HAIRPIN RNA INTERFERENCE CONSTRUCTS AGAINST *Tomato Leaf Curl Sudan Virus*: TRANSFORMATION, AND EVALUATION IN *Nicotiana benthamiana* PLANTS

M. A. Amer[1], Z. Khalid[1], M. T. Shakeel[2], A. Sabra[3], M. Amir[1], M. Zaman[1], K. Hussain[1], I. M. Al-Shahwan[1] and M. A. Al-Saleh [1*]

[1]Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia.
[2]Department of Plant Pathology, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, Pakistan.
[3]College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China.
[*]Corresponding author's email: malsaleh@ksu.edu.sa

ABSTRACT

Tomato Leaf Curl Sudan Virus (ToLCSDV) is a begomovirus that severely damages tomato plants, causing growth retardation, yellowing, and curling of the leaves. Hairpin RNA interference (hpRNAi) is a defense mechanism against viruses and other infectious nucleic acids has been successfully exploited for engineering virus resistance in *Nicotiana benthamiana* plants. In this study, we developed a hpRNAi construct on the conserved region of capsid protein (CP) gene of ToLCSDV to offer protection against tomato-infecting ToLCSDV as a begomoviruses. In order to create the hairpin RNAi construct, the primer pairs CPS-F and CPS-R then CPAS-F and CPAS-R primers were used to amplify the CP gene fragment in sense and antisense orientation. The hairpin RNAi construct (hpCP-pFGC5941) was created by ligating both sense and antisense orientation into pFGC5941 as a binary vector in MCS1 and MCS2, respectively. XhoI and NcoI restriction of the pFGC5941 vector and CPS PCR result reveals the distinct bands for CP sense and plasmid vector. The amplified CP anti-sense segment was restricted and ligated into the vectCPS-pFGC5941 using BamHI and XbaI restriction sites. For confirmation of recombinant clones, restriction analysis displayed required bands at a certain size: pFGC5941 vector (11.406 bp), CPAS (~686 bp), and CPS-pFGC5941 vector (~12.1 Kb). Following PCR validation of CP sense and antisense clones using 35S-Pro-F/chsA-intr-R and chsA-intr-F/OCS-term, respectively, three clones for sense and antisense (1Kb) were obtained. In each experiment, 10 *N. benthamiana* plants were inoculated: 5 plants received ToLCSDV (infectious clone) + hpCP-pFGC5941 (hpRNAi construct) and 5 plants received ToLCSDV (infectious clone) alone. These experiments were performed in triplicates. Plants that were inoculated with the hpRNAi construct showed encouraging resistance when compared to those that were inoculated with the ToLCSDV infectious clone alone. In this study, hpRNAi plants exhibited resistance compared to the controls hpRNAi targeting CP of ToLCSDV reduced infection from 100% to 27% in treated plants. The results of RNAi resistance and their importance in development of RNAi based resistance in elite tomato cultivars are discussed

Keywords: hpRNAi, ToLCSDV, begomovirus, viral resistance, capsid protein, *Nicotiana benthamiana*.

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INTRODUCTION

In the world of vegetable crops, tomato (*Solanum lycopersicum* L.; family: Solanaceae) is one of the most widely consumed and economically significant crops. Growing in various places, tomatoes are regarded as one of the most commercially significant vegetable crops in Saudi Arabia, grown on approximately more than 12,000 hectares of land, yielding about 351,000 tons of fresh tomato fruits annually. Numerous phytopathogens greatly decrease crop production when they infect tomato plants (Agrios, 2005; Jones *et al.*, 2016; FAO, 2020). Viruses belonging to the family *Geminiviridae* and genus *Begomovirus* seriously harm a variety of crops and lower their intended productivity (Malathi *et al*. 2017). Tomato Leaf Curl Sudan Virus (ToLCSDV) is a unique begomovirus that, like other leaf curl viruses, is spread by the whitefly *B. tabaci*, however it is occasionally mistaken for tomato yellow leaf curl virus (TYLCV). Begomoviruses have circular single-stranded DNA genomes that are either mono- (DNA-A) or bipartite (DNA-A and DNA-B) (Zhai *et al*., 2022). According to previous reports, ToLCSDV causes tomato leaf curl disease, which results in notable yield losses because of symptoms including, yellowing, reduced development, and leaf curling. The virus is a danger to tomato production in the area. Depending on the crop type and infection stage, the infection might result in yield losses of up to 100 % (Odeh *et al.,* 2025; Mohammed *et al.,* 2018; Akhtar *et al.,* 2014; Idris *et al.,* 2011, 2012,

