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TRANSIENT EXPRESSION OF SYNTHETIC COAT PROTEIN GENE OF COTTON LEAF CURL BUREWALA VIRUS IN TOBACCO *(NICOTIANA BENTHAMIANA)*

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ABSTRACT

In a study conducted at National Institute for Bio-technology and Genetic Engineering (NIBGE), Faisalabad, Pakistan during 2013-14 a synthetic coat protein (CP) gene from cotton leaf curl Burewala virus was synthesized containing point mutations in its DNA sequence to escape the virus induced gene silencing problem. The synthetic CP (Syn. CP) was cloned and expressed under the control of constitutive Cauliflower mosaic virus 35S promoter. In three independent agro-inoculation experiments, *Nicotiana benthamiana* plants were co-inoculated with Syn. CP construct together with infectious clones of cotton leaf curl Kokhran virus (CLCu KoV) and cotton leaf curl Multan betasatellite which showed significant level for tolerance/resistance (70/30%) against typical downward leaf curl viral symptoms as compared to control plants. This observation was confirmed by PCR in which 14 out of 20 plants were positive and 6 out of 20 plants were negative for CLCuKoV detection. Southern hybridization showed a reduction level of viral DNA accumulation in 10 mild symptomatic plants out of 15 and no detection of viral DNA in 4 asymptomatic plants out of 15 indicating that Syn.CP strategy could be adopted to control CLCuD.

KEYWORDS: Cotton leaf curl disease; Burewala **virus; pathogen derived resistance; synthetic coat protein; transient expression; Pakistan.**

INTRODUCTION

Begomoviruses belongs to the family Geminiviridae of plant DNA viruses, characterized by their circular, single-stranded DNA genome of approx. 2.8 kb size that is encapsidated in twinned icosahedral particles (1). Begomoviruses belonging to the New World (NW) are bipartite, having two components (DNA A and B), while most of the Old World begomoviruses are monopartite having a single component homologous to the DNA A

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component of bipartite begomoviruses. Most of old world monopartite viruses are associated with ssDNA satellites called alphasatellites and betasatellites (2). Monopartite begomoviruses and DNA A of bipartite begomoviruses encode six open reading frames (ORFs), two (AV1 and AV2) in the virion-sense and four ORFs in the complementary-sense (AC1, AC2, AC3 and AC4) while DNA B encodes two ORFs (BV1 and BC1) in virion- and complementary-sense, respectively with an exception of AV2 ORF lacking in old world begomoviruses. The small number of proteins encoded by these ORFs perform several functions including encapsidation, replication, transcription, pathogenicity, and virus movement. Begomoviruses are transmitted exclusively through whitefly (*Bemisia tabaci*) and infect many dicotyledonous plants cultivated for food and fibre (1).

In all geminiviral proteins, coat protein (CP, AV1/V1) is designated as a late gene and considered to be responsible for forming the typical twinned geminate particle structure of viral DNA (3). For encapsidation, CP subunits are involved in specific interaction with the viral genomic ssDNA as well as homotypic interaction leading to multimerization (4). In association with virus structural protein, CP also performs numerous functions like insect transmission, viral DNA shuttling in and out of nucleus, cell-to-cell movement alongwith systemic spread of virions and binds ssDNA and dsDNA (5, 6, 7, 8, 9). Like CP assembly of viral DNA, there is also a fundamental character of CP to disassemble and release viral DNA upon viral infection to the plant cell. The role of CP in assembly and disassembly of capsids for viral DNA is driven by the thermodynamic equilibrium of CP concentration in cell cytoplasm (10). But in bipartite begomoviruses, CP is not required for systemic movement of viral DNA (11). During viral DNA transmission, CP protects it from degardation in the insect vector haemolymph by binding with GroEL protein of genus *Arsenophonus*, a bacterial endosymbiont found in the midgut of *Bemisia tabaci* (12, 13).

