

The antisense 5' end of the V2 gene confers enhanced resistance against the monopartite begomovirus cotton leaf curl Kokhran virus-Burewala strain

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Summary. – Whitefly-transmitted viruses of the genus *Begomovirus* (the family *Geminiviridae*) have become a limiting factor for agricultural productivity in many warmer parts of the world. The economies of Pakistan and India have, since the early 1990s, suffered losses due to cotton leaf curl disease (CLCuD). The disease is caused by begomoviruses, the most important of which at this time is cotton leaf curl Kokhran virus strain Burewala (CLCuKoV-Bu), and a disease-specific betasatellite, cotton leaf curl Multan betasatellite (CLCuMuB). Efforts to minimize losses due to CLCuD rely mainly on the use of insecticides to kill the whitefly vector; no resistant cotton varieties are currently commercially available. The study described here has investigated RNA interference technology for its potential to yield resistance against CLCuKoV-Bu and three other begomoviruses; CLCuKoV, tomato leaf curl New Delhi virus (ToLCNDV) and *Pedilanthus* leaf curl virus (PeLCV). Three fragments of the virion-sense V2 gene of CLCuKoV-Bu were transformed into *Nicotiana benthamiana* in antisense orientation and transgenic lines expressing virus-specific short RNAs were assessed for their ability to yield resistance. Only CLCuKoV-Bu with the V2 sequence closest to the promoter was resistant. Inoculation of CLCuKoV-Bu with CLCuMuB into transgenic plants did not significantly affect the outcome, although viral DNA was detected in number of plants, suggesting that the betasatellite may impair RNAi resistance. Overall the results indicate that targeting the 5' end of V2 gene using antisense-RNA has the potential to deliver resistance against begomoviruses and that RNAi-based resistance imparts some degree of resistance to heterologous viruses.

Keywords: geminivirus; begomovirus; RNAi; resistance; CLCuKoV-Burewala; CLCuMuB

Introduction

Cotton and cotton products are a major source of foreign exchange earnings for Pakistan. However, cotton production

has been severely compromised since the early 1990s due to cotton leaf curl disease (CLCuD). CLCuD is a problem for cotton productivity in Pakistan, India and has spread also to China (Briddon and Markham, 2000; Cai *et al.*, 2010; Zhou *et al.*, 1998). CLCuD is caused by single-stranded DNA viruses of the genus *Begomovirus* (the family *Geminiviridae*) which are exclusively transmitted by the whitefly *Bemisia tabaci* (Briddon and Markham, 2000).

The genomes of begomoviruses consist of either two ~2800 nucleotides (nt) long components, known as DNA A and DNA B, or one component, a homolog of the DNA A of bipartite begomoviruses. Genes are encoded bi-directionally and separated by an ~200 nt long intergenic region (IR). Two genes are encoded by the genomes of monopartite begomoviruses (or the DNA A of bipartite begomoviruses)

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Abbreviations: CLCuD = cotton leaf curl disease; CLCuKoV = cotton leaf curl Kokhran virus; CLCuKoV-Bu = cotton leaf curl Kokhran virus strain Burewala; CLCuMuB = CLCuD-Multan betasatellite; CLCuMuV = CLCu Multan virus; dpi = days post-inoculation; IR = intergenic region; PeLCV = *Pedilanthus* leaf curl virus; PTGS = post-transcriptional gene silencing; Rep = replication-associated protein; TGS = transcriptional gene silencing; ToLCNDV = tomato leaf curl New Delhi virus; TrAP = transcription-activator protein

on the virion-sense strand. These encode the (A)V2 protein (involved in virus movement in plants and suppression of host defense) and the coat protein (involved in genome packaging, insect transmission and systemic spread in plants) (Hanley-Bowdoin *et al.*, 2013; Rojas *et al.*, 2001). Four genes are encoded on the complementary-sense strand which encode the transcription-activator protein (TrAP), the replication-enhancer protein which is involved in virus replication, the replication-associated protein (Rep) and (A)C4 protein (which can be involved in overcoming the host defenses and act as pathogenicity determinant) (Hanley-Bowdoin *et al.*, 2013; Rojas *et al.*, 2001). Two genes are encoded by the DNA B component, the nuclear shuttle protein in the virion-sense and the movement protein in the complementary-sense, which are involved in cell-to-cell and systemic movement (Noueiry *et al.*, 1994).

In the Old World, a majority of begomoviruses are monopartite and associated with single-stranded DNA satellites, known as betasatellites, belonging to the *Betasatellite* genus (the family *Tolecusatellitidae*) (Adams *et al.*, 2017; Briddon *et al.*, 2004, 2008). Betasatellites encode a single protein, β C1, which is a dominant pathogenicity determinant and may be involved in virus movement in plants and in overcoming plant host defenses (Hanley-Bowdoin *et al.*, 2013; Rojas *et al.*, 2001; Zhou, 2013).

In the 1990s, five distinct species of begomoviruses were reported to be involved in the spread of CLCuD in Pakistan and northwestern India, the most important of which were cotton leaf curl Multan virus (CLCuMuV) and cotton leaf curl Kokhran virus (CLCuKoV), in association with a disease-specific betasatellite, cotton leaf curl Multan betasatellite (CLCuMuB; (Sattar *et al.*, 2013)). Losses in cotton production persisted until the late 1990s when the introduction of resistant cotton varieties virtually eradicated the disease. Unfortunately, CLCuD symptoms in cotton reappeared in Pakistan in 2001 and the disease rapidly spread across most of Pakistan and northwestern India (Mansoor *et al.*, 2003). Resistance breaking in cotton was shown to be associated with recombinant strain of CLCuKoV, the Burewala strain (CLCuKoV-Bu), which lacks an intact gene encoding TrAP, and a recombinant strain of CLCuMuB (Amin *et al.*, 2006; Amrao *et al.*, 2010). At this time CLCuD is endemic in Pakistan and northwestern India with no commercially available cotton varieties having resistance to the virus complex that causes the disease (Sattar *et al.*, 2013; Zubair *et al.*, 2017).

Begomoviruses have proven difficult to control by conventional means such as the use of insecticides, agricultural management practices, and the production of resistant plant varieties by conventional breeding approaches (Iqbal *et al.*, 2016). Transgenic approaches to obtain resistance to begomoviruses would appear to provide a possible answer to this problem. Among the available transgenic strategies, those based on RNA interference (RNAi) have been investigated

most widely (Ilyas *et al.*, 2010; Noris *et al.*, 2004; Shepherd *et al.*, 2009; Vanderschuren *et al.*, 2007b).

RNAi (also referred as gene silencing) is a widely conserved process that has a role in many cellular mechanisms including host defense against pathogens, regulation of gene expression, *de novo* DNA methylation and chromatin remodeling (Dalakouras and Wassenegger, 2013). Characteristically RNAi involves the processing of large double-stranded RNAs into 21–24 nt small interfering RNAs (siRNAs). RNAi occurs at both the transcriptional and post-transcriptional levels. Post-transcriptional gene silencing (PTGS) acts on target RNAs, including messenger RNAs and the genomes of RNA viruses, resulting in their degradation. Transcriptional gene silencing (TGS) results from the *de novo* methylation of promoter sequences that may induce chromatin modifications. TGS plays a part in the maintenance of genome integrity by preventing centromeric and telomeric rearrangements, as well as by suppressing the activity of transposons and other invasive DNAs such as DNA viruses (Pumplin and Voinnet, 2013).