2014; Khan *et al.,* 2013a, b; Al-Saleh *et al.,* 2014; Khan *et al.,* 2008; Ajlan *et al.,* 2007). Numerous reports have documented the link between begomovirus and leaf curl disease on a variety of crops. The world's economically significant agricultural crop production and food security are seriously threatened by begomoviruses and their associated ssDNA satellites due to their high diversity, recombination potential, limited knowledge of alternative hosts, and transmission by *B. tabaci*. As a result, further research and deeper understanding are required (Saeed *et al.,* 2017).

Hence, a sequence-specific RNA regulation mechanism called RNA silencing (RNA silencing) has been identified in eukaryotic organisms (Agrawal *et al.,* 2003). It is a crucial protective system for hosts that counters resistance against foreign nucleic acids, including transposons, transgenes, and viruses (Voinnet, 2001; Lippman *et al.,* 2003). It is important in regulating gene expression (Shabalina and Koonin, 2008). Currently, it has been demonstrated that almost every class of virus can encode proteins that interfere with RNA silencing (Li and Ding, 2006). The presence of these suppressors is the strongest evidence supporting the contention that an adaptable defence against infections is RNA silencing. A potential solution for the management of diseases caused by begomovirus is RNA interference (RNAi) (Prins *et al.,* 2008; Chowda-Reddy *et al.,* 2008). RNAi was utilized to help tomatoes become resistant to TYLCV, *Bean golden mosaic virus* (BGMV) (Bonfim *et al.,* 2007) and cotton leaf curl geminivirus complex (Mubin *et al.,* 2011). This resistance lessens the severity of disease and virus titer, but it is not immunity. Additionally, the betasatellite might weaken resistance, which would raise the percentage of plants that eventually exhibit symptoms (Ammara *et al.,* 2015).

Whereas Helper component protease (HC-Pro) stops Dicer from processing dsRNA and prevents duplex siRNA from unwinding (Chapman *et al.,* 2004). These examples illustrate that numerous procedures have emerged in viruses for overcoming RNA silencing by interfering at distinct points in the pathway. A common eukaryotic gene regulatory mechanism called "RNA silencing" hinders the gene expression of a gene by inhibiting transcription and triggering the degradation of RNA specific to a certain region. A specific RNA degradation process called post-transcriptional gene silencing (PTGS) takes place in the cytoplasm and results in the accumulation of 21–24 nt-long siRNAs that correspond to the silenced gene (Fagard and Vaucheret, 2000). Sometimes, because of RNA silencing, plants previously infected with a milder virus strain offer cross-protection (resistance) against a more severe virus with positive sense RNA genomes. Most plant viruses are replicated by using dsRNA intermediates, which significantly induce RNA silencing (Ahlquist, 2002). It has been observed that both DNA and RNA viruses have virus-derived siRNAs (Xie *et al.,* 2004; Akbergenov *et al.,* 2006). When it comes to RNA viruses, secondary structures in the single-stranded viral RNA or replication intermediate can both produce dsRNA (Molnár *et al.,* 2005). In the present study, the major objective was to develop RNAi-based resistance in the model plant, *Nicotiana benthamiana* to prove the efficiency of RNAi technology against ToLCSDV and pave the way for the development of broad-spectrum transgenic resistance against dominant begomovirus species infecting tomatoes and other crops of commercial significance.

MATERIALS AND METHODS

Sense and anti-sense CP gene construction: PCR amplification of the capsid protein gene was performed using the ToLCSDV clone from a previous study (Amer et al. 2025) as a template. A fragment of 686 bp was amplified using Sense (CPS-F/CPS-R) and Anti-Sense (CPAS-F/CPAS-R) primers orientations (Table 1).

