frontiers in Agronomy

ORIGINAL RESEARCH
published: 20 October 2021
doi: 10.3389/fagro.2021.676820

Transgenic Expression of Synthetic Coat Protein and Synthetic Replication Associated Protein Produces Mild Symptoms and Reduce Begomovirus-Betasatellite Accumulation in *Nicotiana benthamiana*

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Cotton (*Gossypium hirsutum L.*) is the most essential fiber crop and is widely cultivated within the tropical regions of the world. It has a major impact on socio-economical get-together in Pakistan. Unfortunately, it has been tormented by cotton leaf curl disease (CLCuD) that is produced by distinct species of begomoviruses (Family; *Geminiviridae)* like *Cotton leaf curl Kokhran virus-Burewala strain* (CLCuKoV-Bu) associated with specific betasatellite—Cotton leaf curl Multan betasatellite (CLCuMuB). Host resistance against CLCuD has not been achieved yet. The present study employed the use of synthetic genes considering the phenomenon of pathogen derived resistance (PDR). The coat protein (CP) and replication associated protein (Rep) genes sequence from CLCuKoV-Bu were codon optimized, synthesized and used to control CLCuKoV-Bu and associated betasatellite related to CLCuD. The *Nicotiana benthamiana* plants were *Agrobacterium* mediated transformed with synthetic CP (CP_{syn}) and synthetic Rep (Rep_{syn}) under the control of *Cauliflower mosaic virus* 35S promoter. The transgenic plants harboring CP_{syn} and Rep_{syn} genes were infiltrated with CLCuKoV-Bu alone and CLCuKoV-Bu- CLCuMuB inoculums showed relatively milder symptoms as compared with wild sorts and low virus concentration as checked by southern hybridization. The results confirmed that CP_{syn}, Rep_{syn} genes may be utilized for the resistance of CLCuKoV-Bu and associated betasatellites related to CLCuD and PDR mechanism may also be developed for the control of CLCuD.

Keywords: cotton leaf curl disease, cotton leaf curl Kokhran virus-Burewala strain, cotton leaf curl Multan betasatellite, synthetic coat protein, synthetic replication associated protein, transgenic resistance

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Disease Management, a section of the journal Frontiers in Agronomy

Received: *06 March 2021*
Accepted: *15 September 2021*
Published: *20 October 2021*

Citation:
Rasool G, Yousaf S, Ammara Ue, Iqbal A, Saeed M, Amin I and Mansoor S (2021) Transgenic Expression of Synthetic Coat Protein and Synthetic Replication Associated Protein Produces Mild Symptoms and Reduce Begomovirus-Betasatellite Accumulation in Nicotiana benthamiana. Front. Agron. 3:676820. doi: 10.3389/fagro.2021.676820

INTRODUCTION

The cotton leaf curl disease (CLCuD), a disease complex associated with diverse begomoviruses, is the most important limiting factor for the production of cotton across the sub-continent. Initially CLCuD was ignored as a major threat until it affected cotton production as during the first 3 years of the epidemics the disease incidence and severity were very high in the Punjab. About 97,580 ha were affected resulting in a loss of 543,294 bales of cotton during 1992–93 in Punjab (Amin et al., 2010). Since 2001 the resistance breaking "Burewala" strain of *Cotton leaf curl Kokhran virus* (CLCuKoV-Bu) has spread to all cotton producing areas of Pakistan (Mansoor et al., 2003a) which is still dominating.

During the past few decades the development of geminivirus resistant germplasm remains a challenge. The natural resistance of host plant is the most effective means of reducing the losses caused by these phytopathogens, however the lack of resistant germplasm has encouraged the interest of scientific community in the development of transgenic resistance. Pathogen derived resistance (PDR) is broadly divided into two classes depending upon prevailing molecular mechanisms protein mediated resistance (PMR) and nucleic acid-mediated resistance (RMR) (Smith et al., 2000; Niu et al., 2006). The PMR generally gives broad spectrum resistance whereas RMR offers highly specific resistance. The endeavors to control geminiviruses by PDR are not highly efficient as for RNA viruses suggestive of less susceptibility of these ssDNA viruses to the resistance mechanism (Lucioli et al., 2008). There is only one report of transgenic immunity against geminiviruses (Aragao and Faria, 2009) hence; exploring novel transgenic resistance approaches is highly desirable.

The effects of mutation in the coat protein (CP) gene was studied by Iqbal et al. (2012) by the introduction of premature stop codon which results the premature termination of translation of CP gene. The transient expression of CLCuKoV CP gene under the control of Cauliflower mosaic virus 35S promoter (35S-KoCP), in *Nicotiana benthamiana* plants was studied and in small number of plants viral DNA could be detected by PCR in tissues distal to the inoculation site.

