro-inoculum into *N. benthamiana* plants. Twelve *N. benthamiana* plants were used for each of the following treatments:

a) ToLCGV+ds1 (C1/C4)

b) ToLCGV+ds2 (V1/V2)

c) ToLCGV+ds3 (C1/C4-V1/V2)

d) ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2)

e) ToLCGV (positive control)

f) Water (negative control)

The disease symptom development was monitored in all treatments daily until 14 dpi. At 7 dpi and 9 dpi, none of the plants from any treatment had developed disease symptoms. At 12 dpi ToLCGV and ToLCGV+ds3 (C1/C4-V1/V2) shows 58% of infection whereas in case of ToLCGV+ds1 (C1/C4) and ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) shows 80% and 63%, respectively but in case of plants received dsRNA for ToLCGV+ds2 (V1/V2) show least infection of 50%. At 14dpi all plants are getting more infection in all the constructs exceptionally ToLCGV+ds2 (V1/V2) shows 58% (Fig. 4.8).

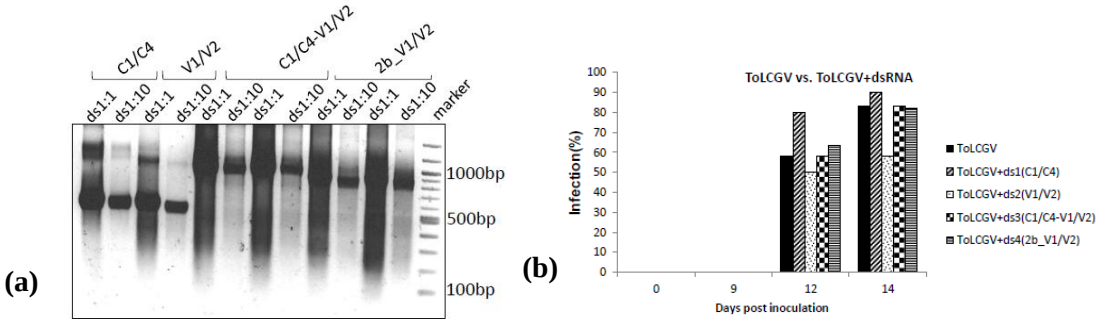

Figure 4.8. Effect of dsRNA exogenous application on disease incidence at 10^4 dilution of ToLCGV on *N. benthamiana* (5^{th} experiment) **(a)** Gel electrophoresis image of dsRNAs for constructs 1, 2, 3 and 4 that were exogenously applied on *N. benthamiana* plants. (**b**) The treatments namely ToLCGV; ToLCGV+ds1 (C1/C4); ToLCGV+ds2 (V1/V2); ToLCGV+ds3 (C1/C4-V1/V2); ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) were applied. Disease incidence was calculated based on the number of plants infected out of 12 plants used in each treatment.

4.2 *S. lycopersicum* (Tomato)

Two experiments were performed using *S. lycopersicum* (tomato) that is the major host plant of *Tomato leaf curl Gujarat virus* (ToLCGV), as the experimental host plant. In the first experiment the plants were infected with ToLCGV by applying *A. tumefaciens* cells (carrying DNA A and DNA B) at an optical density (OD_{600}) brought to 0.005, while at the second experiment the optical density of the cells was brought to 1.0. In case of the second experiment, generally the percentages of infected plants were relatively small for

all treatments, including the positive control group (e.g. plants treated only with ToLCGV). In order to confirm the presence of ToLCGV in the second experiment DNA was extracted from each plant in all treatments and performed PCR to amplify a specific fragment of DNA-A. PCR revealed that in some cases plants failed to exhibit any symptoms but produced a very strong PCR amplified products, suggesting the existence of high viral titer. For the final calculation of infected plants in the second experiment, more accurate PCR results were considered, since this method is extremely sensitive. A general observation from the two experiments in tomato, was that dsRNAs for construct 2 (V1/V2) and construct 4 (CMV-2b_ToLCGV-V1/V2) were found to confer significant resistance (62% and 44%) against ToLCGV in this host plant. The results from these experiments will be described in more details at the following sections.

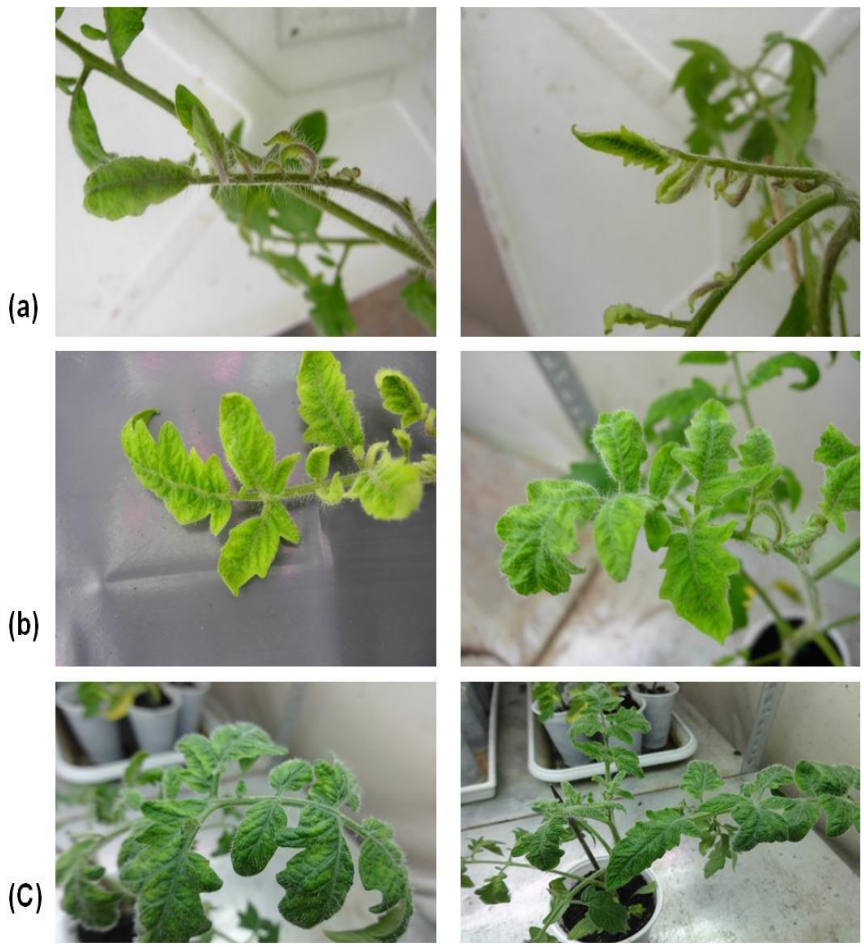

Figure 4.9. The type of symptoms observed the tomato plants infected with ToLCGV. **(a)**small leaves, **(b)** mosaic structure of leaves, **(c)** curling of leaves.