Hairpin RNAi construct of the CP gene and transformation into competent *E. coli:* The restriction enzymes XhoI and NcoI were used to digest the PCR product's CP sense and the RNAi binary plasmid vector pFGC5941 to clone the amplified CP sense. Using the restriction enzymes BamHI and XbaI, clone the amplified CP antisense + RNAi binary plasmid vector (CPS-pFGC5941) that has been inserted with CP sense. Linear vector DNA, insert DNA, and T4 DNA ligase (New England Biolabs) were used in the ligation mixture. On a 1% agarose gel, the quality of the DNA fragments was assessed. Transformation of competent *Escherichia coli* was performed using an adapted calcium chloride procedure (Chang *et al.,* 2017).

Hairpin RNAi construct of the CP gene and transformation into Agrobacterium cells: DNA isolation of the Hairpin RNAi construct of the CP gene was carried out according to the Gene JET Plasmid Miniprep Kit manufactured by Thermo Scientific™. Two pairs of primers were utilized for the sense (35S-Pro-F/chsA-intr-R) and antisense orientation (chsA-intr-F/OCS-term-R). Restriction (XhoI and NcoI for the sense fragment and BamHI and XbaI for the antisense fragment) analysis of three plasmid clones were used to validate and confirm the hairpin CP construct orientation (hp-CP-pFGC5941) (Table 1). After that, 5 µl was added to a 1% agarose gel to validate the recombinant plasmid DNA. Electroporation technique for *Agrobacterium tumefaciens* GV3101 was conducted according to Mersereau *et al.,* 1990 and Kámán-Tóth *et al.,* 2018.

The verified transformed Agrobacterium colony was selected and cultivated in LB broth that was enhanced with a suitable combination of antibiotics (Kanamycin and Rifampicin). The culture grew in a shaker incubator at 28°C for 24

hours to prepare the inoculum. PCR amplification using specific primers (CPSF/CPSR and CPAS-F/CPAS-R) was employed to confirm the transformation of hairpin RNAi construct into Agrobacterium.

Agro-infiltration of plants and symptom observation: A single Agrobacterium colony with a recombinant plasmid was selected, inoculated in a 50-ml LB culture with appropriate antibiotics, vigorously shaken, and kept at 28°C for 48 hours. After centrifuging the culture for 10 min at 4,000 rpm, the pellet was resuspended in a solution containing 100 µM acetosyringone and 10 mM MgCl2 until the optical density (OD600) achieved at 1.0. Before *N. benthamiana* inoculation, the culture was left for two to three hours. Next, a needleless disposable syringe was used to infiltrate the Agrobacterium suspension on the underside of a leaf disc. Ten plants of *N. benthamiana* were inoculated with three different clones in biological experiment to evaluate the resistance response compared to the control plants. For each experiment, five plants were inoculated with ToLCSDV (infectious clone) plus hpCP-pFGC5941 (hpRNAi construct) and five plants were inoculated with ToLCSDV (infectious clone) alone. Five plants were inoculated with pFGC5941 vector without insert and five plants were kept without any inoculation as a negative control. These experiments were carried out three times. All these plants were kept in environmentally controlled and completely isolated in an insect-proof cages in a greenhouse at 25-28 °C and symptoms were observed after 21-30 days. Three weeks after the inoculation, the severity of the symptoms was documented. According to Al-Shahwan *et al.,* 1995, the following scale was used to assess the intensity of the symptoms: mild (+), moderate (++), severe (+++), very severe (++++), and symptomless (-). All the inoculated and control plants were being tested by PCR against ToLCSDV using specific primers (CPSF/CPSR).

Table 1. List of primers used to clone CP sense and antisense orientation and on vector pFGC5941 sequence for clone confirmation.