Cotton Leaf Curl disease (CLCuD) is the most devastating disease of cotton across Pakistan and northwestern India and is caused by several begomoviruses associated with a disease specific satellite (CLCuMuB); (14, 15). The disease elicits typical symptoms of vein swelling, upward and downward curling of leaves, formation of enations underside the leaves and stunted growth (16). The epidemic of CLCuD in Indian subcontinent during the 1990s was associated with multiple begomoviruses (at least six distinct begomovirus species were identified in cotton) and many plants were found to be infected with more than one species. However, since resistance breakdown in cotton in 2001 only a single begomovirus species *cotton leaf*

curl Burewala virus (CLCuBuV), is predominant in cotton growing belt of Punjab, Pakistan (17).

The first successful demonstration of PDR was achieved by expressing CP gene of *tobacco mosaic virus* (TMV) in transgenic tobacco plants (19). PDR broadly categorized as protein mediated resistance (PMR) and RNA mediated resistance (RMR). After the first successful utilization of PDR/PMR for TMV, an RNA virus, there have been many attempts for achieving resistance against geminiviruses. CP gene of Tomato yellow leaf curl virus (TYLCV) and tomato leaf curl virus (ToLCV) (20, 21), Rep protein for TYLCV, tomato leaf curl New Delhi virus (ToLCNDV), tomato yellow leaf curl Sardinia virus (TYLCSV) and mungbean yellow mosaic virus (MYMV) (22-25) and MP protein for tomato mottle virus (ToMoV) (26) are few examples of PMR. However, detailed transgene analysis showed that in many cases where PMR was reported the underlying mechanism was found to be RMR because transgene was silenced by VIGS (27).

Lucioli *et al.* (25) reported that VIGS is a crucial weakness for practical applications of PMR to geminiviruses; however, the authors also showed that VIGS problem can be overcome by synthetic transgene approach. Here CLCuBuV CP gene was selected due to its core functions in causing geminivirus infection, conserved sequence among diverse begomovirus CPs and it has been utilized for development of resistance. The modified Syn.CP by introducing point mutation without disturbing protein sequence to avoid VIGS (28) and to exploit full potential of PDR for interference in viral infection could be the promising solution to develop resistance against begomoviruses. The potential of Syn.CP for resistance against begomoviruses is discussed.

The present study addresses Cotton Leaf Curl disease (CLCuD), a geminiviral disease control by using pathogen derived resistance (PDR) approach that was proposed by Sanford and Johnson (1985) transformation of plants with portions of viral genomes frequently gives rise to plants that are resistant to the virus from which the transformed sequence is derived i.e. a part, or a complete viral gene is introduced into the plant, which subsequently, interferes with one or more essential steps in life cycle of the virus (18).

MATERIALS AND METHODS

This transient experiment was conducted during the year 2012-13 at National Institute of Bio-technology and Genetic Engineering, Faisalabad, Pakistan.