4.2.1 Experiment I

In order to examine if the exogenously applied dsRNA molecules have the capacity to confer resistance against ToLCGV infection, 12 *S. lycopersicum* (tomato) plants cv. Clodin were used for each of the following treatments:

a) ToLCGV+ds1 (C1/C4)
b) ToLCGV+ds2 (V1/V2)
c) ToLCGV+ds3 (C1/C4-V1/V2)
d) ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2)
e) ToLCGV (positive control)
f) Water (negative control)

For the infection of tomato plants with ToLCGV, *A. tumefaciens* cells carrying DNA A and DNA B were first diluted in water at an optical density (OD_{600}) brought to 1.0, then further diluted 200 times at an optical density of 0.005 and the inoculum was agroinfiltrated into tomato plants. The main symptom of ToLCGV-inoculated tomato plants was the characteristic leaf curling, yellow mosaic of infected leaves, smaller leaf size and reduced plant height (Fig. 4.10). The disease symptom development was monitored in all treatments every 3 days until 42 dpi. From the experiment performed, the disease incidence data indicated that that at 42 dpi, 80% of ToLCGV inoculated plants (positive control) were infected. At the same time, 72% of the plants from treatment ToLCGV+ds1 (C1/C4) and 75% of the plants from treatment ToLCGV+ds3 (C1/C4-V1/V2) were infected. These data indicate that dsRNAs for constructs 1 and 3 did not induce any significant resistance against ToLCGV, since the percentages of infected plants at the respective groups are very similar to that in the positive control group.

In contrast, at 42 dpi, ToLCGV+ds2 (V1/V2) and ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) groups exhibited only 44% and 30% infection, respectively. These percentages constitute the 55% and 37% of the percentage of infected plants in the positive control group (if control plants are considered as 100% infected). From the above results, it could be deduced that dsRNAs for constructs 2 and 4 provide significant resistance against ToLCGV infection in tomato: 44% and 62%, respectively. This property was already evident from 28 dpi (Figure 4.10).

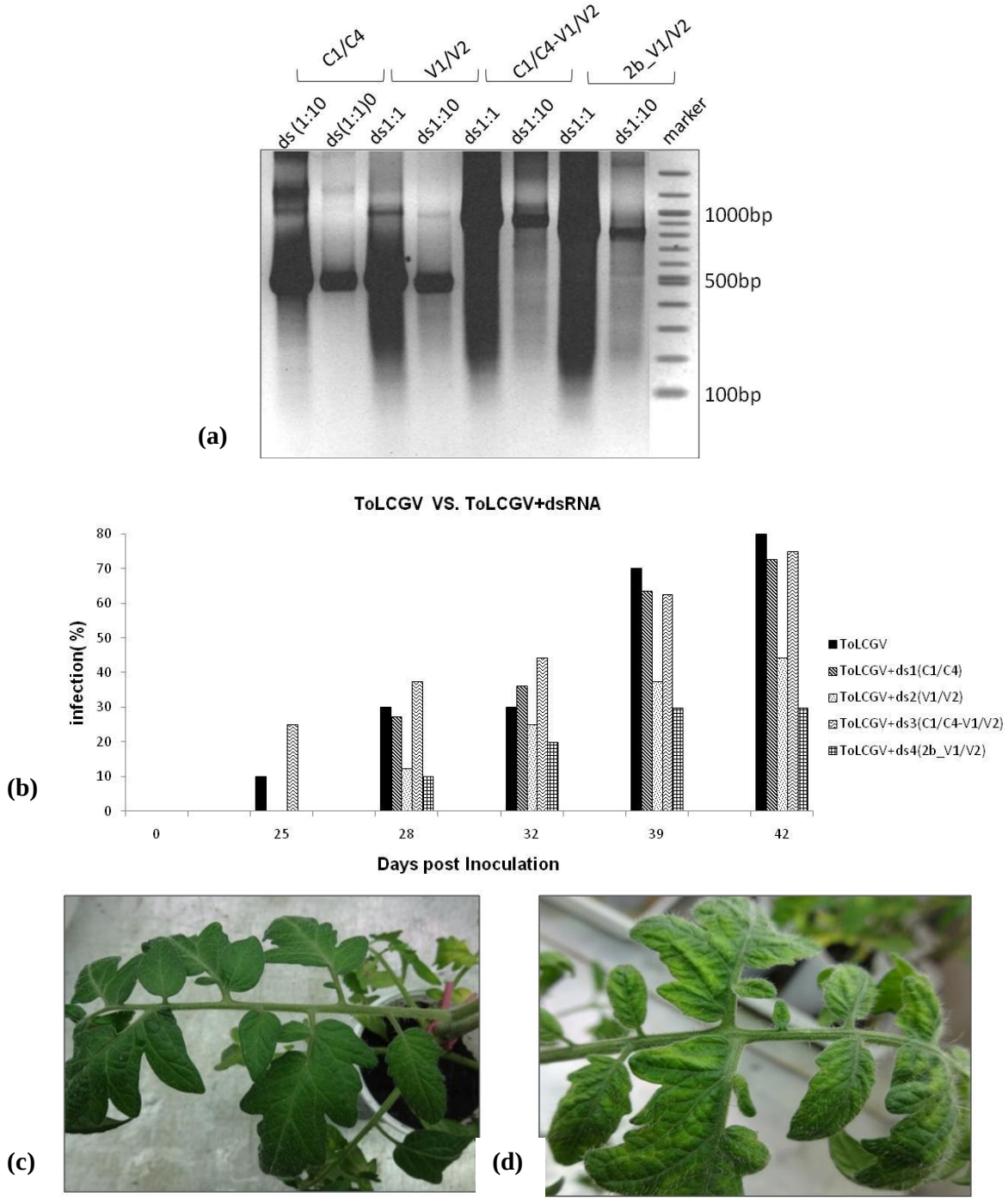

Figure 4.10. Effect of dsRNA exogenous application on disease incidence of ToLCGV on *S. lycopersicum* plants (Experiment I). **(a)** Gel electrophoresis image of dsRNAs for constructs 1, 2, 3 and 4, that were exogenously applied on tomato plants. (**b**) The treatments namely ToLCGV; ToLCGV+ds1 (C1/C4); ToLCGV+ds2 (V1/V2); ToLCGV+ds3 (C1/C4-V1/V2); ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) were applied. Disease incidence was calculated based on the number of plants infected out of 12 plants used in each treatment. **(c)** Phenotype of a healthy non-inoculated tomato plant. **(d)** Phenotype of a tomato plant inoculated with ToLCGV, exhibiting yellow mosaic and curling in leaves.

