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Resistance to cotton leaf curl disease in transgenic tobacco expressing β*C1* gene derived intron-hairpin RNA

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RNA silencing is an adaptive, inducible antiviral defence mechanism in the host against invading viruses. The adaptive antiviral function is characterized by the formation of virus-derived small interfering RNAs (siRNAs) during viral infection. As a counter defense strategy, a number of plant viruses evolve viral suppressors to target antiviral silencing. Cotton leaf curl disease (CLCuD) is a disastrous complex disease caused by presently known five distinct monopartite begomovirus species in association with disease specific betasatellite (DNA-β), which is essential for induction of disease symptoms. Betasatellites are circular, ssDNA molecules that depend on helper viruses for their replication, encapsidation, insect transmission and movement in plants. They possess no appreciable sequence identity to their respective helper viruses except for a conserved hairpin structure necessary for their replication. In this study, siRNA-mediated strategy was applied to generate transgenic tobacco (*Nicotiana tabacum*) against CLCuD infection. A hairpin (hp) RNAi construct capable of expressing dsRNA homologous to the β*C1* gene of Cotton leaf curl Multan betasatellite (CLCuMuB) was designed and developed. A total of eighteen (T_0) and seven (T_1) independent lines of transformed *N. tabacum* plants were developed following *Agrobacterium tumefaciens*-mediated transformation with the β*C1* gene-derived intron-(i)hpRNAi construct. Presence of the potential stretch of βC1 was confirmed by PCR coupled with Southern hybridization. The copy numbers of transgene varied between one and three. The transgenic *N. tabacum* plants of both T_0 and T_1 lines showed high level of resistance following inoculation with viruliferous whiteflies (*Bemisia tabaci*). No symptoms were developed on the five (T_0) and two (T_1) transgenic lines, and remained symptoms free even 90 d post inoculation. The present study has demonstrated that β*C1* gene based RNAi-mediated resistance strategy possesses potential to silence CLCuMuB implicated in the induction of CLCuD symptoms.

Keywords: Silencing suppressor gene, betasatellite, begomovirus, siRNA, cotton leaf curl disease

Introduction

RNA silencing is an evolutionary, conserved mechanism that widely occurs in eukaryotes including plants as posttranscriptional gene silencing (PTGS)[1-3]. Formation of double-stranded RNA (dsRNA) is the main component of the induction of RNA interference (RNAi) silencing pathway. The dsRNAs are processed into 21 to 25 nucleotides, referred to as small interfering RNAs (siRNAs), by DICER—an RNase III-like enzyme. Subsequently, siRNAs guide a nuclease complex, namely, RNA-induced silencing complex, and degrade the target RNAs in a sequence-specific manner. PTGS signal is not restricted to initial cells but it can move to adjacent cells as well as distant parts of the plant. RNA silencing has been demonstrated to have an important antiviral role in plants. As a counter defensive strategy, many plant viruses have evolved viral suppressor proteins of RNA silencing to target different steps of antiviral silencing pathways. The TrAP, C4 and V2 proteins of begomoviruses and the βC1 protein of betasatellites have been shown to possess RNA silencing suppressor activity[4,5].

Cotton leaf curl disease (CLCuD) has become a major risk to cotton cultivation in India[6-8]. The characteristics symptoms of the disease consists of curling of leaves and vein thickening, followed by dark greening and formation of leaf-like structures called enations on the underside of the leaves. This disease is caused by a complex consisting of the monopartite begomovirus *Cotton leaf curl virus* (CLCuV) species of family *Geminiviridae* and associated satellites exclusively transmitted by whitefly (*Bemisia tabaci)*. So far, five distinct begomovirus species along with alfa and betasatellites have been found being associated with