The Cotton Leaf Curl Kokhran Virus-Dabawali recombinant coat protein (rCP) has conserved motif composed of C68, C72, H81, and H85 residues. The mutation in the H85A residue resulted in ∼756-fold reduced the association rate and 3-fold decreased DNA binding affinity (Priyadarshini and Savithri, 2009). After the achievement of CP-mediated resistance (CPMR) it had greatly been developed for many other RNA viruses e.g., tobamoviruses (having CP closely related to TMV; Masuta et al., 1995; Bendahmane et al., 2007). It was also proposed that regulation of viral replication by CP is correlated to the relative strength of CPMR (Bendahmane et al., 2007). Different levels of protection by CPMR indicates the involvement of different mechanisms, broad-spectrum resistance/higher-level resistance was attributed to the co-existence PMR as well as RMR mediated interference (Prins et al., 2008).

First Rep protein-mediated resistance (RPMR) was achieved against TMV expressing TMV Rep gene sequence in transgenic tobacco (Golemboski et al., 1990). Mutations in conserved motifs of Rep acted as *trans*-dominant negative inhibitors of BGMV (Hanson and Maxwell, 1999) and similarly mutations in NTP-binding domain of Rep decreased ACMV but a high level of transgene expression was required to develop this resistance (Sangaré et al., 1999). The use of mutated and truncated Rep gene, resistance was successfully engineered to an African maize pathogen, *Maize streak virus* (MSV) in *Digitaria sanguinalis* (Shepherd et al., 2007a,b). The complete/partial sequence of Rep has been used by researchers to increase resistance in plants (Hanley-Bowdoin et al., 1990; Noris et al., 1996; Brunetti et al., 2001; Lucioli et al., 2003; Antignus et al., 2004). The 210amino acid (aa) from the N-terminal of Rep gene as (Rep_{210}) and 130 aa as (Rep_{130}) were used to develop Rep based resistance, however results exhibited that only 130 aa were sufficient to act as *trans*-dominant negative inhibitor (Lucioli et al., 2003).

As a consequence of viral attack, the pathogen derived transgenes which offer PMR may not work and counterbalanced in a short time due to post transcriptional gene silencing (PTGS)or virus induced gene silencing (VIGS) of the transgene (Lucioli et al., 2008). Furthermore, the modifications by reassortment and recombination of virus sequences (Padidam et al., 1999) put gene silencing (GS) and PDR together and therefore PDR which is a sequence specific mechanism may soon be compromised. To overcome the drawback of GS some other strategies like synthetic gene and non-pathogen derived resistance (NPDR) (Lucioli et al., 2008; Shepherd et al., 2009) are good options to evaluate against begomoviruses.

To overcome the phenomenon VIGS the silent point mutations were introduced in CP and truncated Rep_{130} gene sequence in a way that the protein sequence of CP and Rep_{130} was not disturbed. Considering these facts, the synthetic CP (CP_{syn}) and synthetic Rep_{130} (Rep_{syn130}) were designed to check resistance against CLCuD by overcoming VIGS. The transient expression of CP_{syn} and Rep_{syn130} in *Nicotiana benthamiana* plants showed reduced symptoms and virus titer (Yousaf et al., 2013; Rasool et al., 2016b). The present study deals with the evaluation of transgenic resistance against CLCuKoV-Bu in *N. benthamiana* plants and assesses the resistance responses of CP_{syn} and Rep_{syn130} as well as resistance variations of independent transgenic lines.

MATERIALS AND METHODS

Production of CP_{syn} Expression Cassettes

The coat protein (CP) sequence of CLCuKoV-Bu was assembled and compared with the (accession number HF549183) CPs of other begomoviruses in the database, using BLAST (Mullineaux et al., 1988). The detailed mechanism of point mutations introduced in the CP sequence has been mentioned in Rasool et al. (2016a). The designed mutated CP construct was codon optimized for *Gossypium hirsutum* (http://www.

kazusa.or.jp/codon) and synthesized commercially (GenScript, USA). The synthesized codon optimized CP (CP_{syn}) was cloned between *Sal*I and *Eco*RI sites in pJIT60 vector under the control of *cauliflower mosaic virus* (CaMV) 35S promoter. The expression cassette of CP_{syn} (2.2 kb approx.) contained double CaMV 35S promoter and CaMV 35S terminator. The expression cassette along with promoter/terminator was digested and cloned in the binary vector pGreen0029 at *Kpn*I and *Xho*I sites.

Production of Rep_{syn130} Expression Cassettes

The 130 aa of Rep protein of (CLCuKoV-Bu: FR8197) was assembled and compared with the Rep of other begomoviruses in the database, using BLAST (Mullineaux et al., 1988). The detailed mechanism of point mutations introduced in the Rep sequence has been previously described. The designed Rep construct was codon optimized for *Gossypium hirsutum* (http://www.kazusa.or.jp/codon) and synthesized commercially (GenScript, USA) (Yousaf et al., 2013). The synthesized codon optimized Rep gene (Rep_{syn130}) was cloned at *Hind*III and *Eco*RI restriction sites in plant expression vector pJIT60 under the control of the 35S *Cauliflower mosaic virus* (CaMV) promoter (Guerineau and Mullineaux, 1993). The Rep_{syn130} expression cassette was cloned in plant transformation vector pGreen0029 at *Kpn*I and *Xho*I restriction sites (Hellens et al., 2000).