The (A)V2 gene has structural and positional conservation across most begomoviruses native to the Old World. The (A)V2 protein has a role in virus movement, suppression of host defense, virus accumulation and may be a symptom determinant (Iqbal *et al.*, 2012; Mubin *et al.*, 2007; Rojas *et al.*, 2001). Several studies have investigated the transgenic expression of begomovirus (A)V2 gene sequences as a means of obtaining resistance to begomoviruses (Ammara *et al.*, 2015; Mubin *et al.*, 2007; Yasmeen *et al.*, 2016). The study described here was designed to investigate RNAi-based resistance against CLCuKoV-Bu by targeting the V2 gene. Three regions (N-terminal, middle region and C-terminal) of the V2 gene were used to produce anti-sense RNA constructs to investigate their ability to provide resistance in transgenic *N. benthamiana* plants and determine the most effective sequences for delivering resistance.

Materials and Methods

Production of antisense RNA constructs. Three pairs of oligonucleotide primers (V2NF/V2NR, V2MF/V2MR and V2CF/V2CR (Supplementary Table 1)) were designed to amplify the N-terminal, middle and C-terminal part of almost same size fragments of the V2 gene of CLCuKoV-Bu (AM774301) (Amrao *et al.*, 2010). The restriction sites *Eco*RI and *Hind*III (Thermo Fisher Scientific, USA) were included in the primer sequences to allow directional cloning. The fragments were individually cloned, in antisense orientation, under the 35S promoter of cauliflower mosaic virus in the expression vector pJIT163 (Guerineau and Mullineaux, 1993) to yield constructs BV2^N, BV2^M and BV2^C. The pJIT163 expression cassettes were then cloned as *Kpn*I/*Xho*I (Thermo Fisher Scientific) fragments into the binary vector pGreen0029 (Hellens *et al.*, 2000).

Plant transformation and plant inoculation. *N. benthamiana* was transformed using the leaf disc method (Horsch *et al.*, 1985). Primary transformed plants (T_0) were confirmed to contain the transgene by PCR using specific primers (V2NF/V2NR, V2MF/V2MR and V2CF/V2CR (Supplementary Table 1)) to amplify the fragments of the V2 gene. The plants were then self-pollinated and resulting seeds were collected and germinated on basal MS media (Sigma-Aldrich, Germany) containing kanamycin. Resultant plants were transferred to soil and acclimatized in an insect-free glasshouse at 25°C with supplementary lighting for 16 hours daylight. Transgenic plants were challenged with CLCuKoV-Bu (AM421522), CLCuKoV (AJ496286), CLCuMuB (AJ298903), PeLCV (AM948961) and ToLCNDV (DNA A [U15015] and DNA B [U15017]) by *Agrobacterium*-mediated inoculation as described previously (Hussain *et al.*, 2007).

Extraction of total nucleic acids from plants and detection of viral DNA. Total genomic DNA was extracted from leaf tissue using the CTAB method (Doyle and Doyle, 1990). For PCR-mediated diagnostics of viruses, we have used primer pairs IRVF/IRVR for CLCuKoV, CLCKV2F/CLCKV2R for CLCuKoV-Bu, PedLCVV2F/PedLCVV2R for PeLCV and ToLCNDV2F/ToLCNDV2R for ToLCNDV (Supplementary Table 1). For Southern blot analysis, 10 µg of total DNA was separated on 1% agarose gel in 0.5x TAE buffer and transferred to nylon membrane (Hybond N+, GE Healthcare, USA) (Sambrook *et al.*, 1989). Membranes were hybridized with viral DNA fragments labeled with DIG using specific primers (IRVF/IRVR for CLCuKoV, CLCKV2F/CLCKV2R for CLCuKoV-Bu, PedLCVV2F/PedLCVV2R for PeLCV and ToLCNDV2F/ToLCNDV2R for ToLCNDV) (Supplementary Table 1) and a PCR DIG probe synthesis kit (Roche, Germany) and detected with a DIG luminescent detection kit (Roche) according to manufacturer's instructions.

Detection of small RNAs. Total genomic RNA was isolated from plant tissues using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions and stored at -80°C. Transgene derived small RNAs were detected as described previously (Akbergenov *et al.*, 2006). Small RNAs were transferred to Hybond N+ membrane in 1x TBE using an electroblotter (Transphor unit TE52, Hoefer Inc, USA) at 4°C and run at 10 V for 16–20 h. The RNAs were fixed to the membrane by UV crosslinking and stored dry at 4°C until use. The membrane was first incubated in 5 ml Ultra-hyb-oligo buffer (Thermo Fisher Scientific) in a hybridization oven for 2 h at 45°C. DIG labelled specific probes were produced using specific primers (NterS/NterAS, MPorS/MPorAS and CterS/CterAS) (Supplementary Table 1) and the DIG PCR labelling kit.

Quantification of viral components by quantitative real-time PCR. The quantity and quality of extracted genomic DNA was assessed (NanoDrop ND-1000 spectrophotometer, Thermo Fisher Scientific) and the concentration was adjusted to 10 ng/µl. Reactions were conducted in an iCycler Thermal Cycler with iQ5 Multicolor Real-Time PCR detection system (Bio Rad, USA). Reactions consisted of a total volume of 25 µl with 12.5 µl of SYBR Green super mix (Thermo Fisher Scientific), 0.25 µl of each primer (2.5

pmol/l each), 2.5 µl DNA (25 ng), and 9.5 µl sterile distilled water. The performed cycle profile was: 94°C for 10 min followed by 40 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C. Reactions were performed in 96 well microtitre plates (Bio-Rad, USA). At the end of each run a melt curve analysis was performed from 57°C to 95°C, with an increment of 0.5°C at 10 s intervals. All PCR reactions were performed in triplicates on 96 well-plates with all necessary controls for producing standard curves, as described earlier (Shafiq *et al.*, 2017). Statistical analysis of the quantitative real-time PCR (qRT-PCR) results (two tailed *t* test) were conducted using Excel (Microsoft Corp., USA).

Results

Analysis of transgenic plants

Transgenic plants produced from all independent transformation events were confirmed by growing on media containing kanamycin and by PCR. The T_1 generation plants were found to be segregating and showed differing levels of tolerance on kanamycin. These kanamycin resistant plants were further evaluated for the presence of the transgenes by PCR with 35S promoter (P35F/P35R) as well as gene specific primers. The PCR showed 64.28% plants in line 1, 21.43% in line 2 and 63.63% in line 3 of BV2^N plants to harbor the transgene in the T_1 generation. For BV2^M plants, 75% in line 1, 30% in line 2 and 77.7% in line 3 were found to harbor the transgene. Whereas, 72.22%, 73.33% and 36.84% of plants were confirmed as transgenic for BV2^C lines 1, 2 and 3, respectively.