For this study, the coat protein (CP) sequence of CLCuBuV (accession number HF549183) was re-assembled by using codon usage table of cotton genome Gossypium hirsutum (gbpln): 557 CDS's (190383 codons). In addition the point mutations were also introduced by changing the every third nucleotide (nt) in sequence in such a way that the consecutive resembles of nt sequence between wild type CLCuBuV CP and Syn.CP was remained less or not more than 5 nt alongwith encoding the same amino acids (aa). The designed CP was synthesized commercially. Cost protein (CP) sequence of CLCuBuV was compared with CPs of other begomoviruses in the database, using BLAST (29). The sequences of CPs of cotton leaf curl Kokhran virus (CLCuKoV; AJ496286), cotton leaf curl Multan virus (CLCuMuV; DQ191160), cotton leaf curl Rajasthan virus (CLCuRV; AF363011), cotton leaf curl Alahabad virus (CLCuAlV; GU112081), cotton leaf curl Banglore virus (CLCuBaV; AY705380), Cotton leaf curl Shahdadpur virus (CLCuShV; FN552003), papaya leaf curl virus (PaLCuV; AJ436992) and Pedilenthus leaf curl virus (PedLCuV; GU732204) were obtained from NCBI. Multiple sequence alignments were carried out by using Clustal W (30) in MegAlign program of Lasergene (DNAStar Inc., Madison, WI, USA) (Fig.1). For expression Syn.CP was cloned between *Sal*I and *EcoR*I sites in pJIT60 vector under the control of *cauliflower mosaic virus* (CaMV) 35S promoter. The expression cassette Syn.CP (2.2 kb approx.) containing the duplicated CaMV 35S promoter and CaMV 35S terminator was restriction digested by *Kpn*I and *Xho*I sites and cloned in the binary vector pGreen0029 at *Kpn*I and *Xho*I sites to produce pSyn.CP. The Syn.CP construct was transformed through electroporation into *Agrobacterium tumefaciens* strain GV3101 and confirmed through PCR using specific primers; CPF (5´-ATGTCTAAAAGACCTGCCGACATCAT-3´) and CPR (5´-TTAGTTAGTTACTGAGTCGTAAAAAT-3´). Infectious clones of CLCuKoV, CLCuMuB and pSyn.CP construct were prepared for agro-inoculation as described previously (31). Twenty *N. benthamiana* plants of 4-6 leaves stage maintained in insect free glasshouse containment at 28°C, were inoculated with CLCuKoV/CLCuMuB alone and CLCuKoV/CLCuMuB together with pSyn.CP. Experiments were repeated three times to get the reproducible results. After symptom development in control plants (3 weeks approx.) total genomic DNA was extracted from leaf samples using the CTAB method (32). Virus titer was assessed in inoculated and systemic leaves by Southern hybridization. Genomic DNA was quantified by spectrophotometer and 10 µg was used per sample for Southern hybridization. The DNA probe for hybridization was prepared by using PCR DIG Probe Synthesis Kit (Roche, Germany) and the primer sequences of CLCV1 (5´-CCGTGCTGCTGCCCCCATTGTCCGCGTCAC-3´), CLCV2 (5´-

CTGCCACAACCATGGATTCACGCACAGGG-3´) were used for making DNA probe for CLCuKoV that amplify 1.1 kb fragment in sequence of CLCuKoV (Fig. 3).

RESULTS AND DISCUSSION

A study suggested that the resistance of Rep_{210} was reduced after viral infection due to the activity of VIGS in *Tomato yellow leaf curl Sardinia* (24). Lucioli *et al.* have reported that VIGS is a hurdle in practical application of PMR against geminiviruses however it can be overcome by modifying transgenes through synthetic gene approach (28). As RMR is a sequence homology dependent strategy and has narrow range potential for resistance, in addition, RMR may be compromised by geminiviruses encoded silencing suppressors, like A(C2)/TrAP, C4, V2 and βC1 (33). Recently Catoni *at el.* have reported that RNA-immunity level virus resistance was obtained by a transgene carrying nucleocapsid (N) gene of *Tomato spotted wilt virus*, however, in fifth generation resistance was overcome by TGS (34). If the VIGS issue could be controlled, it is likely to be a highly conserved and important geminivirus gene like CP might become good target for PMR.