Development and Characterization of Transgenic *N. benthamiana* With CP_{syn} and Rep_{syn130} Expression Cassette

N. benthamiana was separately transformed with CP_{syn} and Rep_{syn130} expression cassette by the Agrobacterium tumefaciens-mediated leaf disc method (Horsch et al., 1985). All the primary transformants developed from tissue culture was confirmed by PCR using CaMV 35S promoter primers. These primary transformants were labeled as T_0. All the T_0 transgenic lines were phenotypically normal and kept in insect free glasshouse till flowering stage. The seeds were collected and germinated on MS medium supplemented with 500 mg/l kanamycin to get T1 generation.

Maintenance and Culturing of CLCuKoV-Bu/CLCuMuB Infectious Clones for Agro-Infiltration

Infectious clone CLCKoV-Bu/CLCuMuB (Mansoor et al., 2003b; Saeed et al., 2005) was cultured in LB medium along with appropriate antibiotics. The culture was centrifuged (4,000 rpm) for 15 min to harvest the cells, separately. The cells were resuspended in 10 mM $MgCl_2$, 10 mM MES (pH 5.6) and 100 μM Acetosyringone was added in the culture after adjusting its OD600 to 0.6–1. The culture was left overnight or at least 2–3 h at room temperature before infiltration. Transgenic *N. benthamiana* plants at 5–6 leaves stage were chosen for agroinfiltration. The *Agrobacterium* suspension was infiltrated by 1 ml needleless syringe on the underside of 2–3 leaves. Plants were not watered 24 h before inoculation. After infiltration, plants were kept at 26–28°C with a 16 h photoperiod in an insect-free glasshouse and with 65% relative humidity.

Resistance Assay of CP_{syn} and Rep_{syn130} Expressed Transgenic Plants Challenged With CLCKoV-Bu/CLCuMuB

Agro-inoculated transgenic plants were observed for percentage of symptoms severity, incidence of disease and systemic movement of CLCKoV-Bu/CLCuMuB to evaluate the resistance responses of two transgenic lines each of CP_{syn} and $Repsyn_{130}$. The leaf samples were used to extract total genomic DNA by using the CTAB method (Doyle and Doyle, 1990) and quantified by Nanodrop spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, USA). The presence of inoculated CLCKoV-Bu was checked by PCR using primer set of CLCV1 and CLCV2 (Shahid et al., 2007) whilst CLCuMuB presence was confirmed by using primers β01 and β02 (Briddon et al., 2002; Saeed et al., 2005).

Southern Hybridization for Virus Detection in Agro-Inoculated Transgenic Plants Challenged With CLCKoV-Bu/CLCuMuB

Viral Replication

DNA samples were resolved in 1% agarose gels in 0.5X TAE buffer and blotted on nylon membranes (Roche GmbH, Germany). For the detection of virus replication and its systemic movement in the transgenic leaves were checked by Southern hybridization analysis using DIG labeled specific probes of CLCKoV-Bu DNA and CLCuKB DNA according to the manufacturer instructions (RocheGmbH, Germany).

In transgenic lines, for the detection of virus, 1,100-bp fragment of CP gene of CLCKoV-Bu was used. It was PCR amplified by usingCLCV1 and CLCV2 (Shahid et al., 2007) primers and labeled with digoxigenin using a PCR DIG Probe Synthesis Kit (Roche GmbH, Germany) as described by the manufacturer. For the detection of betasatellite, 380-bp fragment was amplified by using pair of β01 and β02 primers (Briddon et al., 2002; Saeed et al., 2005) and was labeled with digoxigenin using a DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche GmbH, Germany).

RESULTS

Development and Characterization of *N. benthamiana* Transgenic Plants Expressing CP_{syn} and Rep_{syn130} Constructs

The PCR analysis of all putative (primary transformants) transgenic plants raised from tissue culture suggested 20 plants to be transformed with each CP_{syn} and Rep_{syn130} Constructs. However, the PCR analysis with CP_{syn} and Rep_{syn130} gene-specific primers showed only 13 and 11 plants to be transformed. All the primary transformants (T_0) transgenic lines were phenotypically normal, allowed to grow till flowering stage and seeds were collected. The seeds (T1) of all the transgenic lines were germinated on MS growth medium supplemented with 500

TABLE 1 | Transgene response of transgenic plants expressing CP_{syn} and Rep_{syn130} at days post inoculations.

Plant line tested	Inoculum[a]	Infectivity (plants symptomatic/plants inoculated)	Plant symptoms[b] @ 7 days post inoculation			Plant symptoms[b] @ 14 days post inoculation			Plant symptoms[b] @ 21 days post inoculation		
			NS	VMS	SS	NS	VMS	SS	NS	VMS	SS
Nb*	Mock inoculated	0/l0	10	0	0	10	0	0	10	0	0
Nb*	Un-inoculated	0/10	10	0	0	10	0	0	10	0	0
Nb*	CLCuKoV-Bu	10/10	0	0	10		0	10	0	0	10
Nb*	CLCuKoV-Bu-CLCuMuB	10/10	0	0	10		0	10	0	0	10
CP-2	CLCuKoV-Bu	16/20	20	0	0	4	16	0	4	12	4
CP-4	CLCuKoV-Bu	16/20	20	0	0	4	16	0	4	8	8
CP-2	CLCuKoV-Bu-CLCuMuB	18/20	20	0	0	2	18	0	2	12	6
CP-4	CLCuKoV-Bu-CLCuMuB	14/20	20	0	0	6	14	0	6	6	8
R-1	CLCuKoV-Bu	13/20	20	0	0	7	13	0	7	6	7
R-2	CLCuKoV-Bu	12/20	20	0	0	8	12	0	8	6	6
R-1	CLCuKoV-Bu-CLCuMuB	15/20	20	0	0	5	15	0	5	8	7
R-2	CLCuKoV-Bu-CLCuMuB	14/20	20	0	0	6	14	0	6	6	8