Primer Name	Primer Sequence	Restriction site	Amplicon Size
CPS-F	5′-ggctcgagatgttgaagcgtccc-3′	XhoI	686 bp
CPS-R	5′-caccatgggagcgttctcagtatga-3′	NcoI	
CPAS-F	5′-caggatccgagcgttctcagtatga-3′	BamHI	686 bp
CPAS-R	5′-gctctagaatgttgaagcgtcccg-3′	XbaI	
35S-Pro-F	5′-ccactgacgtaagggatgacgcacaat-3′		341 bp
chsA-intr-R	5′-acaattcgtcggccaccccaacccaaa-3′		
chsA-intr-F	5′-gcacctataaacacttacttacacttgccttggag-3′		429 bp
OCS-term-R	5′-ttgttattgtggcgctctatcatagatgtcgc-3′		

RESULTS

PCR Amplification of CP Sense and anti-sense genes Hairpin RNAi construct: PCR amplification of capsid protein gene sense and anti-sense orientations was done using a ToLCSDV clone as a template. The CPS-F and CPS-R and CPAS-F and CPAS-R primers were used to amplify a 686 bp fragment of the CP gene. No PCR amplified product from uninfected sample was used as a negative control. (Figure 1).

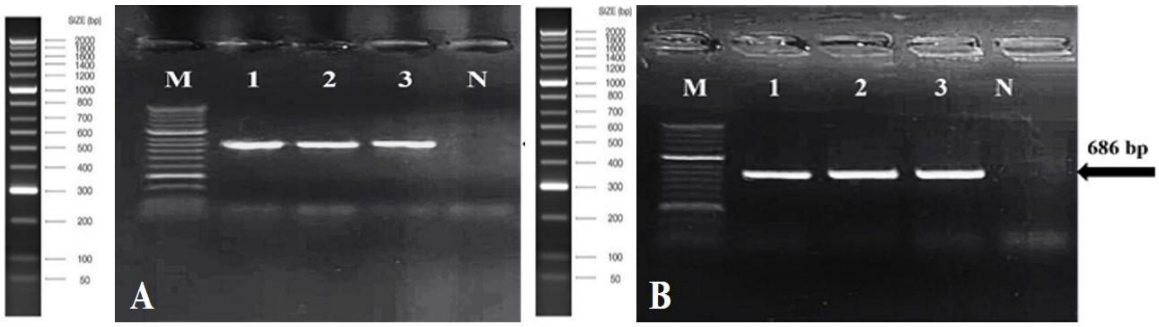

Figure 1: PCR amplification of CP sense (A) and CP antisense (B) using a ToLCSDV clone as a template. Lane M shows the DNA Ladder (Hyper Ladder™ 50 bp; Meridian Bio-Science Inc.), Lanes shows the bands of CP sense and CP antisense (approximately 686 bp), and Lane N shows the negative control.

For the construction of the hairpin RNAi construct of the CP region of ToLCSDV, first we amplified the CP gene fragment in sense orientation and antisense orientation using CPS-F and CPS-R and CPAS-F and CPAS-R pairs of primers. Then both strands (sense and antisense orientation) were ligated into pFGC5941 as a binary vector in MCS1 and MCS2, respectively, to make the hairpin RNAi construct (hpCP-pFGC5941). Restriction of pFGC5941 vector and CPS PCR product with XhoI and NcoI showing the specific bands for vector and coat protein sense. Lane 1 shows pFGC5941 vector (size is 11,406 bp) and lane 2 shows CPS (approx. 686 bp) (Figure 2).