In the T_2 generation, all lines were segregating but with fewer kanamycin susceptible plants than in the T_1 generation. The kanamycin resistant plants were also subjected to PCR confirmation and, except for one plant for line 1 of BV2^N, all plants harbored the respective transgene (Supplementary Table 2).

Plants of the T_3 generation were used for infectivity analysis after germination on kanamycin containing medium and PCR-mediated confirmation. Plants in the T_3 generation were homozygous and no segregation could be detected. These homozygous lines were subjected to transgene derived small RNA analysis (Supplementary Fig. 2). Among the tested transgenic lines, plants of line 2 of BV2^N, line 2 of BV2^M and line 3 of BV2^C produced detectable levels of transgene derived small RNAs and the seeds of these plants were used further for infectivity analysis.

Evaluation of resistance to CLCuKoV-Bu in transgenic N. benthamiana

Wild type (non-transgenic) plants of *N. benthamiana* inoculated with CLCuKoV-Bu showed vein swelling, leaf

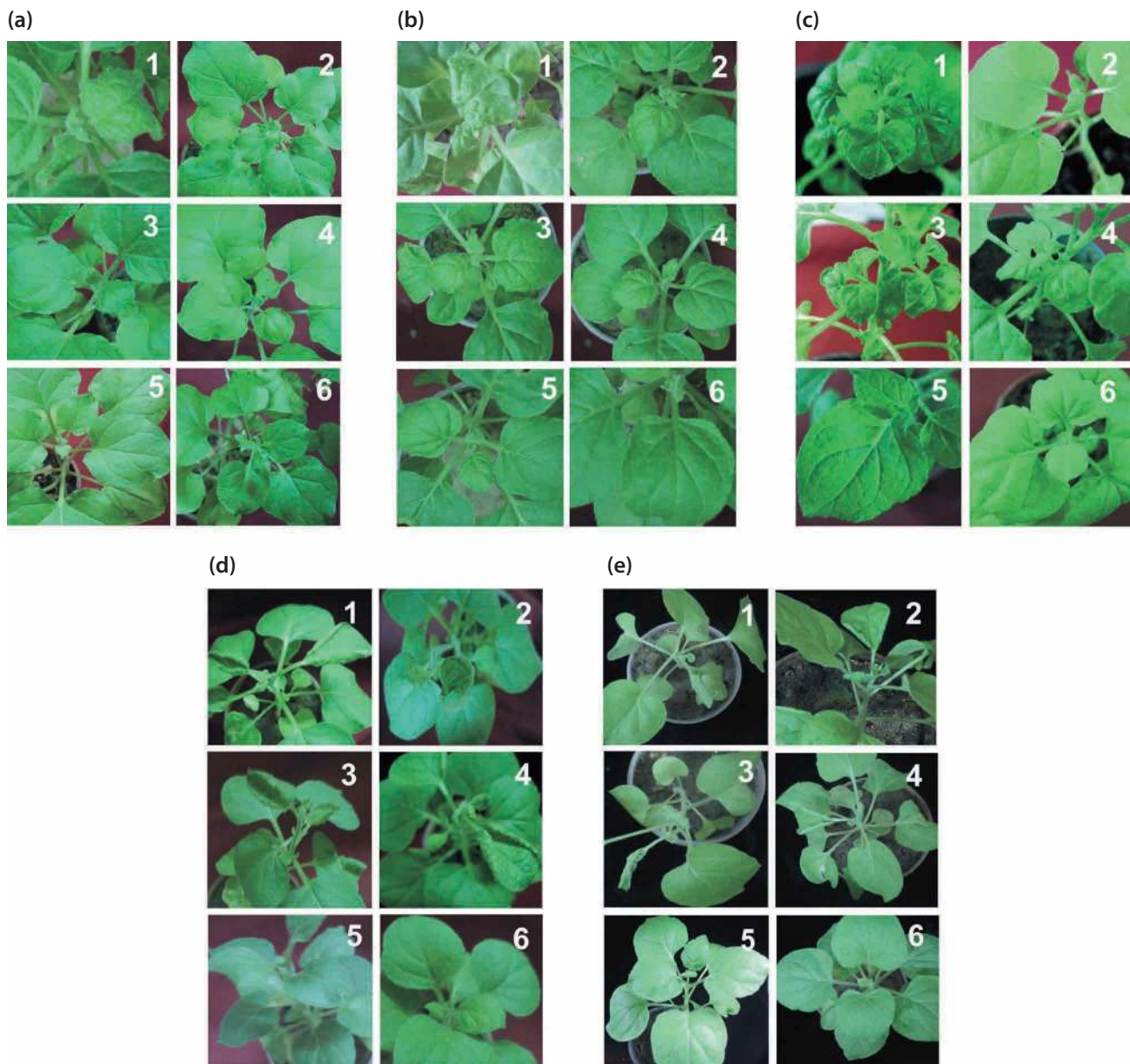


Fig. 1

Symptoms exhibited by transgenic *N. benthamiana* plants after inoculation with begomoviruses

Transgenic plants harboring the BV2^N, BV2^M and BV2^C constructs were inoculated with (a) the CLCuKoV-Bu (2, 3 and 4), (b) CLCuKoV-Bu and CLCuMuB (2, 3 and 4), (c) CLCuKoV (2, 3 and 4), (d) PedLCuV (2, 3 and 4) and (e) ToLCNDV (2, 3 and 4). Non-transgenic plants inoculated with (a) CLCuKoV-Bu (1), (b) CLCuKoV-Bu and CLCuMuB (1), (c) CLCuKoV (1), (d) PedLCuV (1), (e) ToLCNDV (1) are shown for comparison. 5 and 6 show non-inoculated, non-transgenic plants and mock inoculated plants, respectively, in each case. Photographs were taken at 28 dpi.

curling, vein yellowing and a reduction in leaf size symptoms at 9 days post-inoculation (dpi) and by 28 dpi all plants were symptomatic with leaf curling, prominent vein thickening, and a reduction in leaf size (Fig. 1a,1) resulting in stunted plants. Viral DNA was detected in young, newly developing leaves by PCR-mediated diagnostics and by Southern blot

hybridization (Table 2; Supplementary Fig. 1a). In contrast, inoculation of transgenic *N. benthamiana* plants harboring the BV2^N construct did not result in symptoms, even at 28 dpi (Fig. 1a,2). PCR-mediated diagnostics, however, showed the presence of the virus in one plant of the 20 inoculated (Table 2) but no viral DNA was detected by Southern blot

Table 1. Infectivity of inoculated homologous and heterologous begomoviruses in transgenic *N. benthamiana* plants

Plant	Infectivity (plants symptomatic/plants inoculated)									
	CLCuKoV-Bu		CLCuKoV-Bu & CLCuMuB		CLCuKoV		PeLCV		ToLCNDV	
	15 dpi	28 dpi	15dpi	28 dpi	15 dpi	28 dpi	15 dpi	28 dpi	15 dpi	28 dpi
<i>N. b.</i>	19/20	20/20	18/20	20/20	20/10	20/20	20/20	20/20	19/20	20/20
BV2 ^N	0/20	0/20	0/20	0/20	0/20	0/20	18/20	20/20	16/20	20/20
BV2 ^M	12/20	0/20	12/20	0/20	0/20	18/20	12/20	20/20	9/20	20/20
BV2 ^C	7/20	0/20	7/20	0/20	0/20	1/20	13/20	20/20	6/20	20/20
<i>N. b</i> [*]	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
<i>N. b</i> [†]	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

^{*}Non-transgenic *N. benthamiana* plants inoculated with *Agrobacterium* cultures harboring pGreen0029. [†]Non-inoculated plants.