4.2.2 Experiment II

In Experiment 1, the development of symptoms in tomato plants (mainly the yellow mosaic of leaves) was substantially delayed even in the plants inoculated with ToLCGV (positive control). A possible explanation for this could be the 200 times dilution of the Agro-inoculum that was performed. For this reason, 2nd experiment was performed on tomato plants by using *A. tumefaciens* cells at the initial concentration without further dilution, e.g. at an optical density (OD_{600}) of 1.0.

Eleven tomato plants were used for each of the following treatments:

a) ToLCGV+ds1 (C1/C4)
b) ToLCGV+ds2 (V1/V2)
c) ToLCGV+ds3 (C1/C4-V1/V2)
d) ToLCGV +ds4 (CMV-2b_ToLCGV-V1/V2
e) ToLCGV (positive control)
f) Water (negative control)

The disease symptom development was monitored in all treatments every 3 days until 39 dpi. At 19 dpi, 27% of ToLCGV inoculated plants (positive control group) were infected. At the same time, 9% of the plants from treatments ToLCGV+ds1 (C1/C4), ToLCGV+ds3 (C1/C4-V1/V2) and ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) were infected. In case of treatment with ToLCGV+ds2 (AV1/AV2), no visible symptoms observed at 19 dpi. At 39 dpi, the percentage of infected plants in ToLCGV (positive control) group strikingly remained the same (27%), whereas in case of ToLCGV+ds3 (C1/C4-V1/V2), ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) groups, the percentage of infected plants increased to 45% and 36%, respectively. On the other hand, at 39 dpi, the percentage of infected plants in ToLCGV+ds1 (C1/C4) group remained relatively low (18%) and plants from ToLCGV+ds2 (V1/V2) group have not developed any symptoms (Fig. 4.11).

The main problem about the validity of the results of the 2nd experiment in tomato, is that the percentages of infected plants were unexpectedly relatively low for all treatments, including the plants treated only with ToLCGV (positive control group). While at 2nd experiment the development of the symptoms at positive control group began at 19 dpi

(e.g. earlier than at the 1st experiment), the percentage of infected plants remained stable at 27% throughout the whole experiment.

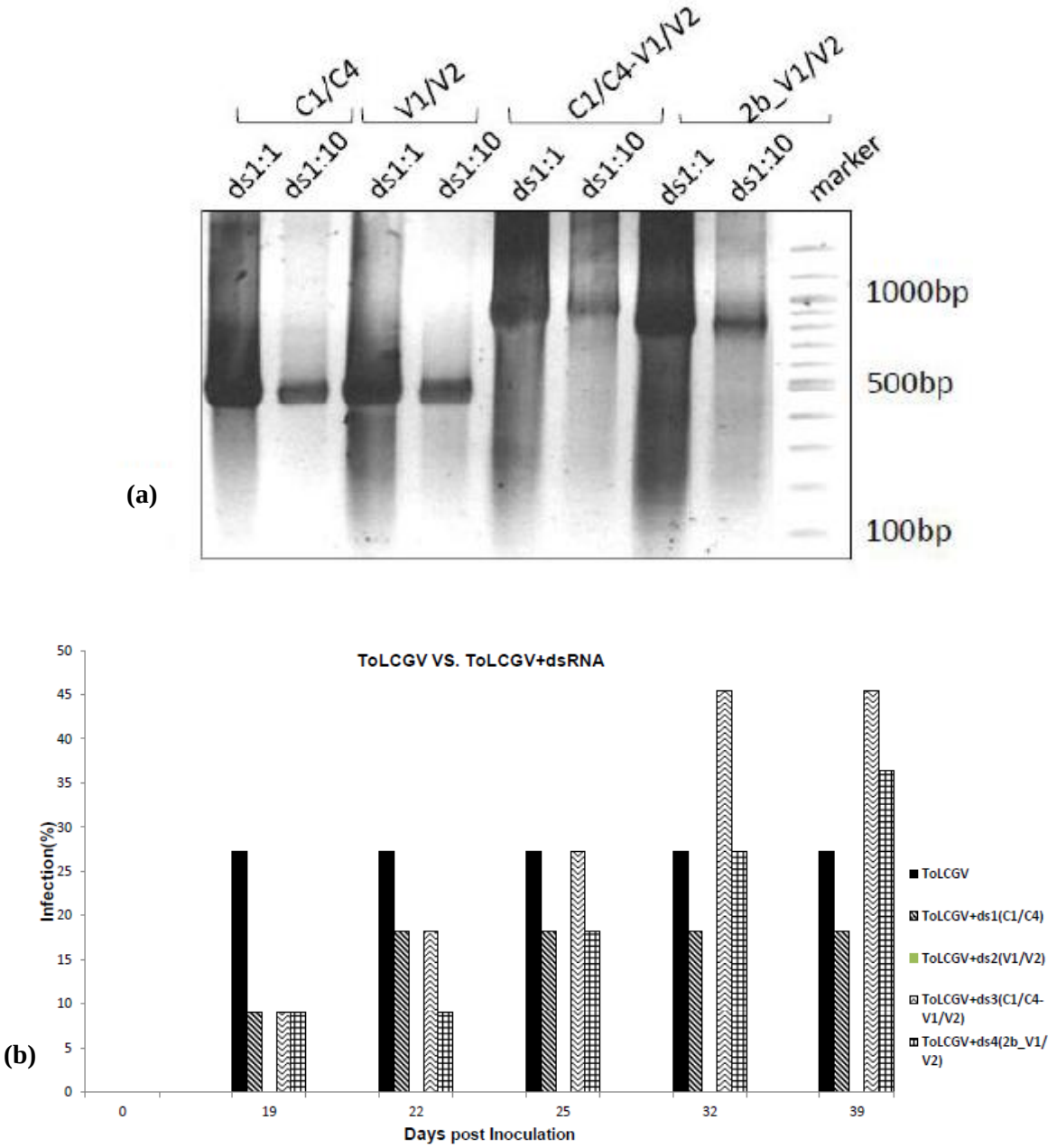

Figure 4.11. Effect of dsRNA exogenous application on disease incidence of ToLCGV on *S. lycopersicum* plants (Experiment II). **(a)** Gel electrophoresis image of dsRNAs for constructs 1, 2, 3 and 4, that were exogenously applied on tomato plants. (**b**) The treatments namely ToLCGV; ToLCGV+ds1 (C1/C4); ToLCGV+ds2 (V1/V2); ToLCGV+ds3 (C1/C4-V1/V2); ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) were applied. Disease incidence was calculated based on the number of plants infected out of the 11 plants used in each treatment.