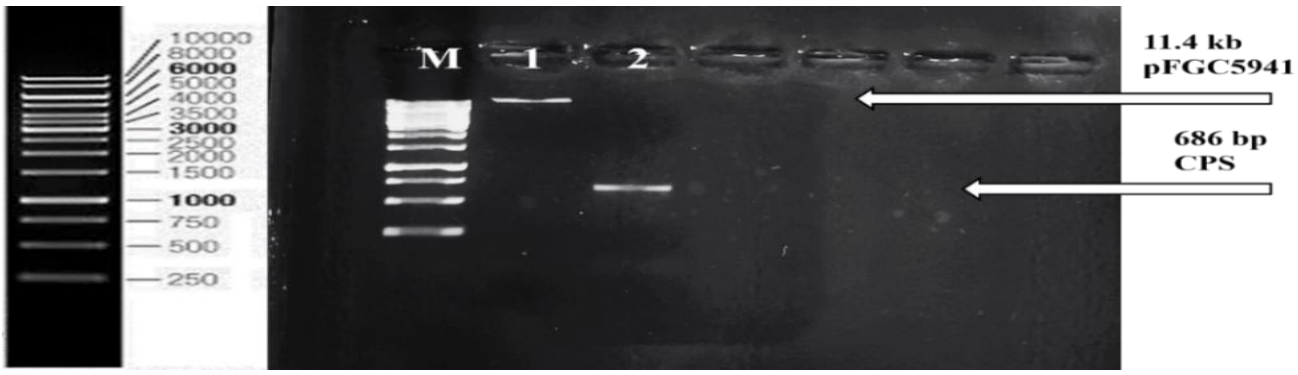

Figure 2: XhoI and NcoI-restricted pFGC5941 vector and CPS PCR product. Lane M is showing DNA Ladder, Lane 1 is showing the pFGC5941 vector (size is 11,406 bp), and Lane 2 is showing CPS (approx. 686 bp).

For restriction of CP antisense in the CPS-pFGC5941941 vector, the amplified CP antisense fragment was restricted into the vectCPS-pFGC5941, which already had the CP gene using BamHI and XbaI. The results obtained showed bands at specific size for each product: pFGC5941 vector (size is 11,406 bp), CPAS (~ 686 bp) and CPS-pFGC5941 vector (~ size is 12.1Kb) (Figure 3).

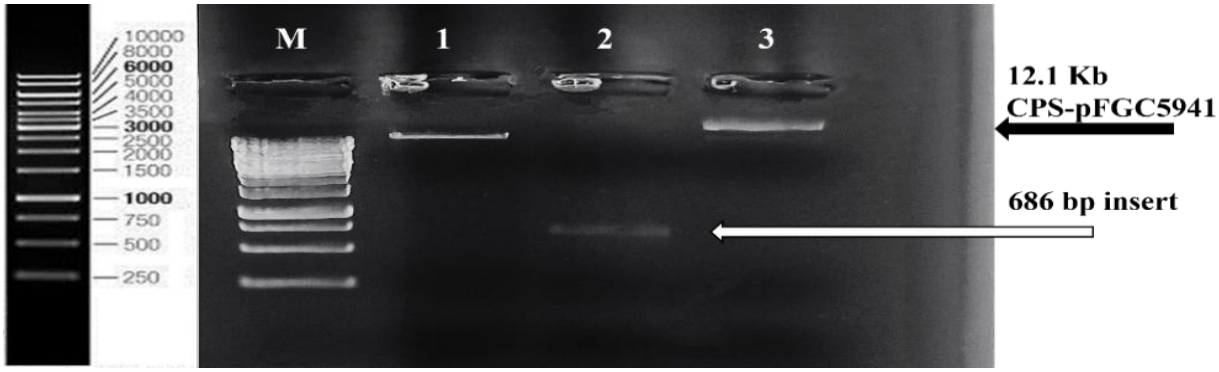

Figure 3: Restriction of CPS-pFGC5941 vector and CPAS PCR product with BamHI and XbaI. Lane M is showing DNA Ladder (Thermo Scientific™ Gene Ruler 1 kb DNA Ladder), lane 1 is showing pFGC5941 vector (size is 11,406 bp), lane 2 showing CPAS (approx. 686 bp) and lane 3 is showing CPS-pFGC5941 vector (approx. size is 12.1Kb).

Clone confirmation of hairpin CP in the pFGC5941 vector using PCR and restriction enzyme analysis: Three clones for sense and antisense are shown in panels 1-3 (1Kb) after PCR validation of clones of CP sense and CP antisense using 35S-Pro-F/chsA-intr-R and chsA-intr-F/OCS-term, respectively. No PCR amplified product (lane N) from uninfected sample was used as a negative control (Figure 4).