Table 2. Detection of inoculated begomoviruses in transgenic *N. benthamiana* plants

Plant	Plants positive/plants inoculated											
	PCR						Southern blot					
	CLCuKoV-Bu	CLCuKoV-Bu	CLCuMuB	CLCuKoV	PeLCV	ToLCNDV	CLCuKoV-Bu	CLCuKoV-Bu	CLCuMB	CLCuKoV	PeLCV	ToLCNDV
<i>N. b.</i>	20/20	20/20	20/20	18/20	20/20	20/20	++++	++++	+++	++++	++++	++++
BV2 ^N	1/20	7/20	0/20	19/20	20/20	20/20	-	+	-	+	++++	++++
BV2 ^M	17/20	16/20	15/20	20/20	20/20	20/20	+	+	-	++	+++	+++
BV2 ^C	8/20	8/20	3/20	20/20	20/20	20/20	+	+	-	+	+++	+++
<i>N. b</i> [*]	0/20	0/20	0/20	0/20	0/20	0/20	ND	ND	ND	ND	ND	ND
<i>N. b</i> [†]	0/10	0/10	0/10	0/10	0/10	0/10	-	-	-	-	-	-

^{*}Non-transgenic *N. benthamiana* plants inoculated with *Agrobacterium* cultures harboring pGreen0029. [†]Non-inoculated plants. Viruses/betasatellites were detected in nucleic acids extracted from plants by PCR using specific primers (Supplementary Table 1). Southern blot hybridization results are given as strong hybridization (++++) through weak hybridization (+++) to no hybridization detected (-). Some plants were not examined for the presence of virus by hybridization (ND).

hybridization (Supplementary Fig. 1a), indicating that viral DNA levels were below the detection threshold.

N. benthamiana plants harboring the BV2^M and BV2^C constructs inoculated with CLCuKoV-Bu started showing symptoms of infection between 12 and 15 dpi which subsequently became mild in newly developing leaves and ultimately the plants recovered, showing no symptoms on new growth (Fig. 1a,3 and 4; Table 1). For the BV2^M plants, viral DNA was identified in most of the asymptomatic plants by PCR analysis (17 out of 20 inoculated) but was not found in Southern blot hybridization (Table 2), suggesting the virus level was below the detection limit (Supplementary Fig. 1a). In contrast, for the BV2^C plants, PCR diagnostics showed far fewer plants (8 out of 20 inoculated) to contain the virus and very weak signals were found by Southern blot hybridization (Supplementary Fig. 1a). qPCR analysis showed that the titer of CLCuKoV-Bu in infected BV2^C transgenic plants was significantly higher than the titer in infected BV2^M transgenic plants. However, for BV2^N

transgenic plants, virus level was below the detection limit (Supplementary Table 3).

Evaluation of resistance to CLCuKoV-Bu and CLCuMuB in transgenic N. benthamiana

Non-transgenic *N. benthamiana* plants co-inoculated with CLCuKoV-Bu and CLCuMuB developed symptoms consisting of leaf curling, vein thickening, yellowing of veins and a reduction in leaf size at 9 dpi (Fig. 1b,1). All inoculated plants developed symptoms and PCR diagnostics showed the presence of both the virus and the betasatellite in all the plants (Table 1 and 2). In contrast, inoculation of transgenic *N. benthamiana* plants harboring the BV2^N construct did not develop symptoms of infection, even at 28 dpi (Fig. 1b,2). PCR-mediated diagnostics, however, showed the presence of the virus, but not the betasatellite, in seven plants of the 20 inoculated. Southern blot hybridization detected neither viral DNA nor betasatellite DNA (Supplementary Fig. 1a and c).

CLCuKoV-Bu and CLCuMuB inoculated *N. benthamiana* plants harboring the BV2^M and BV2^C constructs developed mild leaf curl symptoms at 15 dpi that reduced gradually, and plants ultimately recovered from infection (all plants were non-symptomatic in new growth at ~28 dpi; Fig. 1b,3 and 4). For the BV2^M plants, PCR diagnostics showed the presence of the virus in 16 plants and the betasatellite in 15 (out of 20 inoculated) plants (Table 2). However, possibly very low viral DNA levels (of the linear form) but no betasatellite DNA were detected in Southern blot hybridization (Supplementary Fig. 1b and c). In contrast, for the BV2^C plants, PCR diagnostics showed fewer plants (8 out of 20 inoculated) to contain the virus and only 3 to contain the betasatellite (Table 2). Again, possibly very low viral DNA levels (of the linear form) but no betasatellite DNA were detected in Southern blot hybridization (Supplementary Fig. 1c). qPCR analysis indicated that the titer of virus in CLCuKoV-Bu/CLCuMuB infected BV2^M transgenic plants was significantly higher than the titer of virus in BV2^C and BV2^N transgenic plants, whereas the titer of the betasatellite was highest in BV2^C plants and the lowest in BV2^N plants (Supplementary Table 3).

Evaluation of resistance to CLCuKoV in transgenic N. benthamiana

All non-transgenic *N. benthamiana* plants inoculated with CLCuKoV developed leaf curling, vein thickening and leaf darkening symptoms by 24 dpi (Fig. 1c,1). Except for just one plant, which showed very mild symptoms, none of transgenic *N. benthamiana* plants harboring the BV2^N and BV2^C constructs showed symptoms of infection even at 28 dpi (Fig. 1c,2 and 4). Transgenic plants harboring BV2^M showed mild symptoms consisting of downward leaf curling by 25 dpi. By 28 dpi, most of the plants (18 out of 20) harboring BV2^M started showing symptoms of infection which were milder than non-transgenic *N. benthamiana* plants inoculated with virus (Fig. 1c,3).

PCR-mediated diagnostics for CLCuKoV showed the presence of viral DNA in newly developing leaves of all transgenic plants except one BV2^N plant (Table 2). Southern blot hybridization analysis showed low viral DNA levels in BV2^N and BV2^C plants (Supplementary Fig. 1d). In contrast, the levels of virus detected in BV2^M plants were comparable to those detected in non-transgenic *N. benthamiana* plants. In contrast to CLCuKoV-Bu inoculated plants, the qPCR analysis shows that the titer of CLCuKoV in BV2^C transgenic plants was significantly lower than the titer in BV2^M transgenic plants infected with CLCuKoV. However, in BV2^N transgenic plants, no virus was detected (Supplementary Table 3).