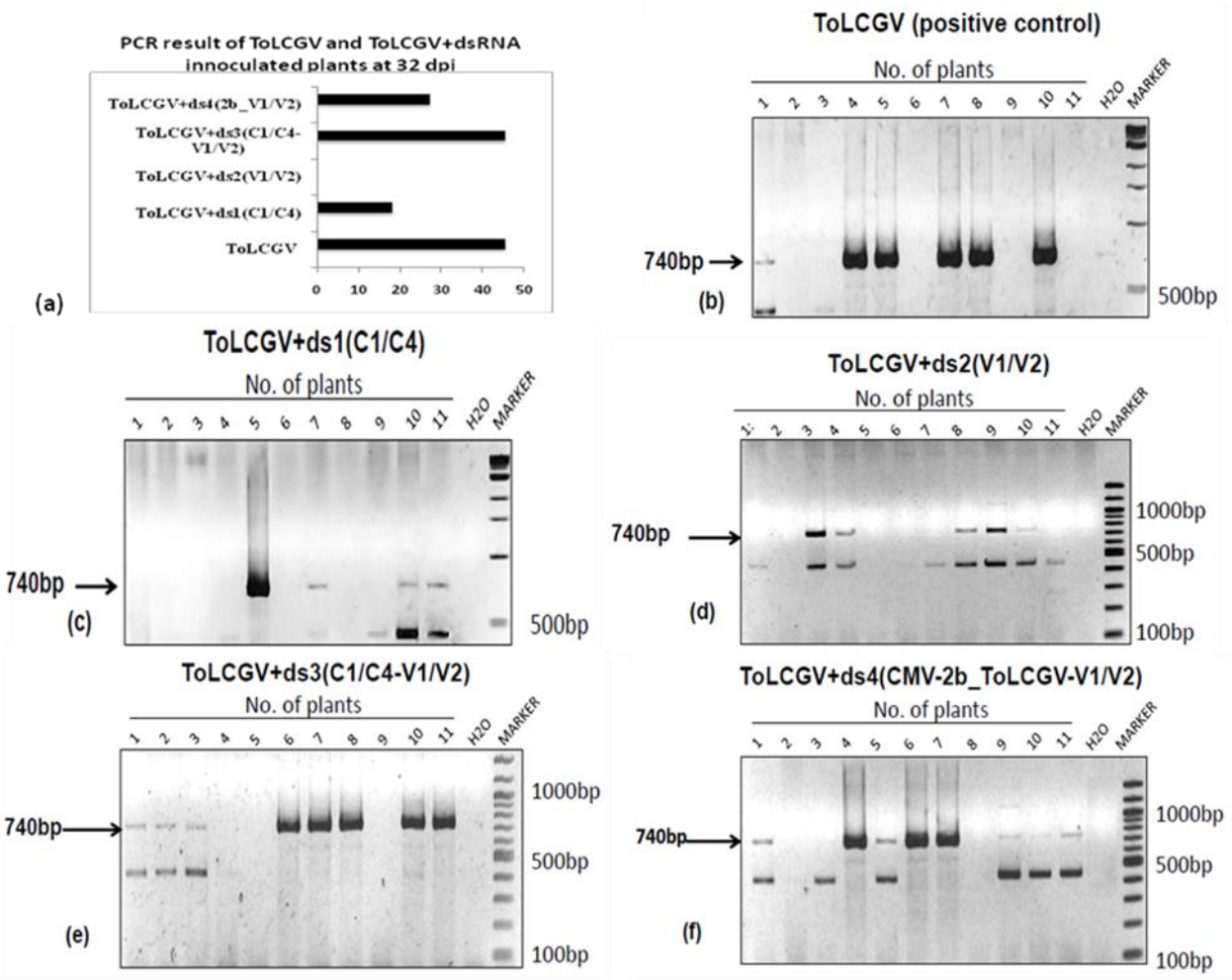

Figure 4.12. Effect of dsRNA exogenous application on disease incidence of ToLCGV on *S. lycopersicum* (Experiment II), as revealed by PCR analysis. **(a)** The treatments namely ToLCGV; ToLCGV+ds1 (C1/C4); ToLCGV+ds2 (V1/V2); ToLCGV+ds3 (C1/C4-V1/V2); ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) were applied. Disease incidence was calculated based on the number of plants infected out of 11 plants used in each treatment. Plants that gave rise to a strong amplification band at 740bp were considered positive to ToLCGV infection. PCR detection of virus in ToLCGV (positive control) inoculated plants **(b)** ToLCGV+ds1 (C1/C4) infiltrated plants **(c)** ToLCGV+ds2 (V1/V2) infiltrated plants **(d)** ToLCGV+ds3 (C1/C4-V1/V2) infiltrated plants **(e)** ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) infiltrated plants **(f)** PCR detection of virus in ToLCGV+ds4(CMV-2b_ToLGV-V1/V2) treated plants.

One possible explanation for this possibly is that the condition of the tomato plants that were used at experiment II was not ideal. Therefore, PCR analysis was done to confirm the results of the experiment II. At 32 dpi, tissue samples from the systemic leaves of each plant in all treatments were collected and subjected to DNA extraction, as described by Weigel and Glazebrook (2002). As a diagnostic tool for the detection of ToLCGV in

these samples, PCR was performed to amplify a specific fragment of DNA-A with a size of 740 bp. The primers used are shown in Table 4.1. Interestingly, the PCR analysis revealed that in some cases plants that had no symptoms of disease (e.g. yellow mosaic), produced a very strong amplification band, suggesting the existence of high viral titer (Fig. 4.12). For the final calculation of infected plants at 2nd experiment in tomato, samples that gave rise to a strong band at 740 bp were considered as positive to ToLCGV infection. The most important finding from PCR analysis is that the percentage of infection in ToLCGV (positive control) group increased to 45%. At the same time, 18% of plants from treatment with ToLCGV+ds1 (C1/C4), 45% of plants from treatment with ToLCGV+ds3 (C1/C4-V1/V2) and 27% of plants from treatment with ToLCGV+ds4 (CMV-2b_ToLCGV-V1/V2) were considered infected from ToLCGV by PCR analysis. Finally, in comparable to other treatments, the virus titer was found to be reduced in the plants treated with ToLCGV+ds2 (AV1/AV2) construct.

4.3 *N. tabacum* cv. Xanthi

Two experiments were performed using *N. tabacum* as our experimental plant to investigate the effect of exogenous application of dsRNAs against CMV. As already mentioned, in the experiments in *N. benthamiana* it was found that concentration of the virus is an important parameter for the final outcome of the interaction between virus and host plant. For this reason two sap dilutions of CMV were used. At 1st experiment 1:25 sap final dilution was used whereas for the experiment II, 1:50 sap final dilution was used. Indeed the results showed better percentages of protection conferred by the external dsRNAs in the experiment II. This suggests that the 1:25 CMV sap dilution seemed to be very strong for studying the induction of resistance against CMV in tobacco. Details of these two experiments will be presented below.