The CP of CLCuBuV sequence after codon optimization and point mutation resulted in Syn.CP sequence that was observed with unchanged amino acids sequence but DNA sequence homology was altered between CPs by point mutation. This modified Syn.CP sequence was used to avoid VIGS by CLCuBuV CP as previously reported for TYLCSV Rep gene (24). Nucleotide and amino acid alignment of Syn.CP with CLCuBuV CP showed 64.7 percent nt homology alongwith 100 percent homology for *aa* sequence. The Syn.CP has shown a range from 64.7 to 70.0 percent and for amino acids ranged from 92.6 to 100 percent homology with other begomoviruses' CP (as described in methodology). Importantly the MSA results of Syn.CP for *nt* sequences showed that patch of 21-23 *nt* was not homologous with begomoviruses' CP that is requisite to trigger VIGS (35). The Syn. CP *nt* sequence showed maximum continuous *nt* homology was just only 5 nt with majority of the begomoviruses' CP in MSA whereas at some places in MSA a range of 7-11 *nt* stretch was observed in CP sequences of CLCuMuV, CLCuRV, PaLCuV, CLCuAV, CLCuBV, PedLCV and CLCuAV. Overall the minimum nt sequence homology of Syn.CP was seen with CLCuBuV and CLCuKoV CP sequences (Fig. 1). These results indicate that Syn.CP *nt* sequence has minimized the chances for VIGS. In this perspective, if VIGS issue is addressed PMR might have advantage over RMR. Here CLCuKoV was used for inoculation as its CP has 99.5 *nt* and 100 percent amino acids

(*aa)* sequence homology with CP of CLCuBuV. Moreover infectious clone of CLCuKoV induced severe symptoms in *N. benthamiana* plants that supports to use CLCuKoV in place of CLCuBuV to evaluate resistance potential of Syn.CP against CLCuD.