Evaluation of resistance to PeLCV in transgenic N. benthamiana

Non-transgenic *N. benthamiana* plants are highly susceptible to PeLCV and all plants developed leaf curling and vein darkening symptoms at 15 dpi which increased in severity, peaking at 21 dpi (Fig. 1d,1). Similarly, transgenic BV2^N, BV2^M and BV2^C plants exhibited symptoms at 15 dpi that culminated at 21 dpi (Fig. 1d,2, 3 and 4).

Viral DNA was detected by diagnostic PCR in all inoculated BV2^N plants (Table 2). Initially fewer BV2^M and BV2^C plants (12/20 and 13/20, respectively) showed symptoms. Nevertheless, ultimately, all plants were symptomatic. Southern blot analysis showed BV2^M and BV2^C to contain significantly less viral DNA in young developing tissues than either inoculated BV2^N or inoculated non-transgenic plants (Supplementary Fig. 1e). The qPCR analysis indicated that the titer of PeLCV in BV2^N transgenic plants infected with PeLCV was significantly higher than the titer of virus in BV2^M and BV2^C transgenic plants (Supplementary Table 3).

Evaluation of resistance to ToLCNDV in transgenic N. benthamiana

The first symptoms of infection appeared on non-transgenic *N. benthamiana* plants inoculated with the DNA A and DNA B components of ToLCNDV at 14 dpi in the form of upward leaf curling, leaf yellowing, vein thickening and a reduction in leaf size (Fig. 1e,1).

Transgenic *N. benthamiana* plants harboring the BV2^N construct inoculated with ToLCNDV showed symptoms comparable to non-transgenic plants (Fig. 1e,2). However, for a few plants (4 out of 20 inoculated) the onset of symptoms was delayed by 1 to 2 days over non-transgenic plants (Table 1). Southern blot hybridization showed the accumulation of approximately the same levels of viral DNA in BV2^N plants as detected in non-transgenic plants (Supplementary Fig. 1f).

Inoculated BV2^M and BV2^C plants responded in the same way as BV2^N plants with some plants showing a delay in the initial appearance of symptoms by 1 to 2 days although a greater number of plants showed the delay (11 out of 20 for BV2^M and 14 out of 20 for BV2^C; Table 1). Eventually all plants showed full symptoms of infection which were indistinguishable from the symptoms exhibited by non-transgenic plants. Southern blot analysis of BV2^M and BV2^C inoculated plants showed the accumulation of viral DNA at levels that were slightly lower than in either infected non-transgenic or BV2^N transgenic plants with the main difference being a lower level of the ssDNA form. The qPCR analysis indicated that the level ToLCNDV DNA A in ToLCNDV infected BV2^N transgenic plants was significantly higher than in BV2^M and BV2^C transgenic plants (Supplementary Table 3).

Discussion

RNAi has been widely investigated as a means of obtaining resistance to plant pathogenic viruses (Ilyas *et al.*, 2010; Shepherd *et al.*, 2009; Vanderschuren *et al.*, 2007b). Despite these successes in model plants, so far only a single RNAi-based resistance against a geminivirus has been approved for cultivation; a common bean variety with resistance against bean golden mosaic virus in Brazil (Aragão and Faria, 2009; Aragão *et al.*, 2013). The work presented here was aimed at engineering RNAi-mediated resistance against monopartite begomoviruses. Specifically, three regions of a gene were examined to determine the best sequence for resistance.

Transgenic plants harboring the BV2^N construct exhibited better resistance against the homologous virus, CLCuKoV-Bu, than the other two constructs. Geminiviruses transcribe in a bi-directional manner using promoter sequences residing in the IR (Frischmuth *et al.*, 1991). Targeting promoter sequences by RNAi may provide an enhanced virus resistance, most likely due to TGS and DNA methylation (Vanderschuren *et al.*, 2007a). Some success has been achieved using this approach (Pooggin *et al.*, 2003; Wang *et al.*, 2014; Yadav and Chattopadhyay, 2011). The better resistance yielded by the N-terminal sequence of the V2 gene (construct BV2^N) may thus be due to these sequences lying adjacent to the IR, with promoter sequences silenced by TGS due to transitive RNAi (Himber *et al.*, 2003; Pooggin *et al.*, 2003).

In the present study, resistance was reduced in transgenic plants when virus was co-inoculated with betasatellite. It was previously suggested that betasatellite can overcome the PTGS mediated resistance (Mubin *et al.*, 2011; Cui *et al.*, 2005). This is likely due to the fact that betasatellites encode a strong suppressor of both TGS and PTGS – the β C1 protein (Li *et al.*, 2014; Shukla *et al.*, 2013; Yang *et al.*, 2011b). Certainly, an effect of the betasatellite was seen here – in the best resistant line (BV2^N) viral DNA levels were raised in the presence of CLCuMuB such that they could be detected by hybridization and the virus was detected in more plants by PCR-diagnostics. Nevertheless, plants remained symptomless. This contrasts with the results of Mubin *et al.* (2011) where, in the presence of the betasatellite, transgenic plants harboring Rep sequences showed symptoms. This difference may indicate that V2-targeted silencing is more efficient than Rep-targeted silencing at preventing suppression of the silencing effect. Maintenance of betasatellites by begomoviruses requires both *trans*-replication by the helper virus-encoded Rep (Iqbal *et al.*, 2012; Rojas *et al.*, 2001) and *trans*-movement by the helper virus movement functions (which for monopartite begomoviruses include the V2 protein, CP and possibly C4) (Iqbal *et al.*, 2012; Qazi *et al.*, 2007). The lack of symptoms for CLCuKoV-Bu/CLCuMuB inoculated transgenic plants here may be due to suppression of expression of the V2 protein, which is re-

quired for symptoms even in the presence of the dominant symptom determinant encoded by β C1 (Iqbal *et al.*, 2012). Alternatively, slowing down the spread of virus in plants may be due to the V2-mediated resistance, allowing transgene-mediated silencing to more effectively counter the virus. In Rep silenced plants replication is slowed (but apparently not abolished), possibly allowing the virus to spread and induce symptoms.

Across the three regions of V2 sequence used, the CLCuKoV clone shows only one nucleotide change (in the N-terminal V2 fragment) with respect to CLCuKoV-Bu; the sequences of the middle and C-terminal fragments are identical (Supplementary Fig. 3). Despite this the resistance to CLCuKoV is poorer than to CLCuKoV-Bu. Overall more transgenic plants are infected by CLCuKoV (as judged by PCR diagnostics), virus levels in plants were possibly higher, for the BV2^C construct, and plants did not recover. Since these differences in responses to infection by CLCuKoV-Bu and CLCuKoV cannot be attributed to sequence differences they must be due to the viruses concerned. No work has been conducted to examine the relative potencies of homologous suppressor from different geminiviruses, or the relative abilities of different geminiviruses to replicate and spread in plants. The poorer response of transgenic plants to CLCuKoV, in comparison to CLCuKoV-Bu, may thus be due to CLCuKoV encoding more effective suppressors, which counter the resistance, or the virus is better able to replicate in, and/or spread throughout plants more efficiently – thereby possibly being less affected by the transgene mediated silencing effect. This is an important question, which will need to be addressed in the future if broad spectrum resistance (resistance from one construct against a number of different viruses) is to be achieved.