4.3.1 Experiment I

In order to examine if the exogenously applied dsRNA molecules have the capacity to confer resistance against CMV infection, 12 *N. tobacum* plants were used for each of the following treatments: a) CMV+ds (CMV_2b)

b) CMV+ds4 (CMV-2b_ToLCGV-V1/V2)

c) CMV (positive control)

d) Water (negative control)

For *Cucumber mosaic virus* (CMV), the host plant was *N. tabacum* cv. Xanthi and the inoculation consisted of solution containing dsRNAs [for construct 4 (CMV_2b-ToLCGV-V1/V2) and CMV_2b] and CMV-infected plant sap; was prepared by adding 4 µl of *in vitro* produced dsRNA and 16 µl of sap (at 1:25 dilution) that was extracted from 14 days post infection (dpi) CMV-infected tobacco plants. The positive control group comprised of inoculating single leaves of experimental tobacco plants with CMV sap alone (without dsRNA). Twelve tobacco plants per treatment were inoculated by gently rubbing the inoculation mixtures onto single fully expanded carborundum-dusted leaves with 20 µl inoculation mixture per leaf and disease symptom development was monitored daily until 8 dpi. At 6 dpi, CMV (positive control) inoculated plants showed 100% of infection whereas CMV+ds (CMV_2b) and CMV+ds4 (2b_V1/V2) treatments showed 25% and 66% of infection respectively. At 8 dpi all the plants from CMV+ds (CMV_2b) and CMV+ds4(2b_V1/V2) were infected (100%).

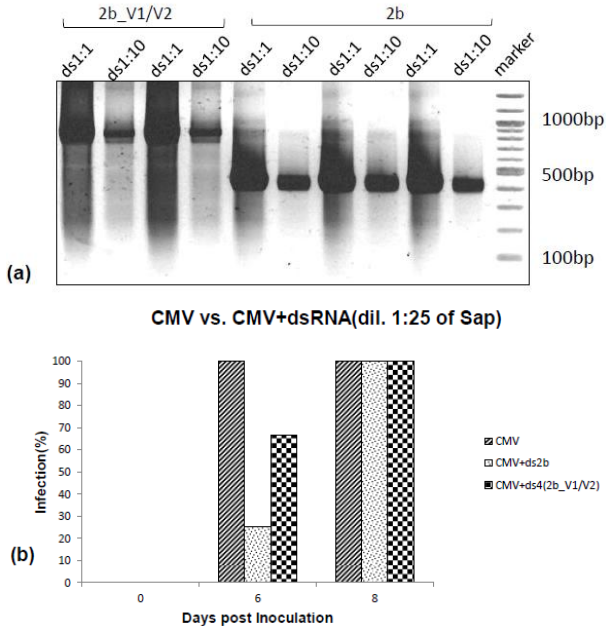

Figure 4.13. Effect of dsRNA construct on disease incidence of CMV on *N. tabacum* plants (Experiment I). **(a)** Gel electrophoresis image of dsRNAs for constructs CMV_2b and ds4 (2b_V1/V2), that were exogenously applied on tobacco plants. (**b**) The treatments namely, CMV, CMV+ ds (CMV_2b) and CMV+ds4 (CMV-2b_ToLCGV-V1/V2) were applied. Disease incidence was calculated based on the number of plants infected out of 12 plants used in each treatment

4.3.2 Experiment II

As in case of experiment II, twelve *N. tabacum* plants for each of the following treatments:

a) CMV+ds (CMV_2b)

b) CMV+ds4 (CMV-2b_ToLCGV-V1/V2)

c) CMV (positive control)

d) Water (negative control)

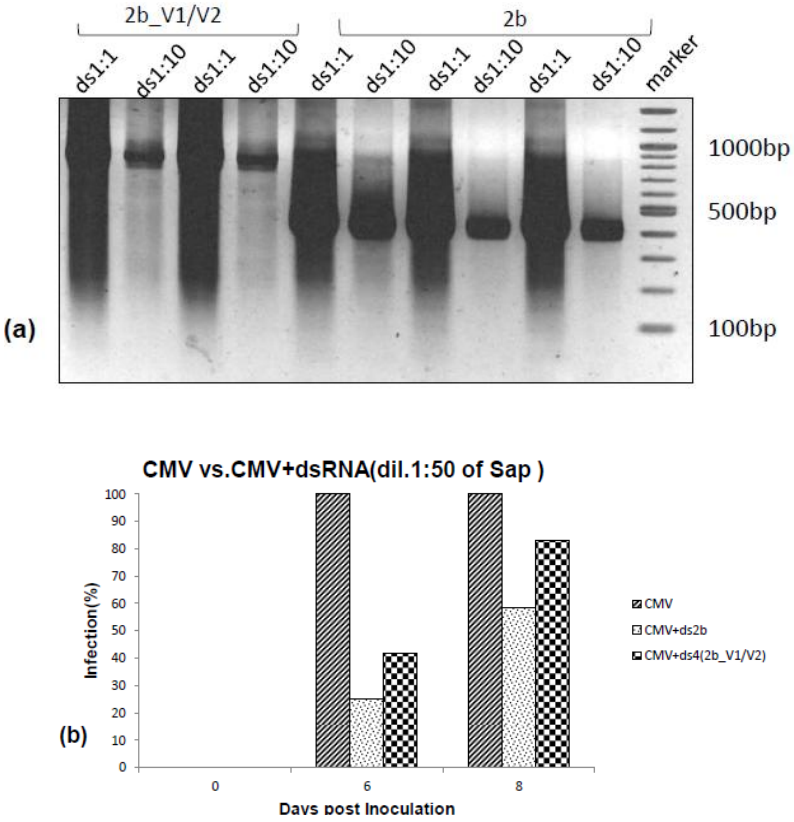

Figure 4.14. Effect of dsRNA construct on disease incidence of CMV on *N. tabacum* plants (Experiment II). **(a)** Gel electrophoresis image of dsRNAs for constructs CMV_2b and ds4(2b_V1/V2), that were exogenously applied on tobacco plants. (**b**) The treatments namely CMV, CMV+ds (CMV_2b) and CMV+ds4 (CMV-2b_ToLCGV-V1/V2) were applied. Disease incidence was calculated based on the number of plants infected out of 12 plants used in each treatment.