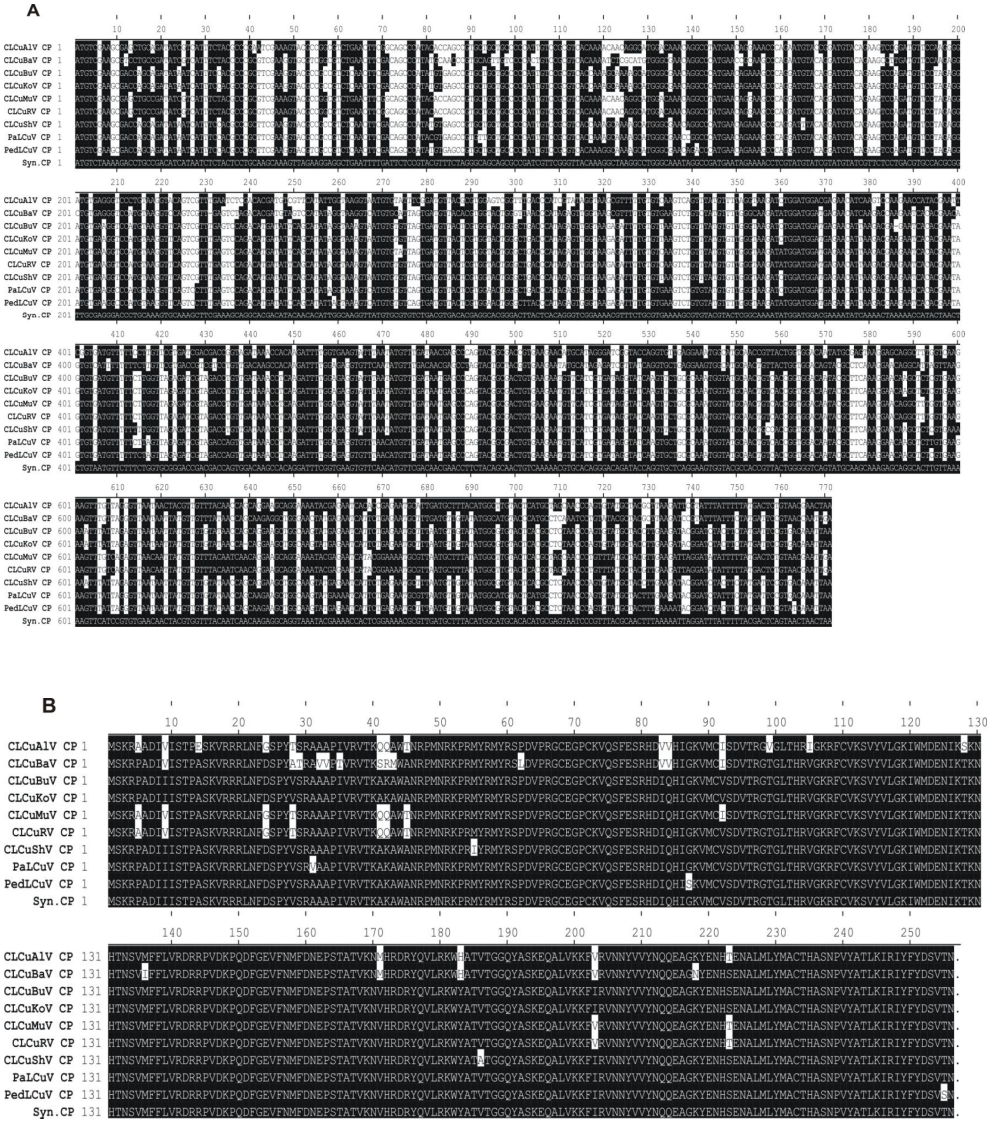

Fig 1. Multiple sequence alignment of Syn.CP for nucleotide (A) and amino acid (B) with CP sequences of CLCuKoV, CLCuMuV, CLCuRV, CLCuAV, CLCuBV, CLCuShV, PaLCuV and PedLCV that are involved in cotton leaf curl disease.

Agro-inoculation with CLCuKoV/CLCuMuB resulted in typical leaf curl symptoms of downward leaf curling, vein thickening, darkening and stunting growth in controls plants 14 days after inoculation (dpi) which turned severe by 25 dpi. Twenty plants inoculated with CLCuKoV/CLCuMuB together with pSyn.CP construct showed resistance response by delaying the downward leaf curl and vein thickening and producing very mild symptoms even some plants developed no symptoms at 21-25 dpi (Fig. 2).

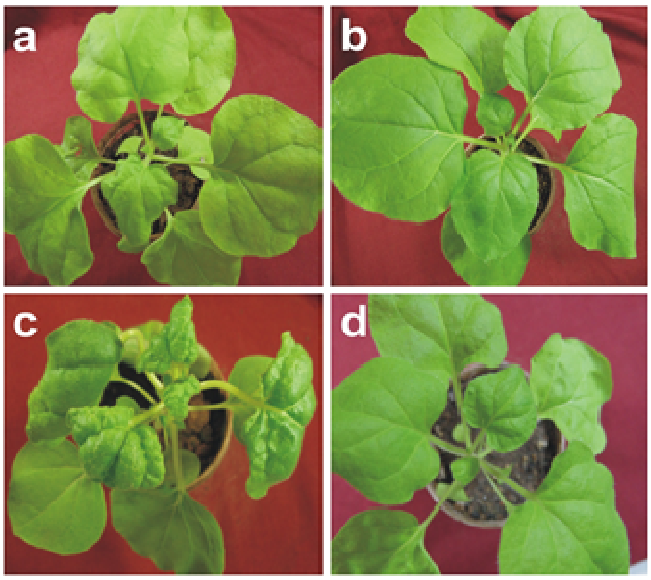

Fig. 2. Agro-inoculation of *N. benthamiana* plants. Panel (a & b) represents the plants inoculated with infectious clone of CLCuKoV/CLCuMuB +Syn.CP. Panel (c) shows the virus symptomatic control plants inoculated with CLCuKoV/CLCuMuB. Panel (d) remained un-inoculated as healthy control plants.