Analysis of restriction of the hp-CP RNAi construct in the pFGC5941 vector. The samples were digested using XhoI and NcoI for the sense fragment and BamHI and XbaI for the antisense fragment. The restriction reactions were analyzed on a 1% agarose gel and the expected size of bands for the vector as well as the insert were obtained. Samples of sense and antisense segments are displayed in lanes 1–7, respectively. (Figure 5). Hyper Ladder^{TM} 50bp (Meridian Bioscience Inc.) was utilized to determine the product sizes. Finally, one clone was chosen for transformation into the *A. tumefaciens* GV3101 strain, and the clone was named hpCP-pFGC5941.

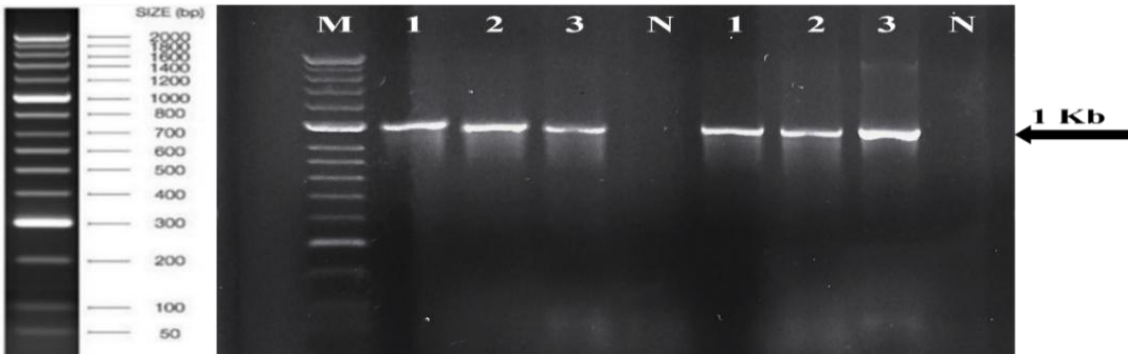

Figure 4: PCR confirmation of clones of CP sense and CP antisense using 35S-Pro-F and chsA-intr-R primers (for sense) and chsA-intr-F and OCS-term-R primers (for antisense). The panel M is showing DNA Ladder (Hyper Ladder™ 50bp; Meridian Bioscience Inc.) and panels 1-3 are showing three clones for sense and antisense respectively. Panel N is showing negative control.

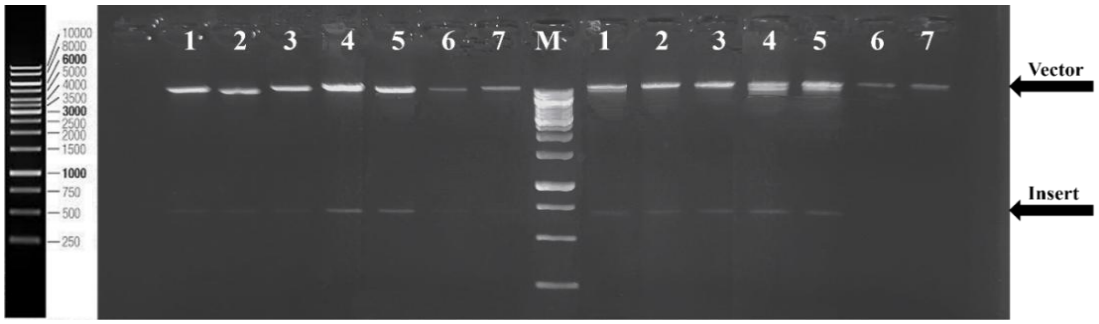

Figure 5: Restriction analysis of hp-CP RNAi construct in pFGC5941 vector. For sense fragment the samples were digested with XhoI and NcoI and for antisense fragment the samples were digested with BamHI and XbaI. The panel M is showing DNA Ladder (Thermo Scientific™ Gene Ruler 1 kb DNA Ladder) and panels 1-7 are showing samples for sense and antisense fragments respectively.