The inoculation consisted of solution containing dsRNAs [construct 4 (2b_V1/V2) and CMV-b] and CMV-infected plant sap; was prepared by adding 4 µl of *in vitro* produced dsRNA and 16 µl of sap (at 1:50 dilution) that was extracted from 14 dpi CMV-infected tobacco plants. Twelve tobacco plants per treatment were inoculated by gently rubbing

the inoculation mixtures onto single fully expanded carborundum-dusted leaves with 20 µl inoculation mixture per leaf and disease symptom development was monitored daily until 8 dpi. At 6 dpi, CMV (positive control) inoculated plants showed 100% of infection whereas CMV+ds (CMV_2b) and CMV+ds4 (2b_V1/V2) treatments showed 25% and 41% of infection respectively. At 8 dpi, CMV+ds (CMV_2b) and CMV+ds4 (2b_V1/V2) treatments showed 58% and 83% of infection, respectively. In conclusion, when 1:50 dilution of CMV sap is used dsRNA for CMV_2b and construct 4 (2b_V1/V2) confer resistance at 41% and 16% of the plants, respectively.

5. DISCUSSION

5.1. Two important tomato viral pathogens

Geminiviruses compose one of the largest plant-infecting groups of viruses, which are transmitted through insect vectors. These viruses are ssDNA having one or two DNA components and are well known to infect several economically important crops causing high yield losses. *Tomato leaf curl Gujarat virus* (ToLCGV) is a bipartite geminivirus causing serious damage in tomato in various parts of India. On the other hand, CMV that belongs to another family, *Bromoviridae*, differs from *Tomato leaf curl virus* in many aspects, the most important of which is that it constitutes an RNA virus. More precisely, CMV is a single stranded RNA virus of three strands, which are enclosed by the coat protein and is known to have a wide range of host plants, including tomato. One of the most important ORFs of CMV is that encoding for 2b protein that inhibits the ability of the plant's RNAi mechanism. DNA and RNA viruses differ substantially in their mode of replication. For example, for their replication DNA viruses are based mainly on the host's intracellular machinery of DNA replication that resides into the nucleus and takes places during mitosis. On the contrary, the replication of RNA viruses is much simpler, it occurs into specific cytoplasmic organelles called VRCs (Virus Replicating Complexes) and it only necessitates the function of either the host- or virus-encoded RNA dependent RNA polymerases for the production of the complementary RNA strand. In this aspect, one interesting point of the present study was to investigate if the proposed method of exogenous dsRNA application has similar degree of effectiveness against either a DNA or an RNA virus, or if exist significant differences between the two categories.

5.2. Development of novel approach to confer resistance against two different viruses infecting tomato

The mechanism that is being attempted to exploit is a natural defence mechanism of the plants, called RNA silencing or RNA interference (RNAi) that is activated for the inhibition of invading viruses. RNAi is triggered by double stranded RNA (dsRNA) molecules and has been established as a powerful means to control plant viruses in transgenic plants (Waterhouse *et al.*, 1998; Smith *et al.*, 2000; Liu *et al.*, 2007; Zhu *et al.*, 2009). One of the most successful transgenic approaches has been the incorporation of hairpin constructs that contain viral target sequences (Leibman *et al.*, 2011). Hairpin

constructs when transcribed can continuously produce large amounts of dsRNAs which are cleaved by DICER-like proteins (DCL) into small-interfering RNAs (siRNAs) or vsiRNAs (viral derived siRNAs). The whole population of vsiRNAs in transgenic plants that possess hairpin constructs is huge, it is produced in all parts of the plant and has been proved very efficient in conferring resistance against the cognate virus (from which they are derived and to which they show complementarity) (Zhu *et al.*, 2009). However, transgenesis is not approved worldwide and there is great concern about possible negative effects of transgenic plants to the natural environment. Thus in order to produce plants that are resistant to several types of viruses, there is a need for the development of a more environmentally safe technology. A non-transgenic approach exploiting RNAi has been developed previously to control plant viruses and was called 'RNA based vaccination' (Tenllado *et al*., 2004). In the current study, we followed the same concept and designed the production of dsRNA molecules against specific target genes of ToLCGV and CMV. More precisely, dsRNAs were made for overlapping regions of the ToLCGV genome, namely, C1/C4, V1/V2, C1/C4-V1AV4 (fusion construct) and the hybrid of CMV-2b joined to ToLCGV-V1/V2 (hybrid construct).

5.3. Advantages and disadvantages of RNA based vaccination

For the production of above dsRNAs, a previously described method based on two sequential PCR reactions coupled with *in vitro* transcription was followed (Voloudakis *et al.*, 2015). DsRNAs were manually applied by rubbing on the upper surface of carborundum dusted leaves of 35-days old *N. benthamiana* or *S. lycopersicum* plants, that both constitute hosts of ToLCGV. Both ToLCGV and dsRNA construct were agro-infiltrated in the lower surface of the same leaf at same day. The proposed method of RNA-based vaccination may have advantages and disadvantages relative to the transgenic technology. One such advantage is that the method is much simpler and avoids the time consuming, laborious and expensive production of transgenic plants. Moreover, because in natural conditions many arising diseases originate from mixed viral populations, this method could be used for the application of dsRNAs not only for one virus but for many viruses simultaneously. On the other hand, there are possible disadvantages of RNA based vaccination. For example, as mentioned above the application of dsRNAs is executed by gently rubbing on one or two carborundum dusted leaves. The dsRNA molecules are expected to move systemically from the inoculated leaves to the whole

plant, and then will be recognized by the DCL proteins, giving rise to siRNAs and activating the RNAi response. Indeed, recently in Prof. Voloudakis' lab, it was found that dsRNA molecules targeting specific genes of TMV genome after exogenous application to tobacco plants move very rapidly from the local inoculated leaves to systemic parts of the plants. However, it was also found that dsRNA is prone to degradation *in planta*, thus it is expected that after a period of several days the pool of the applied dsRNAs will be exhausted (Konakalla *et al.*, 2016). Possibly, one solution could be the repetitive application of exogenous dsRNAs at fixed periods of time.

5.4. RNA based vaccination against ToLCGV in *N. benthamiana*

Several experiments were performed using *N. benthamiana* as experimental plant for ToLCGV. From the initial experiments it became evident that *N. benthamiana* were extremely susceptible to ToLCGV, since all plants developed disease symptoms within 12 days post inoculation (dpi) and the only effect of exogenous dsRNA application was just a three-day delay in ToLCGV symptom development. One possible explanation for these results is that the virus concentration, induced by agro-infiltration was so high that overcame the plants defense mechanism. The final outcome of the battle between a virus and a host plant will depend on the balance between the virus multiplication rate from the one side and the degree of activation of RNAi on the other side. If multiplication of the virus into plant cells is high, then the multiplication of the virus will be very rapid and difficult to be inhibited even by an efficient mechanism such as RNAi.