**Non-transformed N. benthamiana.*

[a]Plants were either not inoculated, inoculated with Agrobacterium cultures harboring the empty pGreen0029 vector (mock), or inoculated with cultures harboring infectious constructs for CLCuKoV-Bu (KoV) and CLCuMuB (MuB).

[b]N. benthamiana physically observed symptoms were classified as no symptoms (NS), very mild symptoms (VMS), mild symptoms (MS), and severe symptoms (SS), The inoculation results of CLCuKoV-Bu and CLCuMuB indicated at 7 days post inoculation, 14 days post inoculation and 21 days post inoculation.

TABLE 2 | Transgene response of transgenic plants expressing CP_{syn} to challenge with CLCuKoV-Bu/CLCuMuB.

Plant line tested	Inoculum[a]	Infectivity (plants symptomatic/plants inoculated)	Plant symptoms[b]			Southern Blot hybridization[c] (CLCuKoV-Bu/CLCuMuB)	Infectivity %age	Resistance %age
			NS	MS	SS			
Nb*	Mock inoculated	0/l0	10	0	0	N	–	–
Nb*	Un-inoculated	0/10	10	0	0	N	–	–
Nb*-1	CLCuKoV-Bu	10/10	0	0	10	H	100%	0%
Nb*-2	CLCuKoV-Bu	10/10	0	0	10	H	100%	0%
Nb*-1	CLCuKoV-Bu- CLCuMuB	10/10	0	0	10	H	100%	0%
Nb*-2	CLCuKoV-Bu-CLCuMuB	10/10	0	0	10	M	100%	0%
CP-2	CLCuKoV-Bu	16/20	4	12	4	M	20%	80%
CP-4	CLCuKoV-Bu	16/20	4	8	8	M	40%	60%
CP-2	CLCuKoV-Bu-CLCuMuB	18/20	2	12	6	M	30%	70%
CP-4	CLCuKoV-Bu-CLCuMuB	14/20	6	6	8	M	40%	60%

**Non-transformed N. benthamiana.*

[a]Plants were either not inoculated, inoculated with Agrobacterium cultures harboring the empty pGreen0029 vector (mock), or inoculated with cultures harboring infectious constructs for CLCuKoV-Bu (KoV) and CLCuMuB (MuB).

[b]N. benthamiana physically observed symptoms were classified as no symptoms (NS), mild symptoms (MS), and severe symptoms (SS).

[c]The results for the Southern hybridization of CLCuKoV-Bu and CLCuMuB are indicated as no hybridization (N), moderate hybridization (M), or high levels of hybridization (H). Levels of hybridization segregated with symptoms, with plants with severe symptoms showing high levels of hybridization, plants with moderate symptoms showing moderate hybridization, and plants with no symptoms showing no hybridization.

mg/l kanamycin. On the selection medium, four lines of CP_{syn} (CP-2 and CP-4) and Rep_{syn130} (R1 and R2) constructs showed 3:1 of segregation pattern which is suggestive of a single insertion (data not shown). The seeds of all putative and singly inserted transformed CP_{syn} and Rep_{syn130} were collected and evaluated for resistance.

Resistance Evaluation of CP_{syn} Transgenic Lines Challenged With CLCKoV-Bu/CLCuMuB

The resistance evaluation of CP_{syn} transgenic *N. benthamiana* plants was observed in two lines (CP-2 and CP-4) at T2 generation. These lines were checked for resistance against the infectious clone of CLCKoV-Bu and CLCKoV-Bu/CLCuMuB.