In three repeated experiments, it was clearly observed that at 21-25 dpi, there were severe leaf curl symptoms in all ten control plants as compared to plants inoculated with virus plus pSyn.CP in which approx. Ten out of 20 showed mild symptoms, 4 out of 20 appeared with delayed symptoms and 6 out of 20 plants remained asymptomatic (Table 1). In all treatments, subjectively, 70 percent mixed (CLCuKoV/CLCuMuB+pSyn.CP) inoculated plants showed tolerance and 30 percent plants remained resistant to CLCuKoV/CLCuMuB by developing mild to delayed symptoms and remained asymptomatic, respectively. The inoculated plants via *Agrobacterium* cultures showed variation in symptoms that were relatively mild. Although the plants were infiltrated with viral and Syn.CP in equal ratio, the variation in yet symptoms could be attributed to the possibility that some of cells in infiltrated

leaves either only received viral construct or viral construct rapidly mobilize virus DNA systemically and developed mild or delayed symptoms. Total genomic DNA was extracted from the systemic leaves of plants for PCR and southern hybridization to assess virus replication. Southern blot analysis showed higher level of viral DNA in all positive control plants with severe symptoms (Lane +) and negative control plants remained symptomless (Lane). However, there was detected significantly low viral DNA accumulation in pSyn.CP inoculated plants that are 9 out of 15 with mild symptoms and 2 are severe symptomatic (Lane 1 and 8) and four are remained asymptomatic (Lane 3, 4, 5 and 10) (Fig. 3). PCR analysis showed presence of viral components in all positive control plants as well as in more than 70 percent (14/20) for pSyn.CP treated plants (Table 1). Southern blot and PCR analysis showed low virus accumulation level detection in mild to delayed viral

Table 1. Viral symptoms severity, molecular analysis (PCR/Southern hybridization) and resistance percentage infected *N. benthamiana* plants by CLCuKoV/CLCuMuB with pSyn.CP construct.

Agro-inoculum	Infected plants	PCR (CLCuKoV)	Southern hybridization	Tolerance/resistance %age
	Exp.1			
CLCuKoV+CLCuM betastellite+Syn.CP	10/20 (mild) 6/20 (late to severe) 4/20 (asymptomatic)	16/20 (+) 4/20 (-)	+++ ----	80%/20%
CLCuKoV+CLCuM betastellite	10/10 (severe)	10/10 (+)	++++ ----	0%
Un-inoculation	5/5 (asymptomatic)	0/5	----	0%
	Exp.2			
CLCuKoV+CLCuM betastellite+Syn.CP	10/20 (mild) 4/20 (late to severe) 6/20 (asymptomatic)	14/20 (+) 6/20 (-)	+++ ----	70%/30%
CLCuKoV+CLCuM betastellite	10/10 (severe)	10/10 (+)	++++ ----	0%
Un-inoculation	5/5 (asymptomatic)	0/5	----	0%
	Exp. 3			
CLCuKoV+CLCuM betastellite+Syn.CP	11/20 (mild) 3/20 (late to severe) 6/20 (asymptomatic)	14/20 (+) 6/20 (-)	+++ ----	70%/30%
CLCuKoV+CLCuM betastellite	10/10 (severe)	10/10 (+)	++++ ----	0%
Un-inoculation	5/5 (asymptomatic)	0/5	----	0%

++++ = Severe symptoms, +++ = Mild symptoms, ---- = Asymptomatic

symptomatic plants (tolerant) and implied no detection in asymptomatic plants (resistant) against virus inoculation that revealed reduce viral DNA replication. These results showed that co-delivery of pSyn.CP and CLCuKoV/CLCuMuB could not prevent viral spread in the systemic leave.

However, virus DNA accumulation was significantly reduced as shown by Southern blot analysis Lane 2-7 and 9-15; (Fig. 3). This type of results have been reported earlier by using CP of TMV which showed the delayed symptoms after TMV infection to transgenic tobacco plants (19). CP transgene has also been used against two begomoviruses (TYLCV and TLCV) and delayed viral symptoms were observed in transgenic tomato plants after being challenged with the cognate viruses (20, 21).