For these reasons, it was felt necessary to use dilutions of ToLCGV agro-inoculum in order to test the efficacy of the RNA-based vaccination method in *N. benthamiana* that could be induced by exogenous application of the dsRNA molecules made in this study targeting ToLCGV. For this, an experiment was performed using serial dilutions of ToLCGV agro-inoculum to find out the highest dilution at which all plants from the positive control group (ToLCGV only) become infected. It was observed that when ToLCGV agro-inoculum is diluted 2×10^3 times, then 100% of the plants develop the symptoms at 14 dpi. On the other hand, when ToLCGV agro-inoculum was further diluted (2×10^4 times), then only 60% of the plants develop symptoms at 14 dpi, suggesting that the 2×10^4 is an extremely high dilution of agro-inoculum. For this kind of experiments, if the effectiveness is to be determined, if any, of the applied dsRNAs, it is

absolutely necessary for the positive control group (ToLCGV only) to exhibit a percentage of infection very close to 100%. Thus, two more dilution experiments were performed using ToLCGV agro-inoculum. In the case of 2×10^3 dilution of ToLCGV agro-inoculum, there was a few days delay in ToLCGV symptoms development, but finally all plants got infected to ToLCGV at 14 dpi. ToLCGV+ds2 (V1/V2) inoculated plants always exhibited the lowest percentage of ToLCGV infection, as compared to the other treatments. The results suggest that dsRNA for construct 2, namely ds (V1/V2) confers resistance in *N. benthamiana* plants against ToLCGV when compared to dsRNAs for the remaining three constructs. Surprisingly, dsRNAs for the fusion and the hybrid constructs does not seem to provide any significance protection against ToLCGV. One possible explanation for this failure could be the lower amounts of ds3 (C1/C4-V1/V2) and ds4 (CMV-2b_ToLCGV-V1/V2) as compared to ds1 (C1/C4) and ds2 (V1/V2). From the results it is clear that the bands corresponding to 10 times diluted dsRNA of construct 3 (C1/C4-V1/V2) and construct 4 (CMV-2b_ToLCGV-V1/V2) are thinner than the respective bands of construct 1 (C1/C4) and construct 2 (V1/V2) (Fig. 4.7a). Also *in vitro* transcription reactions for ds3 and ds4 were successful producing in gel electrophoresis very strong bands, comparable to the bands of ds1 and ds2. It is possible that constructs 3 and 4 because of their length constitute inaccessible substrates for the function of T7-RNA polymerase and the subsequent annealing of the two complementary RNA strands. Importantly, at 14 dpi apart from ToLCGV+ds2 (V1/V2) treatment that exhibited 58% of infection, all the remaining treatments including ToLCGV positive control showed percentages of infection closed to 80%. If we consider the percentage of infection of ToLCGV positive control group as 100%, then the respective percentage of ToLCGV+ds2 (V1/V2) treatment is reaches 72 %. This means that ds2 (V1/V2) confers protection at 27 % of the plants.

Two are the main conclusions that can be drawn from the application of RNA-based vaccination against ToLCGV in *N. benthamiana*. First, dsRNA from construct 2 (V1/V2) seems to possess the higher capacity among all four constructs developed to activate the RNA silencing against ToLCGV. Second, *N. benthamiana* is not a suitable experimental plant for this kind of experiments because it has been proved to be extremely sensitive to ToLCGV infection in the way the inoculum is provided. Possible explanations for the extreme sensitivity of *N. benthamiana* to ToLCGV could be that some important factor(s)

in the defense mechanism against geniniviruses are missing or naturally ineffective in this host plant. These factors may be some specific members of the DICER, RDR or AGO protein families that have crucial role in different steps of the RNA silencing mechanism.

5.5. RNA based vaccination against ToLCGV in tomato (*Solanum lycopersicum*)

Because of the above discussed reasons, we have further tested RNA-based vaccination against ToLCGV on tomato plants. On the basis of results obtained from infiltration of *N. benthamiana* plants, we have used a dilution of ToLCGV agro-inoculum (2×10^2 times) (experiment 1) and the disease symptoms were continued till 42 dpi, to find out the final percentages of infected plants for every treatment. The first observation is that the development of symptoms from ToLCGV infection in tomato takes much longer time as compared to *N. benthamiana*. This may indicate a slower multiplication rate of the virus in tomato, or may be a property of the specific variety of tomato plants (cv. Clodin) that was used in these experiments. The results from this experiment in tomato clearly indicate that dsRNAs for constructs 2 and 4 provide significant resistance against ToLCGV infection in tomato, while dsRNAs for constructs 1 and 3 do not possess this capacity. In another experiment (OD of 1.0; experiment 2) in tomato, generally the percentages of infected plants were relatively smaller for all treatments, including the positive control group (plants treated only with ToLCGV). Importantly, in case of treatment using ToLCGV+ds2 (AV1/AV2), none of the plants exhibited disease symptoms and also PCR was failed to detect any viral components and thus, all plants from this group were considered as negative for ToLCGV infection. In case of experiment 1, the most effective was ds4 (CMV-2b_ToLCGV-V1/V2), while experiment 2, the most effective was ds2 (V1/V2). These constructs share a common region (291 bp) that is the overlapping region of DNA-A genome that contains the whole V2 ORF and the initial part of V1 ORF. Thus, it is reasonable to assume that this part of the genome may play a crucial role in the activation of RNA silencing mechanism that is directed against ToLCGV. V1 ORF encodes the viral coat protein and plays a predominant role in virus transmission. In begomoviruses, V2, which partially overlaps with the coat protein (CP) is present only in the old world viruses and encodes for pre-coat protein (PCP). The pre coat-protein of *Tomato yellow leaf curl virus* also suppresses RNA silencing by binding to the host's SGS3 protein (Glick *et al*., 2008). Interesting research has been done regarding the properties of Ty-1 locus from wild tomato species that confers resistance against TYLCV

and several bipartite begomovirus species. Ty-1 locus encodes for an RNA-dependent RNA polymerase (RDR) with similarity to RDR3, 4, 5 of *Arabidopsis thaliana*. Recently it was found that Ty-1 confers resistance to geminiviruses by activating the transcriptional gene silencing mechanism (TGS) that increases cytosine methylation of the V1 promoter region at the viral genome (Butterbach *et al.*, 2014). Interestingly, the V1 promoter sequence is included in the 432 nt overlapping region of V1/V2 ORFs that was used as template for the production of ds2 in the present study. Thus, it is tempting to speculate that the exogenous application of dsRNA for V1/V2 may promote the production of siRNAs from the crucial region of V1 promoter, leading to the activation of TGS against ToLCGV. Such a hypothesis could be tested in the future. This interpretation could explain the higher efficacy of ds2 (V1/V2) and ds4 (CMV-2b_ToLCGV-V1/V2), as compared to the other constructs.