Transient analysis of the efficiency of the hairpin RNAi construct to develop resistance against ToLCSDV: To assess the capacity of the hairpin RNAi construct to resist the infection of ToLCSDV, a transient assay was designed, and the construct was transformed by Agrobacterium into *N. benthamiana*. The transient assay was done by co-inoculation of the agro-inoculum of the hairpin RNAi construct as well as the agro-inoculum of ToLCSDV infectious clones into *N. benthamiana*. Agroinfiltration of *N. benthamiana* plants was done in three different experiments. 10 plants of *N. benthamiana* were inoculated in each experiment: 5 plants with hpCP-pFGC5941 (hpRNAi construct) + ToLCSDV (infectious clone) and 5 plants with ToLCSDV (infectious clone) only (Table 2). After 30 days of inoculation, 15/15 plants (100%) plants were found to be positive for ToLCSDV inoculated with infectious clone of ToLCSDV only. The plants developed typical disease symptoms, including leaf curling, vein swelling, and stunted growth (Figure 6). However, 4/15 (27%) plants developed mild symptoms that were inoculated with hpCP-pFGC5941 (hpRNAi construct) + ToLCSDV (infectious clone) while the 11/15 plants (73%) remained asymptomatic (Figure 7 A, B). The results obtained revealed that hpRNAi plants exhibited resistance compared to the controls hpRNAi targeting CP of ToLCSDV reduced infection from 100% to 27% in treated plants.

Figure 6: *N. benthamiana* plants inoculated with ToLCSDV-Infectious clone only showing leaf curling, vein swelling, and stunted growth symptoms. After 30 post inoculation, all plants (100%) inoculated with ToLCSDV (infectious clone) were found positive for ToLCSDV.

Figure 7: *N. benthamiana* plants co-inoculated with hpCP-pFGC5941 (hpRNAi construct and ToLCSDV-Infectious Clone showing asymptomatic and mild symptoms. After 30 post inoculation, 4/15 plants (27%) were found positive for ToLCSDV (A). However, 11/15 plants (73%) had remained asymptomatic (B).

Table 2. Transient assay of hairpin RNAi construct against ToLCSDV in *N. benthamiana* plants

Inoculum	Infected plants/Inoculated plants experiments				PCR detection of ToLCSDV	PCR results
	I	II	III			
hpRNAiconstruct+ToLCSDV (Infectious Clone)	1/5	1/5	2/5		4/15	4/15
ToLCSDV (Infectious Clone only)	5/5	5/5	5/5		15/15	15/15
pFGC5941 vector (without insert)	0/5	0/5	0/5		0/15	0/15
Healthy plants	0/5	0/5	0/5		0/15	0/15

DISCUSSION

The primary pathogens that significantly reduce tomato crop quality and productivity are plant viruses. *Begomovirus*, *Tobamovirus*, *Orthotospovirus*, *Crinivirus*, and *Potyvirus* are the five genera that comprise the major viruses that infect tomatoes globally. Through hybrid breeding, tomato resistance genes against viruses, such as the Ty gene resistance against begomoviruses, the Sw gene resistance against orthotospoviruses, the Tm gene resistance against tobamoviruses, and the Pot 1 gene resistance against potyviruses, have been detected from indigenous germplasm and introduced into commercial cultivars. Tomato antiviral breeding is severely restricted by the fact that these resistance genes mostly display qualitative resistance driven by single genes, which cannot defend against virus mutations, recombination, mixed infection, or new viruses. Future research on tomato viral resistance breeding should concentrate on quickly, safely, and effectively producing broad-spectrum genetic materials resistant to several viruses based on the epidemic features of tomato viruses.