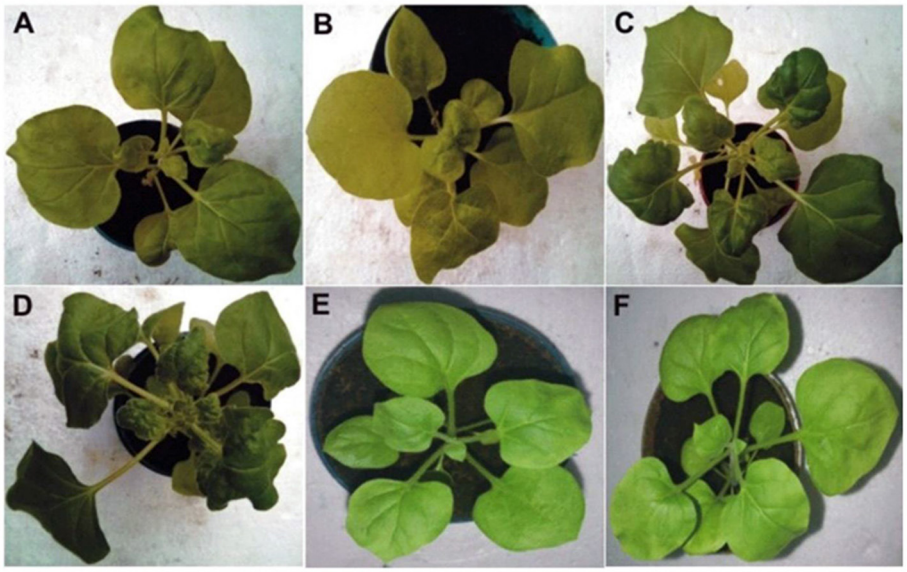

FIGURE 1 | The photographs represents the phenotypic symptoms observed in the agro-inoculated two CP_{syn} transgenic *N. benthamiana*lines compared with control plants. The CP-2 transgenic line was inoculated with CLCuKoV-Bu **(A)** and CLCuKoV-Bu/CLCuMuB showed mild leaf curl symptoms **(B)**. The CP-4 line was inoculated with CLCuKoV-Bu **(C)** and inoculated with CLCuKoV-Bu/CLCuMuB showed severe symptoms **(D)**. For mock inoculation the non-transgenic *N. benthamiana* plants were either inoculated with the empty pGreen0029 vector **(E)** or not inoculated **(F)**. All the pictures were taken at 25 dpi.

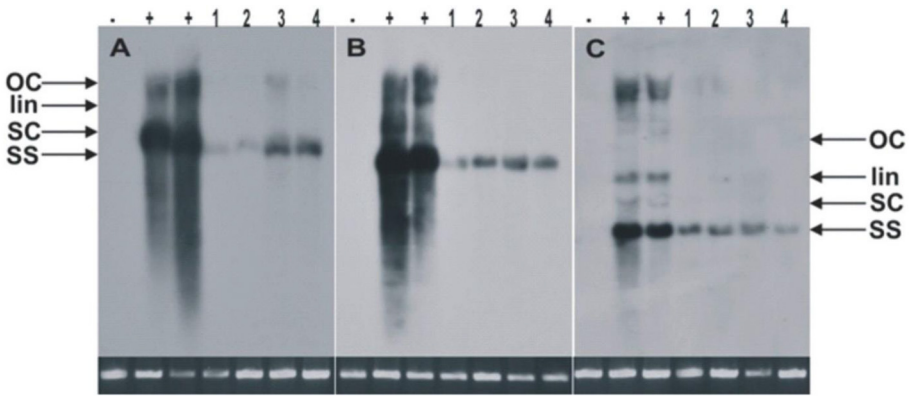

FIGURE 2 | The Southern blots for the detection of CLCuKoV-Bu alone **(A)** or with the CLCuKo-Bu/CLCuMuB **(B,C)**. The total genomic DNA samples were extracted from two transgenic replicates of CP-2 transgenic line (line 1 and 2 as indicated), and two replicates from CP-4 line as indicated in (line 3 and 4) for the expression of CP_{syn}. For all the blots the one negative uninoculated control plant in (- lanes), and two positive non-transgenic symptomatic control plants in (+ lanes). All the DNA samples were extracted from leaves at 21–25 dpi and ∼10 μg of total genomic DNA was used in each case. The position of all replicative forms of DNA like single-stranded (ss), supercoiled (sc), linear (lin) and open circular (OC) have shown in all three blots. In each case, a photograph of the ethidium bromide stained genomic DNA bands on the agarose gel are shown below the blot to confirm.

Twenty plants (confirmed by PCR for the presence of CP_{syn}) of each line and wild-type healthy *N. benthamiana* were agro-inoculated and kept under observation for 50 dpi. The periodic development of phenotypic symptoms was observed at three different intervals (7, 14, and 21 dpi). All the replicates of both CP-2 and CP-4 transgenic lines were remained asymptomatic at 7 dpi, however very mild symptoms (VMS) started to appear at 14 dpi (**Table 1**). The majority of replicates showed VMS at 21 dpi and few remained asymptomatic, the severe symptoms (SS) were observed in 4/20 in CP-2 replicates and 6/20 in CP-4 replicates (**Table 1**). The phenotypic data showed that the majority of CP-2 and CP-4 replicates were able to silence the CLCKoV-Bu along with the CLCuMuB. The data for evaluation of the resistance is presented in **Table 2**. Resistant phenotypes and symptom development as shown in **Figure 1**, panel were observed for all CP_{syn} transgenic replicates at the 21 dpi. The CP-2 and CP-4 transgenic lines replicates were inoculated with CLCuKoV-Bu alone showed mild to asymptomatic viral