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CLCuD[9]. The genome of CLCuV is monopartite, circular, ssDNA of about 2.8 kb and associated with beta and alphasatellites (Figs 1A & B). Begomoviruses are characterized by having single-stranded, circular, either monopartite or bipartite DNA genomes of 2.5-3.0 kb that are encapsidated in characteristic twinned icosahedral geminate capsids. The replication of begomoviruses, including (CLCuV), occurs *via* rolling circle replication (RCR) mechanism transcribing ssDNA into double-stranded DNA (dsDNA) intermediates, and then using dsDNA as a template mature ssDNA genomes are produced. Gene products of begomoviruses are transcribed *via* intergenic region (IR), which acts as a bidirectional promoter present between the first ORF of the virion sense DNA and the complementary DNA[10]. The IR contains DNA elements that are required for replication of begomoviruses and its transcriptional regulation with the presence of Rep-binding site, TATA boxes, and stem-loop elements. It contains a conserved nonanucleotide (TAATATTAC) sequence and origin of replication (*ori* site) for viral genome. ORFs like *V1* and *V2* are transcribed from virion strand, and *C1, C2, C3, C4* and *C5* from complementary strands. Replication-associated protein (Rep) is encoded by *C1* gene and plays a key role in rolling circle replication and regulation of gene expression of a begomovirus. The *C2* encodes the transcriptional activator protein, a multifunctional protein, which acts as a transactivational regulatory factor and regulates transcription of coat protein and movement protein encoding genes besides suppressing gene silencing. The *C3* encodes the replication enhancer protein (REn), which leads to accumulation of viral DNA followed by symptom development. It interacts with Rep to increase Rep-mediated ATPase activity. The C4 protein suppresses miRNA and siRNAs-mediated RNA silencing. The C5, an uncommon protein for begomoviruses, has been reported to contribute to viral DNA replication but is not essential for viral infectivity. The coat protein V1, a structural multifunctional protein, is involved in genome packaging, insect transmission and systemic spread of virus. The pre-coat protein V2 interacts with silencing suppressor genes (C2 or C4) and maintains high level of viral DNA in infected tissues.

On the basis of genome organization, insect vector and host range, the family *Geminiviridae* is differentiated into nine genera[12] with *Begomovirus* being the largest genus. Members of the genus *Begomovirus* are transmitted by *Bemisia tabaci* vector (Dijkstra & Khan)[13]. In the New World (NW), most begomoviruses have bipartite genomes (DNA-A and DNA-B), while in the Old World both bipartite and monopartite begomoviruses have been reported. Most monopartite begomoviruses are associated with a satellite molecule called betasatellite, which is often required for wild type symptom development in naturally-infected host plants. Betasatellites are ssDNA, circular molecules of *ca*. 1,350 nucleotides that are responsible for development of symptoms in their natural hosts[14]. They depend on its helper virus for its replication, encapsidation, movement within plants and insect-mediated transmission. Interestingly, they do not reveal sequence identity with their helper viruses, except for a conserved hairpin structure (TAATATTAC loop) necessary for their replication.

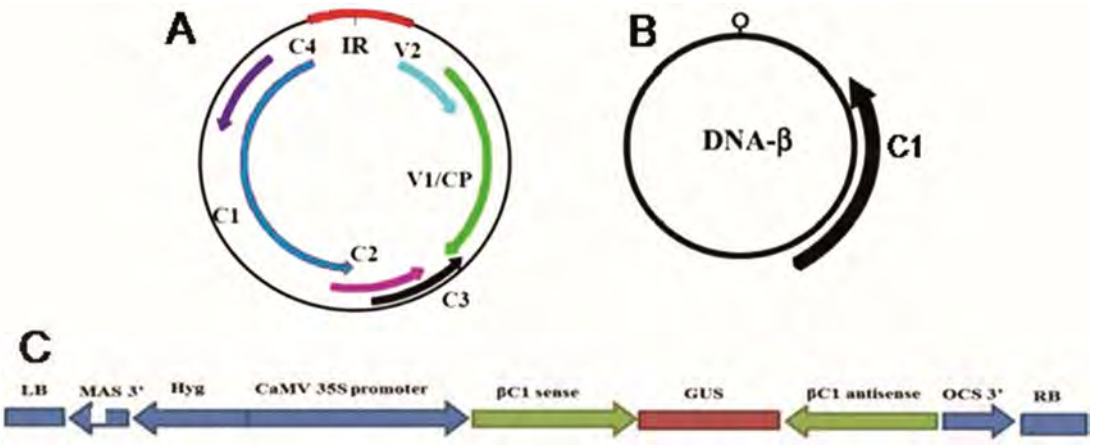

Fig. 1 (A-C) — (a) Schematic diagram of a monopartite begomovirus showing various ORFs; (b). Schematic diagram of a betasatellite ORF C1; & (c). T-DNA map of RNAi vector pFGC 1008 with β*C1* gene in sense and antisense orientation.

Some begomovirus-betasatellite disease complexes are associated with a nanovirus-like component named alphasatellite[15]. Alphasatellites molecules, which are slightly larger than betasatellites, have a single gene, which encodes a Rep protein, an A-rich sequence having a hairpin structure with a nonanucleotide sequence (TAGTATTAC)[14,16]. The role of the alphasatellite is not yet fully understood. However, its Rep protein behaves as silencing suppressor.

Recently, a novel class of DNA satellites (deltasatellites) has been identified in association with NW begomoviruses that depend on a limited range of begomoviruses for maintenance *in planta*[17]. These satellites are approx one quarter the size of a begomovirus genome/genomic component and possess a stem-loop structure with the nonanucleotide forming part of the loop, a putative second predicted stem-loop structure, an A-rich region[18].