To develop resistant germplasm against tomato viruses, modern methods of tomato breeding are recommended. These methods include gene editing based on CRISPR/Cas, transgenic breeding based on RNA interference and hybrid breeding based on marker-assisted selection (MAS) (Shahriari *et al.,* 2023). The results presented here have shown the potential of hpRNAi approach for generating resistance in model plants, *N. benthamiana* targeted against conserved part of CP gene the virus genome, revealed the promising resistance against the ToLCSDV; a begomovirus virus causing huge losses to tomato production in Arabian Peninsula (Ammara *et al.,* 2015). Increasing tomato crop yield is crucial to minimizing production losses caused by various viral diseases. Conventional methods for developing resistance are either confined to selective breeding or are not sustainable over time, making it difficult to find a line that is resistant to various viral infections (Sajid and Elçi, 2024). The results presented involve the cloning of hairpin RNAi construct targeting CP gene of ToLCSDV, cloned in a binary vector pFGC5941 and evaluation of the efficiency of the construct against the virus in model plants *N. benthamiana*. pFGC5941 binary vector was used for cloning the hpRNAi construct to develop the RNAi resistance against Tomato spotted wilt orthotospovirus (TSWV) species (Sajid and Elçi, 2024). In a related investigation, the conserved sequences corresponding to the two ORFs begomovirus genome, AC1 (which codes for replication associated protein), AC2 (which codes for transcriptional activator protein), and the bC1 ORF of betasatellite genome (which determines pathogenicity) were found. These ORFs encode proteins that are crucial for viral pathogenicity, movement, replication, and suppression of gene silencing. These proteins' ability to multitask makes their transcript suitable targets for begomovirus resistance development. Off-target effects are frequently linked to RNAi-mediated gene silencing, where transgene- generated siRNA may silence the transcript of additional host genes that are not targeted, resulting in aberrant and harmful phenotypes (Fedorov *et al.,* 2006; Jackson *et al.,* 2003).

In our study we targeted of CP region of ToLCSDV, this is a novel approach to develop resistance against this virus. For other viruses' similar approaches has also been used to develop RNA interference-based resistance for example Ammara *et al.,* (2015) used similar method for Tomato yellow leaf curl virus-Oman (TYLCV-OM) and its related betasatellite (Ammara *et al.,* 2015). A potential technique that has been proven to be successful against a few infections is RNA interference (RNAi). To achieve long-lasting resistance, various geminivirus genomic regions have been targeted via RNA interference. However, one limiting factor is the transgene's silence after virus infection. In this instance, we have developed the first amplicon-based RNA interference construct to target the βC1 gene of the betasatellite linked to cotton leaf curl virus.

Rep-based activation or looping out of the construct caused by virus infection not only produces short interfering (si) RNAs but also multiple copies of transgene, which leads to the accumulation of defective betasatellite molecules. Subsequent transcription gives rise to increased number of siRNAs that gives enhanced resistance. Cotton leaf curl Multan betasatellite (CLCuMB) and Cotton leaf curl Khokran virus (CLCuKV) were tested against transgenic N. benthamiana plants carrying RCβ (RNAi construct for betasatellite). Southern blot hybridization revealed a decreased

titter of the virus and betasatellite (Akhtar *et al.,* 2021). In another promising study, to express dsRNA homologous to the intergenic region (IR) of CLCuRV, an intron hairpin (ihp) RNAi construct was developed. Nine independent lines of transformed cotton were generated when transformation of cotton plant carried out using ihpRNAi construct with the help of *Agrobacterium tumefaciens*. PCR paired with Southern hybridization verified the existence of the possible IR stretch in the transformed cotton. The transgenic plants demonstrated a high level of resistance when infected with viruliferous whiteflies (Khatoon *et al.,* 2016). Further studies and large experiment will be needed to develop hpRNAi-CP based resistance against ToLCSDV in elite tomato varieties to get economic benefits of RNAi-based broad-spectrum resistance.

Conclusion: The current study results are highly promising for developing resistance against begomoviruses like TLCSVD and may be useful in the transformation of tomato varieties to develop broad-spectrum transgenic resistance against begomoviruses. Further studies are recommended to develop RNAi constructs targeting multiple segments of the genome of begomoviruses, and stacking multiple RNAi constructs under a single promoter may be initiated to make durable and broad-spectrum resistance. Different inducible and tissue-specific promoters can be investigated to avoid off-targeting of RNAi constructs. In a study concerning TLCSDV infection, the implementation of hairpin RNA interference (hpRNAi) targeting the coat protein (CP) resulted in a significant reduction of infection. Specifically, in treated plants, the infection rate decreased from 100% in the untreated control group to 27% in the plants that received the hpRNAi treatment. More research is required to enhance the design of RNA interference (RNAi) constructs for more precise and efficient gene silencing. The development of combinatorial RNAi (coRNAi) tactics to overcome viral resistance, the creation of improved viral vectors for precise delivery and tissue specificity, and the optimization of RNAi trigger design including the use of longer small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs)-are all examples of this.

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