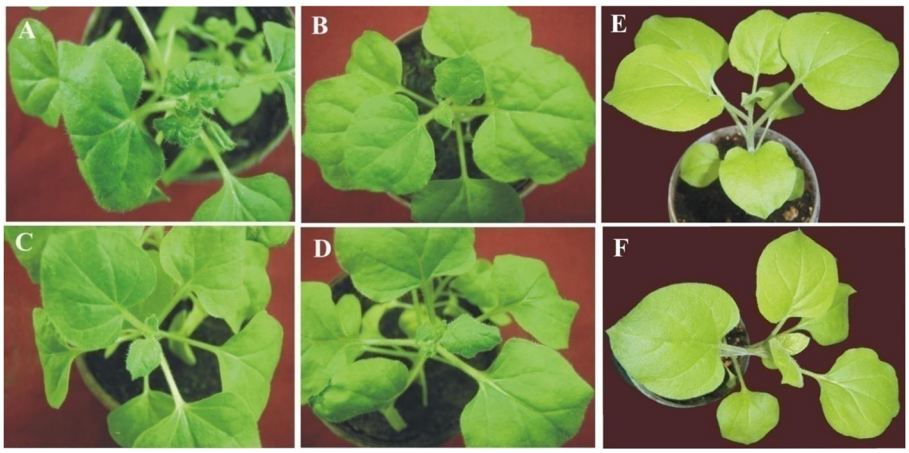

FIGURE 3 | The photographs represents the phenotypic symptoms in agro-inoculated Rep_{syn130}transgenic *N. benthamiana* compared with control plants and were taken at 21 dpi. In **(A)** the transgenic *N. benthamiana* line R1 and in **(B)** R2 transgenic line inoculated with CLCKoV-Bu. The plant shown in **(C,D)** are R1 and R2 Rep_{syn130} transgenic plants, respectively, coinoculated with CLCKoV-Bu/CLCuMuB. **(E,F)** are the non-transgenic *N. benthamiana* plants were either not inoculated or with the empty pGreen0029 vector, respectively.

symptoms (12/20 and 10/20) whereas, the transgenic replicates (12/20 and 10/20) inoculated with CLCuKoV-Bu/CLCuMuB displayed mild to asymptomatic viral symptoms (**Figure 1**; **Table 2**). The PCR analysis showed the presence of CLCuKoV-Bu in all samples of CLCuKoV-Bu inoculated CP-2 and CP-4 transgenic replicates. Nevertheless, the transgenic replicates of both CP-2 and CP-4 transgenic lines showed the presence of both virus and betasatellite in all DNA samples when inoculated with of CLCuKoV-Bu/CLCuMuB (**Table 2**). The Southern blot analysis verified that when CP-2 and CP-4 transgenic replicates were inoculated with CLCuKoV-Bu showed moderate to lower level of CLCuKoV-Bu, respectively (**Figure 2**; **Table 2**). The same replicates when inoculated with CLCuKoV-Bu/CLCuMuB showed moderate to high level of CLCuKoV-Bu/CLCuMuB accumulation as compared to positive control plants by Southern analysis (**Figure 2**; **Table 2**).

Resistance Evaluation of Rep_{syn130} Transgenic Plants Challenged With CLCKoV-Bu/CLCuMuB

The resistance evaluation of Rep_{syn130} transgenic plants was performed on two lines (R1 and R2) at T_2 generation. Resistance against CLCKoV-Bu/CLCuMuB was checked on twenty plants (confirmed by PCR for the presence of Rep_{syn130}) of each line and ten plants of wild-type healthy *N. benthamiana*. These plants were inoculated by CLCKoV-Bu and CLCKoV-Bu/CLCuMuB complex and kept under observation for 50 dpi. The data for evaluation of the resistance potential was recorded at three different intervals (7, 14, and 21 dpi) and presented in **Table 1**. All the transgenic replicates of lines R1 and R2 remained asymptomatic at 7 dpi, however the development of VMS was observed at 14 dpi. The severity in the symptom development was observed in 7/20 in R1 line and 6/20 in R2 line. The overall effect of CLCuMuB on the development of disease severity symptom was not very significant as 8/20 replicates developed SS whereas 6/20 exhibited SS without CLCuMuB. Resistant phenotypes as shown in **Figures 3C,D** were observed for Rep_{syn130} transgenic plants. The transgenic replicates 7/20 plants of R1 and 8/20 plants of R2, no symptoms were observed and these plants remained symptomless even at 50 dpi as well as till senescence (**Table 3**). The southern blot shown in **Figure 4** is representing the titer of CLCuKoV-Bu in nine plants of R1 (lane 2–11) and 8 plants of R2 (lane 12–19). The lane 1 and 20 are representing the DNA from negative and positive control plants, respectively. The R1 and R2 transgenic replicates inoculated with CLCuKoV-Bu alone exhibited mild to asymptomatic viral symptoms (6/20 and 8/20) while plants (8/20 and 7/20) inoculated with CLCuKoV-Bu/CLCuMuB exhibited mild to asymptomatic viral symptoms (**Figure 3**; **Table 3**). A considerable decrease in the titer of CLCuKoV-Bu DNA was observed in both R1 and R2 lines compared to control (**Figure 4** lane 20). All plants of R1 and four plants of line R2 also showed decrease in the level of CLCuKoV-Bu DNA.