RNA interference is a potential approach, which has been successfully applied to generate resistance against geminiviruses[19,20]. With a view to develop transgenic resistance against CLCuD, we have attempted to silence betasatellite associated with CLCuD through RNAi-mediated approach in this study. Tobacco (*Nicotiana tabacum*) plants were successfully transformed by betasatellite (βC1)-based intron hairpin (ihp) RNAi construct through *Agrobacterium tumifaciens*. The level of resistance in the transformed *N. tabacum* plant was evaluated following inoculation with viruliferous *B. tabaci* insect vector. The transformed plants showed high level of resistance and remained virus free even after 3 months of inoculation.

Materials and Methods

Development of ihpRNAi Construct and Its Mobilization into *A. tumefaceins* Strain GV3101

An ihpRNAi vector carrying *C1* gene sequence of Cotton leaf curl Multan betasatellite (CLCuMB) was developed (Fig. 1C). The *C1* gene of CLCuMB was amplified through PCR in sense orientation using oligo primers: Forward S (5′ ggatccatttccctctctgcga-gcttattatc 3′)/Reverse S (5′ actagtggcgcgccgc-tagttccttaatgatagttcattg 3′) and Reverse AS (5′ CTTAGTGCGCAAGTACTT 3′). Appropriate restriction sites were incorporated in oligo primers. The PCR amplicons were ligated in pFGC1008 vector using PCR Cloning kit (Qiagen) according to manufacturer's instructions. For the insertion of β*C1* gene in antisense orientation, the recombinant plasmid carrying *C1* gene were digested with *BamH*I and *Spe*I restriction enzymes and ligated with *C1* gene in antisense orientation to obtain the RNAi construct containing *βC1* gene in sense and antisense orientation with GUS as intron (Fig. 1C), and mobilised into *A. tumefaciens* strain GV3101.

Plant Material and Culture Conditions

Seeds of *N. tabacum* plants were germinated on Petri plates containing moist filter paper at 28±2°C (Fig. 2A). After 2 d, the germinated seeds were transferred onto MS medium supplemented with 15 g/L sucrose, 50 mg/L myoinositol and 0.8% agar, pH 5.8 (Fig. 2B), and maintained at 28±2°C under 16 h photoperiods at light intensity of 60 μmol m^{-2} s^{-1} .

N. tabacum Plant Transformation

Leaf disc explants were taken from 3-4-wk-old *in vitro* grown *N. tabacum* seedlings and precultured on MS medium[21]. The leaf discs were infected with *A. tumifaciens* strain GV3101 harbouring βC1-derived ihpRNAi construct in liquid MS medium for 20 min. These infected explants were cocultivated on solid MS medium supplemented with NAA (0.01 mg/L) and BAP (1 mg/L) and incubated at 25ºC for 48 h in

Fig. 2 (A-I) — Generation of transgenic tobacco (*N. tabacum*) plants expressing β*C1* RNAi gene construct: (A-F) Stages in the regeneration of putative transgenic tobacco plants transformed with β*C1* RNAi gene construct on shooting and rooting medium supplemented with hygromycin, cefotaxime; (G) Putative transgenic lines of tobacco undergoing hardening; & (H, I) T_0 transgenic tobacco plants growing in the glasshouse.

dark. The explants were then washed with distilled water and cefotaxime (500 mg/L), and were cultured on selection medium containing MS salts, NAA, BAP, hygromycin (25 mg/L) and cefotaxime (500 mg/L). Transformed calli were regenerated within 2-3 wk on selection medium. These calli were subcultured on the same medium, where they gave rise to shoots. Individual shoots were then transferred to shooting medium (MS medium containing 1.0 mg/L kinetin, hygromycin (25 mg/L) and cefotaxime (500 mg/L) till definite shoots appeared. For rooting, developed shoots were further transferred onto rooting medium (1/2 MS media with 0.5 mg/L NAA with hygromycin & cefotaxime). The rooted plants were acclimatized in Hogland's solution for 2 wk before transferred to pots containing agropeat and soil (2:1). Ultimately, the established plants were shifted to the glasshouse.

Evaluation of CLCuD Resistance in Transformed (T_0) *N. tabacum* Plants

Total genomic DNA was extracted from 29 hygromycin resistant shoots of transformed (T_0) and untransformed *N. tabacum* plants as described earlier[22]. For the screening of putative transformed shoots, βC1 gene of betasatellite was amplified by PCR using βC1 specific primers. Each PCR was performed in 25 µL of reaction mixture consisting of: 1 µL (150-200 ng) of total plant genomic DNA, 2.5 µL of 2 mM dNTPs, 50 pmol each of primers, 2.5 µL of 10× Taq buffer, 0.25 µL of *Taq* DNA polymerase (5 U/µL) and 17 µL of sterile double distilled water. The PCR conditions included an initial denaturation step at 94°C for 2 min and 25 amplification cycles consisting of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and elongation at 72°C for 1 min. Reactions also included a final elongation step of 5 min at 72°C. The amplified products were electrophoresed on 1% agarose gel and visualized under UV light by ethidium bromide staining.