There is one significant difference between these two viruses which might be responsible for the poorer resistance to CLCuKoV than CLCuKoV-Bu. CLCuKoV-Bu associated with resistance breaking in cotton across Pakistan and northwestern India is the only begomovirus known to lack an intact TrAP-encoding gene (Amrao *et al.*, 2010) that has been found to be important in maintenance of betasatellite (Iqbal *et al.*, 2017). Although one might think that a virus lacking a full TrAP might be impaired, CLCuKoV-Bu is nevertheless infectious (Amrao *et al.*, 2010; Rajagopalan *et al.*, 2012; Zaffalon *et al.*, 2012) and present across a large area of southern Asia (Amrao *et al.*, 2010; Rajagopalan *et al.*, 2012; Zaffalon *et al.*, 2012). Although the resistance to PeLCV provided by the three constructs was very poor, BV2^M and BV2^C appeared to provide marginally better resistance than the overall best construct BV2^N. Comparisons of the sequence of PeLCV homologous to the three transgenes (Supplementary Fig. 3), showed the N-terminal fragment to have 13 mismatches, whereas the middle and C-terminal fragments have 3 and 4 respectively. This result is thus con-

sistent with the idea that RNAi is a homology-dependent phenomenon (Baulcombe, 1996; Chellappan *et al.*, 2004). The same is also the case for ToLCNDV.

For a number of the transgenic plants inoculated as part of this study, there were initial symptoms which gradually declined with, ultimately, new growth on plants showing no symptoms. This is a phenomenon known as recovery and is attributed to a build-up of siRNA (Chellappan *et al.*, 2004; Yang *et al.*, 2011a). The recovery seen thus likely indicates that, although the virus is initially able to replicate to levels which induce symptoms in the plant, ultimately the transgene derived siRNAs are able to reduce virus levels such that they are no longer able to affect plant growth and development. An analysis of the viral siRNA produced in response to infection in tomato and *N. benthamiana* by the monopartite begomovirus tomato yellow leaf curl China virus showed the distribution of siRNA to be non-uniform, with a greater proportion of the siRNA produced against the region of the genome containing the V2 gene (Yang *et al.*, 2011a). The analyses indicated that the greater proportion of siRNA in this region was due to the higher expression levels (higher levels of transcription) across this region, rather than due to transcripts in this region having more secondary structures (leading to hairpin structures [dsRNA]) which might act as substrate for processing into siRNA. However, what is unclear from the work of Yang *et al.* (2011a) is whether, with more siRNA targeting the V2 region during infection, this makes the V2 sequences a more or less effective target for RNAi-mediated engineered resistance. Certainly, the results obtained in the study presented here suggest that the V2 sequence, and specifically the N-terminal end of V2, is a good sequence for delivering resistance.

The question of which region of the genome of monopartite begomoviruses makes the best target for resistance has been addressed previously (Lin *et al.*, 2012). A not dissimilar study of the monopartite begomovirus CLCuMuV also concluded that the sequences of the overlapping Rep and TrAP genes formed the best target for resistance (Mubin *et al.*, 2011). Since these two studies and that of Yang *et al.* (2011) used distinct viruses, it is not possible to draw definitive conclusions. Such studies would need to be done using one virus to assess whether the region of the genome that induces the greatest number of siRNA during a normal infection is also the best target for RNAi-mediated resistance. This is something that will need to be addressed in the future, so that the best RNAi-mediated resistance can be achieved.

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Supplementary information is available in the online version of paper.

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Supplementary information

The antisense 5' end of the V2 gene confers enhanced resistance against the monopartite begomovirus cotton leaf curl Kokhran virus-Burewala strain

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Table S1. Oligonucleotide primers used in the study

Primer	Sequence (5'-3')*	Predicted product size
V2NF	AATGAAGCTTATGAGTAAGTTTCTCTACTAACTG	94 bp
V2NR	CGTTGAATTCCTAATGGGATCCACTGTAAATGAGT	
V2MF	CGGCAAGCTTATGGGCTGTCGAAGTTGAGACGGC	93 bp
V2MR	ATTTGAATTCGTAAATAAGGGCTAGGAATTATGT	
V2CF	TTCAAAGCTTATGGGAACATCTGGACTTCTGTAC	95 bp
V2CR	CGTGAATTCCTAGCCATTGTCCGCGTCACCAAAG	
PedLCVV2F	ATGTGGGATCCGTTATTGAAC	358 bp
PedLCVV2R	CTAGGAACATCTGGACTTCTG	
ToLCNDV2F	GGTCGACAAACATGTGGGATCC	361 bp
ToLCNDV2R	CCCGGGCTTCTATACATTCTGTAC	
CLCKV2F	GTCGACAAGTATGCGTTTGAAAAATGTG	354 bp
CLCKV2R	GGATCCACCTTCACATCCTCTAGGAAC	
NterS	ATGAGTAAGTTTCTCTACTAACTG	94 bp
NterAS	TAATGGGATCCACTGTAAATGAGT	
MPorS	ATGGGCTGTCGAAGTTGAGACGGC	94 bp
MPorAS	TGAAATAAGGGCTAGGAATTATGT	
CterS	ATGGGAACATCTGGACTTCTGTAC	93 bp
CterAS	TAGCCATTGTCCGCGTCACCAAAG	
P35F	GACAGTGGTCCCAAAGATGGA	650 bp
P35R	CAGTGGAGATATCACATCAATCCA	
IRVF	CGTGAATTCATGTGGGATCCACTGTAAATGAG	128 bp
IRVR	TTCGTCGACGAACATCTGGACTTCTGTA	
DNAAqPCRF	CCTTTAATCATGACTGGCTT	1445 bp
DNAAqPCRR	CATTTCCATCCGAACATTC	
BetaqPCRF	GATTTGACTTATATTGGGCCAATTTAAT	132 bp
BetaqPCRR	GATACTATCCACAAAGTCACCATCGCTAAT	
18s rRNAF	TCTGCCCTATCAACTTTCGATGGTA	137 bp
18s rRNAR	AATTTGCGCGCTGCTGCCTTCCTT	

*EcoRI (GAATTC) and HindIII (AAGCTT) recognitions sequences used for cloning the V2 gene fragments are underlined.