DISCUSSION

The engineered resistance approaches are potentially offering an alternative and rapid method for the transfer of resistant genes to the traditional cultivars (Vanderschuren et al., 2007; Loriato et al., 2020). For engineered resistance the CP gene was selected in this study due to its function in assembly/dis-assembly of viral DNA during plant cell infection. The constitutive expression of CP elevated its levels that may protect the plant from secondary infection by the same virus (Powell et al., 1990). The similar mechanism of inhibition or interference may be involved in this study as the resistance response against CLCuKoV-Bu and CLCuMuB was observed. The resistance observed in transgenic plants supports the previous finding of Kunik et al. (1994) and Raj et al. (2005) who extended the use of CPMR

TABLE 3 | Transgene response of transgenic plants expressing Rep_{syn130} to challenge with CLCuKoV-Bu/CLCuMuB.

Plant line tested	Inoculum[a]	Infectivity (plants symptomatic/plants inoculated)	Plant symptoms[b]			Southern Blot hybridization[c] (CLCuKoV-Bu/CLCuMuB)	Infectivity %age	Resistance %age
			NS	MS	SS			
Nb*	Mock inoculated	0/l0	10	0	0	N	–	–
Nb*	Un-inoculated	0/10	10	0	0	N	–	–
Nb*-1	CLCuKoV-Bu	10/10	0	0	10	H	100%	0%
Nb*-2	CLCuKoV-Bu	10/10	0	0	10	H	100%	0%
Nb*-1	CLCuKoV-Bu- CLCuMuB	10/10	0	0	10	H	100%	0%
Nb*-2	CLCuKoV-Bu-CLCuMuB	10/10	0	0	10	M	100%	0%
R-1	CLCuKoV-Bu	13/20	7	6	7	M	35%	65%
R-2	CLCuKoV-Bu	12/20	8	6	6	M	30%	70%
R-1	CLCuKoV-Bu-CLCuMuB	15/20	5	8	7	M	35%	65%
R-2	CLCuKoV-Bu-CLCuMuB	14/20	6	6	8	M	40%	60%

**Non-transformed N. benthamiana.*

[a]Plants were either not inoculated, inoculated with Agrobacterium cultures harboring the empty pGreen0029 vector (mock), or inoculated with cultures harboring infectious constructs for CLCuKoV-Bu (KoV) and CLCuMuB (MuB).

[b]N. benthamiana physically observed symptoms were classified as no symptoms (NS), mild symptoms (MS), and severe symptoms (SS).

[c]The results for the Southern hybridization of CLCuKoV-Bu and CLCuMuB are indicated as no hybridization (N), moderate hybridization (M), or high levels of hybridization (H). Levels of hybridization segregated with symptoms, with plants with severe symptoms showing high levels of hybridization, plants with moderate symptoms showing moderate hybridization, and plants with no symptoms showing no hybridization.

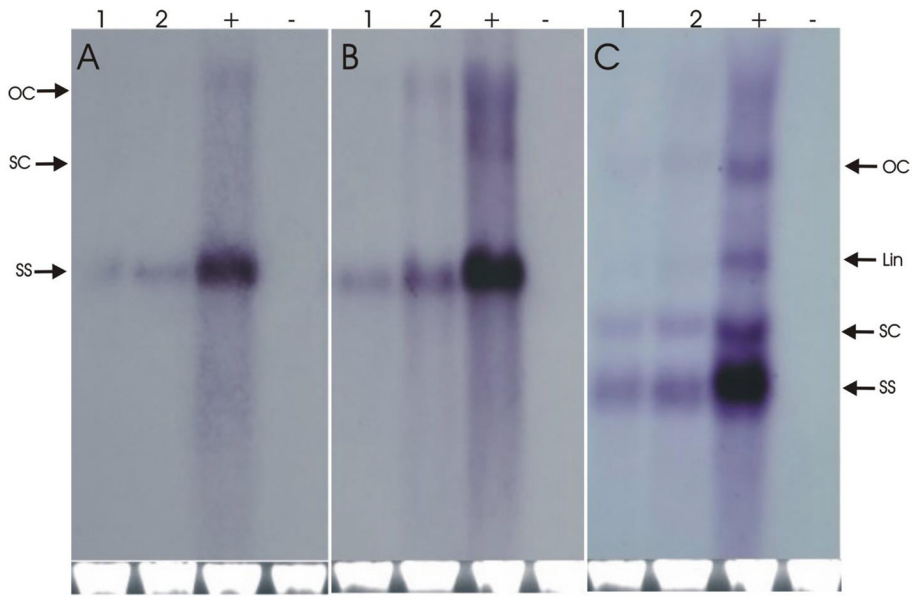

FIGURE 4 | The Southern blot analysis for the detection of CLCuKoV-Bu alone **(A)** and CLCuKo-Bu/CLCuMuB **(B,C)**. The total DNA was extracted from three transgenic replicates from R1 transgenic line in (lane R1-R3). For all three blots one negative uninoculated control plant in (H lanes), and one positive non-transgenic symptomatic control plants in (P lanes). The position of all replicative forms of DNA like single-stranded (ss), supercoiled (sc), linear (lin) and open circular (OC) have shown in all three blots. In each case, a photograph of the ethidium bromide stained genomic DNA bands on the agarose gel are shown below the blot to confirm equal loading of DNA samples.