In the majority of experiments performed, dsRNA for construct 1 (C1/C4) was not found to confer any significant resistance against ToLCGV. One possible explanation for this could be that the specific overlapping region from C1/C4 ORFs that was selected for the production of dsRNA does not give rise to a substantial population of siRNAs or that the siRNAs produced have reduced capacity to inhibit the multiplication of the virus. One unexpected result was also the incapacity of the dsRNA for the fusion construct (C1/C4-V1/V2), a construct that contains the V1/V2 region that was observed that provides resistance, to protect *N. benthamiana* or tomato plants from ToLCGV infection. One possible explanation could be that the simultaneous production of siRNAs from C1/C4 region together with siRNAs from V1/V2 region may have a negative effect in the effectiveness of the latter on RNAi pathway. For example, if a large population of siRNAs exists, there must be an antagonism between them for incorporation into the RISC complexes. Another possible explanation for the low efficiency of construct ds3 (C1/C4-V1/V2) to protect from ToLCGV infection could be its lower concentration relative to other dsRNAs for other constructs such as V1/V2, something that was already discussed in previous sections.

5.6. RNA based vaccination against CMV in tobacco (*N. tobacum*)

From the two experiments using RNA-based vaccination against CMV in tobacco plants, it is concluded that dsRNA for CMV-2b exhibits greater capacity to confer resistance

against the virus as compared to dsRNA for the hybrid construct (2b-V1/V2). One possible explanation is that the hybrid construct may produce a mixed population of siRNAs (derived from both 2b and V1/V2 regions) that antagonize between them for incorporation into RICS complexes. This may diminish the effectiveness of siRNAs derived from 2b region to find the complementary parts of CMV genome and to inhibit the multiplication of the virus.

In summary, the work described here investigated the efficiency of the exogenous application of dsRNA molecules to confer resistance in host plants against *Tomato leaf curl Gujarat virus* (ToLCGV) and *Cucumber mosaic virus* (CMV). The generation of transgenic plants has been used widely to confer resistance against several viruses. The major point of concern with transgenic resistance is the presence of selectable marker genes which may be detrimental to environment and human health. Moreover, these genes may be transferred to other crops by cross pollination. Therefore the present study emphasizes that using this RNA-based vaccination method, it is now possible to confer virus resistance and subsequently, address the issues raised by public concerns over the environmental safety.

6. SUMMARY

The present study was aimed to develop dsRNA mediated protection in *N. benthamiana* and *S. lycopersicum* against Cucumber mosaic virus and Tomato leaf curl virus. Following achievements were made:

1. Identification of target region of ToLCGV was performed based on the sequence conservation. The conserved region of AV1/AV2 and AC1/AC4 were selected through sequence alignment using different isolates of ToLCVs and different region were selected as the target region. Similarly, the target regions from CMV were also selected. The following targets region were considered for developing different constructs : Construct 1st - ToLCGV-C1/C4 (Overlapping region), Construct 2nd - ToLCGV-V1/V2 (Overlapping region), Construct 3rd - ToLGV-C1/C4-V1/V2 (Fusion construct), Construct 4th - CMV_2b-ToLCGV-V1/V2 (Hybrid construct) and Construct 5th - CMV-2b
2. The oligonucleotides (primers) were designed to amplify the target region from CMV and ToLGCV.
3. In case of construct 4, the target region was cloned by amplifying the specific region from CMV and ToLCV using polymerase chain reaction (PCR). A DNA construct joining the target regions of both CMV and ToLCGV was generated.
4. In this study for the first time construction of a hybrid construct (CMV_2b-ToLCGV-V1/V2) to generate dsRNA for the concomitant protection of two viruses.
5. For all four constructs, the T7 RNA polymerase promoter sequence was introduced at both 5' and 3' ends in the second PCR reaction using a specific T7 linker primer.
6. dsRNA specific to the target regions of CMV and ToLCGV were synthesized *in vitro*.
7. The efficacy of the generated dsRNA molecules were checked by applying exogenously onto leaves of *N. benthamiana* and *S. lycopersicum*, followed by virus inoculation in case of ToLCGV. ToLCGV was agro-infiltrated onto leaves previously applied with dsRNA.

8. In case of CMV, infected sap from the disease plants was prepared from 14 days CMV inoculated tobacco plant and efficacy of the generated dsRNA molecules was checked by applying onto leaves of tobacco.
9. In case of tomato plants, the construct 2nd (ToLCGV-V1/V2) and construct 4th (CMV_2b-ToLCGV-V1/V2) gave protection against ToLCGV.
10. In case of *N. tabacum* plants, the construct 5th (CMV-2b) provided more protection than the construct 4th (CMV_2b-ToLCGV-V1/V2).

Taken together, it can be concluded that in the case of *N. benthamiana* plant, that is highly sensitive to ToLCGV, application of dsRNA produced for all the constructs delayed appearance of symptom 3 to 4 days following ToLCGV inoculation. In case of tomato plants the construct 2 and construct 4 induced some degree of resistance against ToLCGV where as in the case of *N. tabacum,* the construct 5 provided more resistance than construct 4 against CMV.

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8. APPENDIX

For agarose gel electrophoresis

- 10X TAE buffer : 48.4g of Tris base, 11.42 ml of Glacial acetic acid, 20 ml EDTA 0.5 M (pH 8) was added and the volume was adjusted to 1liter with distilled H_2O. The pH was adjusted to 8-8.3. For gel electrophoresis 0.5 X TAE buffer is used.
- 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 4 ºC.
- DNA Molecular Marker (New England Biolabs, USA) - (ladder size in bp) 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100.
- Ethidium Bromide solution (10 mg/ml, AppliChem, USA) is stored at room temperature. 15 ug of the solution are added to 250 ml 0.5 X TAE buffer and used for gel staining.

For cloning

- Ampicillin Stock solution (100 mg/ml) was made in double distilled H_2O, filter sterilized and aliquoted in 1.5 ml tubes and stored at -20 ºC.
- Kanamycin Stock solution (100 mg/ml) was made in double distilled H_2O, filter sterilized and aliquoted in 1.5 ml tubes and stored at -20 ºC.
- Rifampicin Stock solution (10 mg/ml) was made in double distilled H_2O, filter sterilized and aliquoted in 1.5 ml tubes and stored at -20 ºC.
- Lennox L broth base media: 2g are dissolved in 100 ml of H_2O and sterilized by autoclaving.
- Lennox L broth base agar media: 2 g are dissolved in 100 ml of H_2O, then 1.7 g of bacto-agar is added and sterilized by autoclaving.

For plasmid isolation

For plasmid isolation, the Nucleospin Plasmid kit was used (Macherey-Nagel, Germany) following the manufacturer's instructions.

For total DNA extraction

For DNA isolation the Quick DNA Prep for PCR adapted from Weigel and Glazebrook (2002) was used, as described in the materials and methods section.

Vector map of pGEM-T Vector