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Table S2. Analysis of transgenic *N. benthamiana* plants

Construct	Line	Kanamycin resistance (plants resistant/plants examined)		PCR-mediated detection of construct ^s (plants positive/plants examined)		Transgene specific sRNA*
		T ₂		T ₂		
				35S	Tg	
BV2 ^N	1	6/7		5/5	4/5	(-)
	2	5/5		5/5	5/5	(+)
	3	7/7		5/5	5/5	(-)
BV2 ^M	1	5/5		5/5	5/5	(-)
	2	6/6		4/4	4/4	(+)
	3	5/5		3/5	4/5	(-)
BV2 ^C	1	5/5		5/5	5/5	(-)
	2	4/4		5/5	5/5	(-)
	3	5/5		5/5	5/5	(+)

*The results of the detection of transgene-derived sRNAs in T₃ generation plants by hybridization are given as either positive (+) or negative (-). ^sTransgenic lines were examined for incorporation of the expression constructs by PCR-mediated amplification of CaMV 35S promoter sequences (35S) using primer pair P35F/P35R (Table S1) and virus sequences (Tg) using primer pairs V2NF/V2NR (for the N-terminal fragment), V2MF/V2MR (for the middle fragment) and V2CF/V2CR (for the C-terminal fragment).

Table S3. Quantitative real time PCR analysis of virus/betasatellite DNA levels in infected transgenic *N. benthamiana* plants

Construct*	CLCuKoV-Bu		CLCuKoV-Bu/CLCuMuB				CLCuKoV		PeLCV		ToLCNDV	
	virus (ng/μg) [†]	SD [‡]	virus (ng/μg) [‡]	SD [‡]	beta- satellite (ng/μg) [‡]	SD [‡]	virus (ng/μg) [‡]	SD [‡]	virus (ng/μg) [‡]	SD [‡]	virus (ng/μg) [‡]	SD [‡]
NB	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
INB	0.007 ^A	0.001	0.068 ^A	0.004	0.598 ^A	0.040	0.007 ^A	0.001	0.045 ^A	0.004	0.048 ^A	0.004
BV2 ^N	0.000	0.000	0.002 ^B	0.001	0.062 ^B	0.022	0.000	0.000	0.025 ^B	0.001	0.026 ^B	0.002
BV2 ^N	0.000	0.000	0.002 ^B	0.001	0.062 ^B	0.022	0.000	0.000	0.023 ^B	0.001	0.025 ^B	0.002
BV2 ^M	0.001 ^B	0.000	0.045 ^C	0.003	0.105 ^C	0.015	0.005 ^B	0.000	0.014 ^C	0.001	0.009 ^C	0.001
BV2 ^M	0.001 ^B	0.000	0.053 ^C	0.003	0.114 ^C	0.021	0.005 ^B	0.000	0.016 ^C	0.001	0.010 ^C	0.001
BV2 ^C	0.004 ^C	0.001	0.009 ^D	0.001	0.225 ^D	0.021	0.002 ^C	0.000	0.011 ^D	0.001	0.016 ^D	0.002
BV2 ^C	0.004 ^C	0.001	0.010 ^D	0.002	0.293 ^D	0.025	0.002 ^C	0.000	0.012 ^D	0.001	0.017 ^D	0.001

*The DNA samples examined were either extracted from healthy *N. benthamiana* plants (NB), virus infected *N. benthamiana* plants (INB) or transgenic *N. benthamiana* plants (BV2^N, BV2^M or BV2^C). [‡]Standard deviation. [†]Values followed by the same letter are not significantly different at the 5% level using a two tailed *t* test.

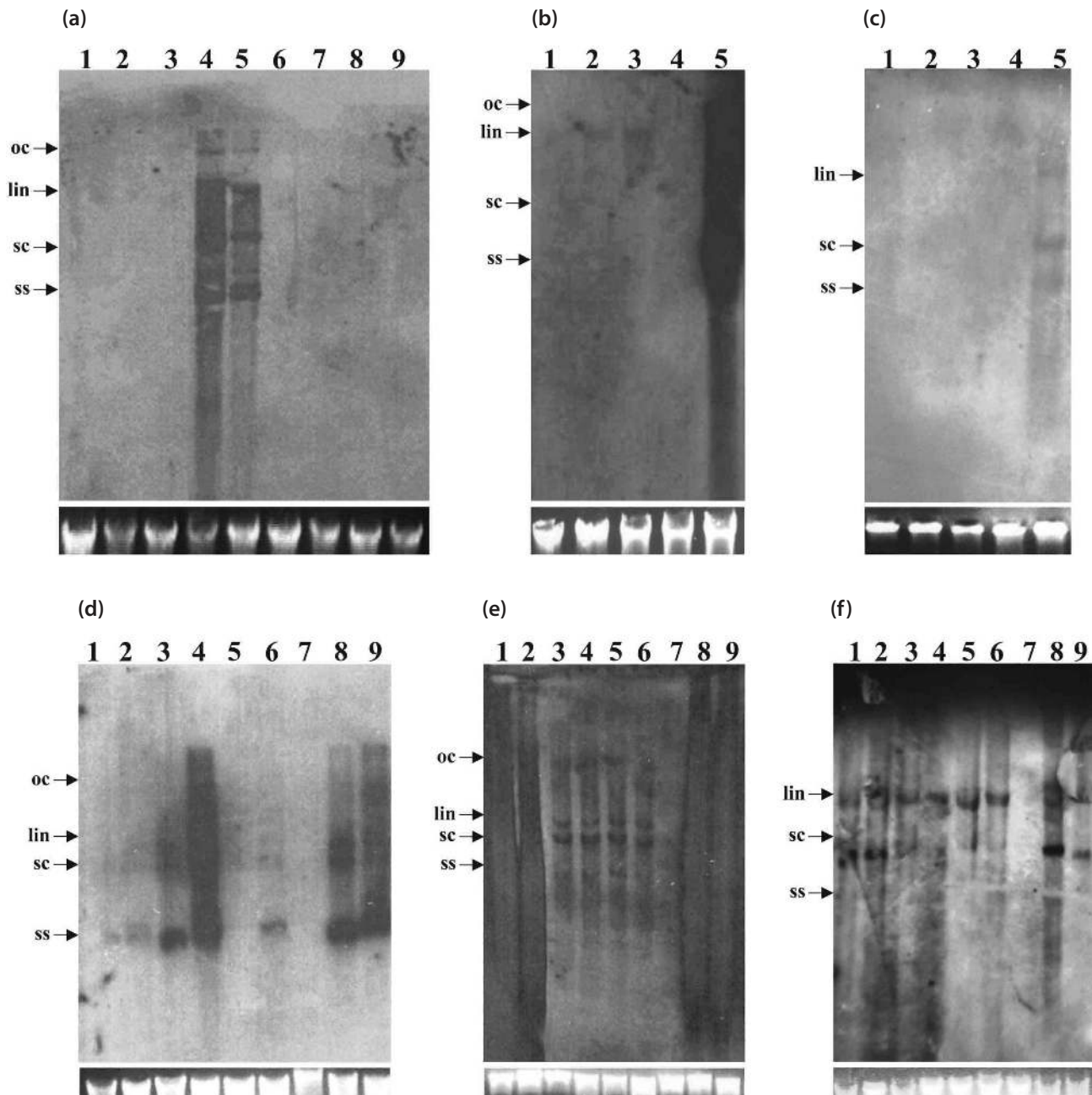


Fig. S1

Southern blot detection of begomoviruses (genomes or DNA A components) and betasatellite in inoculated *N. benthamiana* plants