from RNA to DNA viruses (geminiviruses). Similarly, codon optimized synthetic CP and RNAi construct has been used to develop resistance against Tomato yellow leaf curl virus Oman (TYLCV-OM) (Ammara et al., 2015, 2020). The CP_{syn} used in this study showed maximum aa sequence homology with different begomovirus strains associated with CLCuD complex. These results indicate that CP_{syn}nt sequences have minimized the chances for VIGS (Rasool et al., 2016a,b). The resistance evaluation of transgenic *N. benthamiana* plants expressing CP_{syn} against CLCuKoV-Bu/CLCuMuB showed mild to delayed symptoms. The transgenic plants inoculated with CLCuKoV-Bu exhibited milder symptoms than the plants coinoculated with CLCuKoV-Bu and CLCuMuB. The southern hybridization results showed increased hybridization in plants coinoculated

with CLCuKoV-Bu and CLCuMuB as compared to plants inoculated with CLCuKoV-Bu (**Figures 1A–D**). There has been many reports describing the role of betasatellite in relation to increased virus titer (Saunders et al., 2000). Some replicates when phenotypically observed displayed milder symptoms while some were asymptomatic (**Table 1**). These results have indicated that the presence of CP_{syn} have significantly reduced virus DNA accumulation however, the virus spread in the systemic leave was not reduced as shown by southern blot analysis (**Figure 2**).

The multifunctional property of Rep makes it well-studied for developing resistance against homologous as well as heterologous viruses and the mechanism of its action has also been described (Brunetti et al., 2001; Lucioli et al., 2003; Shivaprasad et al., 2006). However, it did not provide durable and enhanced resistance to DNA viruses as to RNA viruses (Ribeiro et al., 2007) because of GS. The Rep_{syn130} used in this study encompasses all the essential domains and its multifunctional property related with the oligomerization nature (Orozco et al., 2000).

The transgenic *N. benthamiana* plants constitutively expressing Rep_{syn130} controlled the accumulation of homologous CLCuKoV-Bu as well as its associated CLCuMuB by trans-dominant inhibition. Several reports of trans-dominant negative inhibition of both animal (Smith and Adeluca, 1992) and plant viruses particularly geminiviruses (Hou et al., 2000; Chatterji et al., 2001) have been described. The resistance response of transgenic *N. benthamiana* plants expressing Rep_{syn130} against CLCuKoV-Bu showed mild to delayed symptoms (**Figure 3**) as compared to plants coinoculated with CLCuKoV-Bu/CLCuMuB. However, the plants not showing the resistant phenotype may be due to some differences in the gene expression. The observed resistance is likely to be achieved as the expression of Rep_{syn130} might have acted as *trans*-dominant negative inhibitor and stopped the replication of attacking CLCuKoV-Bu as well as CLCuMuB. As the replication of CLCuMuB is dependent on helper begomovirus thus its titer was also reduced. Different studies had indicated that RPMR is positively correlated to the levels of transgene expression (Noris et al., 1996; Lucioli et al., 2003) whereas in some cases although a correlation between transcript level and resistance existed (Hong and Stanley, 1995).

Transgenic *N. benthamiana* harboring Rep_{syn130} showed that the gene has no toxic effect when constitutively expressed and do not cause any developmental abnormalities that are earlier described for complete Rep genes of several begomoviruses during plant transformation (Hong and Stanley, 1996). The Rep proteins are highly important and relatively conserved in begomoviruses, moreover due to codon optimization and introduction of point mutations Rep_{syn130} is viewed as a good candidate of trans-dominant negative inhibition of CLCuD. However, tomato plants expressing TYLCSV synthetic Rep have revealed some transcriptional changes similar to stress-related responses showing that this domain has interfered with viral infections (Lucioli et al., 2014). Resistance response of stably transformed Rep_{syn130} transgenic *N. benthamiana* plants was analogous to the transiently expressed Rep_{syn130} as previously described by the authors. Since synthetic Rep circumvented GS of the transgene on the attack of TYLCSV (Lucioli et al., 2008) in the same way this study confirmed the functioning of truncated Rep_{syn130} against CLCuKo-Bu. The resistance is correlated with the presence of transgene although in some cases the presence of the transgene was also associated with the infected plants expressing usual but mild symptoms e.g., some replicates of transgenic lines R1 and R2 showing tolerant behavior (**Table 2**) that might be due to differential expression of the transgene.

It may be predicted that CP_{syn} and Rep_{syn130} constructs can be used to achieve resistance against other viruses associated with CLCuD and its associated betasatellites. Moreover, further studies are required to check the combined resistance potential of both genes, CP_{syn} and Rep_{syn130}, by gene staking or pyramiding that could offer broad spectrum resistance. Consequently, the synthetic gene approach alone, or in combination with other PDR or non-pathogen derived (NPDR) strategies, may provide effective control strategy for begomoviruses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

This research article is a part of the Ph.D. thesis of GR and SY. SM and MS conceived the study and designed the experiments. SY and GR performed the experiments. SY, GR, UA, and AI analyzed the data. UA and AI wrote the first draft of the manuscript which was edited by SY and GR. All authors read and approved the final manuscript.

FUNDING

This work was supported by the Indigenous Ph.D. Fellowship Programme of the Higher Education Commission, Government of Pakistan.

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