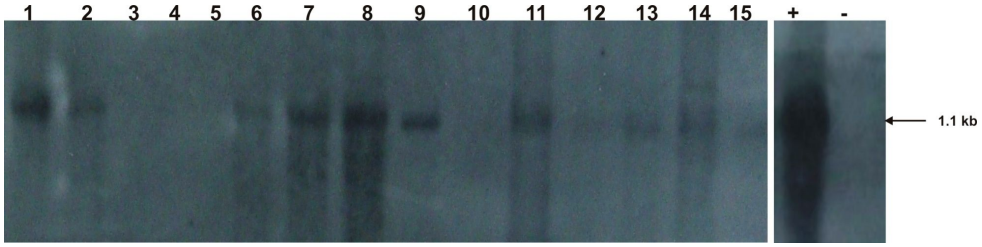

Fig. 3. Southern blots hybridized with CLCuKoV probe showing detection of 1.1 kb fragment of CLCuKoV DNA levels in different lanes. DNA (10ug) was loaded in each lane was extracted from *N. benthamiana* plants inoculated with; CLCuKoV/CLCuMuB showing high virus titer (+), un-inoculated as healthy control plants (-) and plants inoculated with CLCuKoV/CLCuMuB +Syn.CP represent different CLCuKoV levels (1-15).

In this study, the choice of CP gene is based on already described functions like assembly and dis-assembly for viral DNA during its plant cell infection. This assembly and disassembly of capsids is driven by the thermodynamic equilibrium of the CP concentration in cell cytoplasm (10). This uncoating of capsids inhibition or interference might be due to the previous CP concentration that expressed in the cell in which viral infection to be initiated. The expression of CP in transgenic plants results in higher CP concentration that may inhibit the plant from secondary infection. In this way, geminiviral CP as transgene can be used to confer resistance against geminiviruses in transgenic plants. It was proposed that viral CP expressed in transgenic plant cell may inhibit viral infection by preventing capsids disassembly to release viral DNA and cell to cell movement (36, 37).

Syn.CP gene was analysed via *Agrobacterium* transient expression assay in *N. benthamiana*. The *Agrobacterium*-mediated transient expression assay has become well known as a speedy and functional method of analyzing genes in inoculated tissues of plants (38). In spite of some limitations, we can analyze the gene expression in limited time even in 3-4 days that could be taken 4-6 months in regeneration of gene in plant by stable transformation. By transient assay, we can extract the DNA, RNA and protein

from agro-infiltrated leaf patches and can be used for Southern, Northern and Western hybridization blot for further analysis (39). In this transient assay, the expression through agro-infiltration of Syn.CP showed effective control of CLCuKoV/CLCuMuB in plant tissues. Recently Medina-Hernández *et al.* (28) also reported *Agrobacterium* transient expression assay to evaluate two RNAi constructs for developing transient resistance to pepper golden mosaic virus in *N. benthamiana* plants (40). In this context, codon optimized synthetic Rep protein of CLCuBuV is newly reported to deal with resistance against geminivirus (CLCuKoV/CLCuMuB) by Agrobacterium transformation in a transient experiment (41).

Hence it can be concluded that Syn.CP of CLCuBuV may be used to develop broad-spectrum resistance against diverse geminiviruses as Syn.CP gene has higher level of amino acids (aa) sequence homology with other CLCuD begomoviruses' CP. Here also the transient expression assay results have been proved that use of Syn.CP gene could be an effective PDR/PMR strategy for controlling begomovirus infections by interfering in some steps of viral infections including capsid uncoating, cell to cell movement and thermodynamic equilibrium of CP concentration in cell and also to develop resistant transgenic plants. For future aspects, transformation in cotton genome, the codon usage table for *Gossypium hirsutum* was used to design synthetic CP gene for optimal CP expression. Presently Syn.CP construct is being transformed in elite cotton varieties to see whether the results obtained by transient analysis can be obtained in stable transgenic plants. The expression of Syn.CP transgene in transgenic plants may confirm and validate these transient assay findings that could provide better environment to test the resistance against begomoviruses that provided by Syn.CP construct.

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Shahid Mansoor	:	Reviewed the research work and the writeup.
Muhammad Saeed	:	Supervisor. Provided guideline about experimental designs.