Membranes were probed to detect the presence of CLCuKoV-Bu (a and b), CLCuMuB (c), CLCuKoV (d), PeLCuV (e) and ToLCNDV DNA A (f). The DNA samples run on the gel were extracted from CLCuKoV-Bu (a), CLCuKoV-Bu with CLCuMuB (b and c), CLCuKoV (d), PeLCuV (e) and ToLCNDV (f) inoculated transgenic plants harboring the BV2^N (lanes 1 and 2 in a, d, e, f and lane 1 in b and c), BV2^M (lanes 6 and 7 in a, lanes 3 and 4 in d, e, f and lane 2 in b and c), and BV2^C (lanes 8 and 9 in a, lanes 5 and 6 in d, e and f and lane 3 in b and c) constructs. A DNA sample extracted from a non-inoculated *N. benthamiana* plant (lane 3 in a, lane 7 in d, e and f and lane 4 in b and c) was included as a negative control. Samples from inoculated non-transgenic plants (lanes 4 and 5 in a, 8 and 9 in d, e, f and lane 5 in b and c) were included as positive controls. Approximately 10 µg DNA were loaded in each case. The positions of viral single-stranded (ss), super-coiled (sc), linear (lin) and open-circular (oc) DNA forms are indicated. The ethidium bromide stained genomic DNA band is shown below each blot to confirm equal loading.

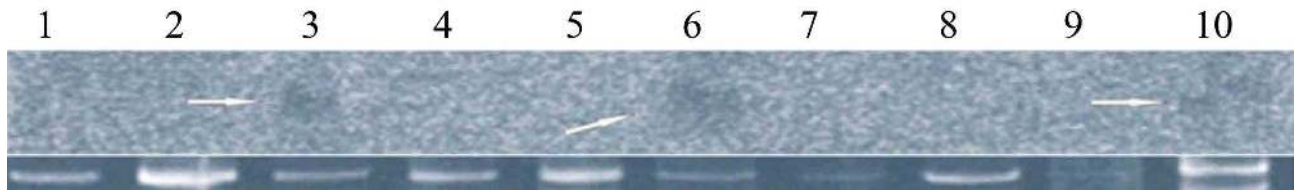


Fig. S2

Detection of CLCuKoV-Bu-specific small RNAs in transgenic *N. benthamiana* plants

Small RNA samples were extracted from a non-transgenic *N. benthamiana* plant (lane 1) and transgenic *N. benthamiana* plants (from lines 1 to 3 in each case) transformed with constructs BV2^N (lanes 2-4), BV2^M (lanes 5-7) and BV2^C (lanes 8-10). Approximately 10µg of RNA were loaded in each well. A photograph of the ribosomal RNA band on the ethidium-stained acrylamide gel is shown below the blot to confirm equal loading

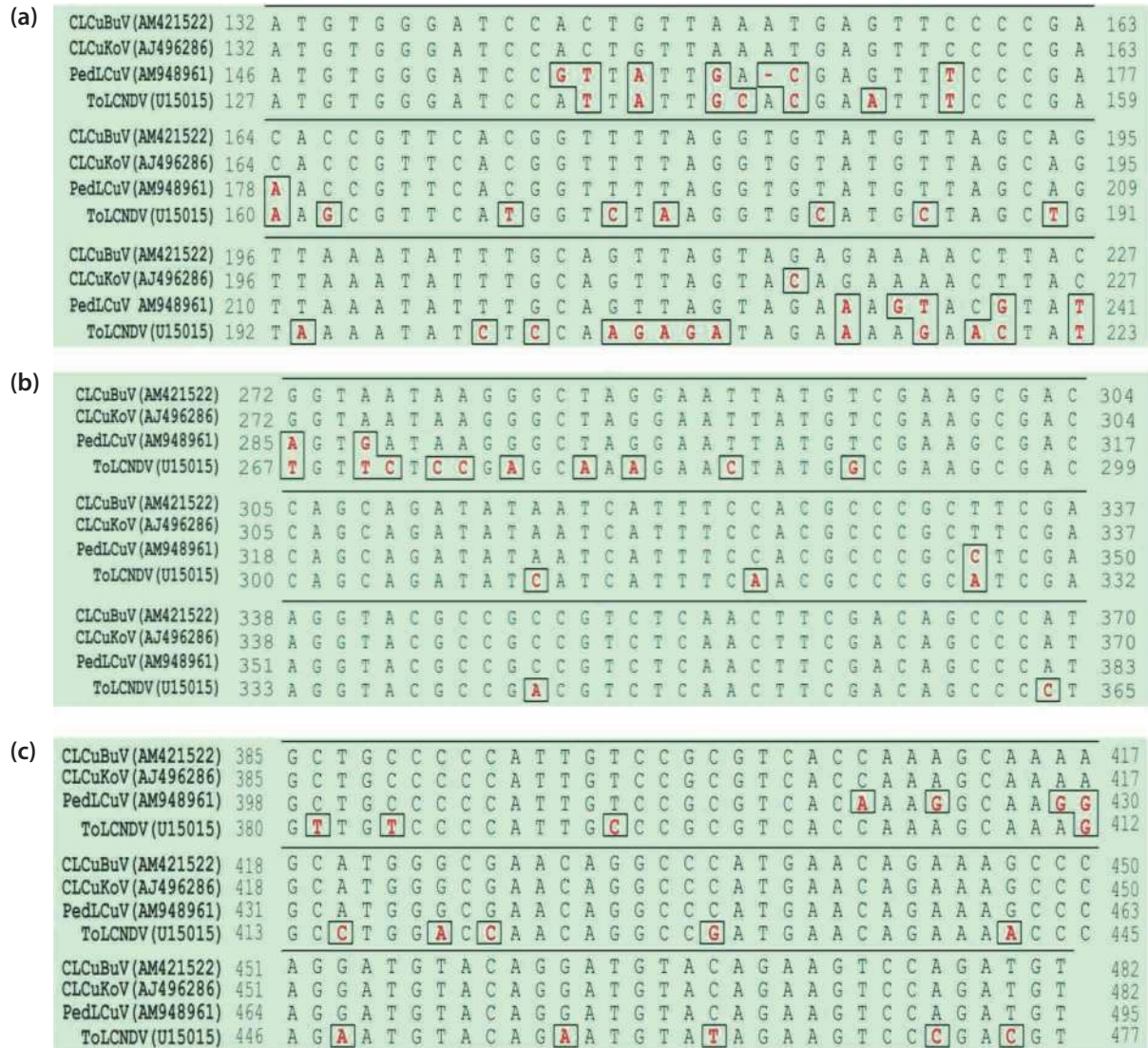


Fig. S3

Alignment of the N-terminal (a), middle portion (b) and C-terminal (c) V2 gene sequences, homologous to the cotton leaf curl Kokhran virus-Bu (CLCuBuV) fragment introduced into *N. benthamiana*, of the viruses used

Nucleotide sequences differing from cotton leaf curl Kokhran virus-Bu (top line in each case) are shown in red text and are boxed. The nucleotide coordinates of the sequences are shown in each case.