PCR Based Detection of β*C1* Gene in Transformed (T_1) *N. tabacum* Plants

T_1 *N. tabacum* plants were raised under glasshouse conditions from the seeds collected from five PCR positive T_0 transformed *N. tabacum* plants. Total genomic DNA was isolated from T_1 transformed *N. tabacum* plants and subjected to βC1 amplification by PCR as discussed in the previous section.

Southern Blot Analysis

To evaluate the integration of the transgene and its copy numbers in the genome of the T_1 transgenic *N. tabacum* lines, Southern hybridization was performed. The plasmid DNA having ihpRNAi cassette was used as positive control and DNA isolated from leaves of non-transformed *N. tabacum* plant was used as the negative control. Out of eighteen, seven lines were subjected to Southern hybridization. About 10 µg of genomic DNA isolated from seven independently transformed plants (T_1) and digested with *Bam*HI was electrophoresed on 0.8% agarose gel and transferred to Hybond-N+ membrane using standard protocols (Sambrook *et al*)[23]. The blot was hybridized with a random primed non-radiolabelled probe developed from the DNA fragment amplified from the *C1* gene of CLCuMB genome using DIG DNA labeling detection kit (Roche Diagnostics, USA) as per manufacturer's instructions.

Evaluation of CLCuD Resistance in Transgenic *N. tabacum* (T_1) Lines

In order to check the level of CLCuD resistance in transgenic *N. tabacum* plants, they were inoculated with *B. tabaci* harboring CLCuCV as essentially described earlier[11]. Healthy (virus free) *B. tabaci* were fed on the CLCuV-infected cotton plants for a period of 24 h. A total of seven (T_0) and three (T_1) transgenic lines at 4-5 leaf stage were exposed to viruliferous *B. tabaci* (50-60 numbers/plant) for a period of 3 d. Non transformed *N. tabacum* plants were also inoculated with viruliferous *B. tabaci*. The plants were maintained in glasshouse and examined for several weeks to check the development of symptoms.

Detection of CLCuV in Transgenic *N. tabacum* (T_0) Plants

Total viral genomic DNA was isolated from transgenic as well as nontransformed *N. tabacum* plants, following inoculation with viruliferous *B. tabaci* using DNaeasy mini kit (Qiagen, USA). It was subjected to PCR amplification employing *Begomovirus* DNA A specific primers[24].

Results

Agrobacterium-mediated Transformation of *N. tabacum* Plants

An ihpRNAi construct containing β*C1* gene in sense and antisense orientation separated by an intron for producing a hairpin loop-like structure was designed and developed (Fig. 1C). *A. tumefaciens* strain GV3101 was transformed with pFGC1008 vector carrying the β*C1* gene based ihpRNAi construct. The mobilization of the RNAi construct into *A. tumifaciens* strain was confirmed by colony PCR yielding DNA fragment of desired size

(~350 bp). A total of 100 leaf discs were treated with *A. tumifaciens* suspension. Only 35 numbers of agro-transformed leaf discs showed callus induction and hygromycin-resistant shoots were regenerated from only 29 callus-induced leaf discs (Fig. 2).

PCR Analysis of Transformed (T_0) *N. tabacum* Plants

Initial screening of the putative transgenic plants was done by PCR based amplification of the β*C1* gene using specific oligo primers. Of 19 plantlets screened, 18 of them showed the presence of DNA fragment of expected size (~350 bp) corresponding to that of positive DNA. PCR amplification thus demonstrated the presence of the desired DNA fragment in the transgenic plantlets (Fig. 3). There was, however, no amplification of the DNA isolated from the non-transformed control plants.

Southern Blot Analysis

PCR positive transgenic (T_0) plants were further confirmed for the integration of the βC1 sequence into the plant genome through Southern hybridization. The non-radiolabelled probe was prepared from the βC1 of CLCuMB. Then β*C1* gene based DNA probe was hybridized with *Eco*R1 digested genomic DNA isolated from the transformed *N. tabacum* plants. Hybridization gave positive signals in all the seven PCR positive transformed *N. tabacum* plants (Fig. 4). The copy number of the integrated transgene in the transgenic *N. tabacum* plants varied among different lines. Three lines (2, 3, 4 and 6) possessed the transgene in three copies, while the plants of the event (line 5) had two copies. Four hybridizing bands were seen in the plants of event (line 1). A single copy of the transgene was witnessed in the plants of event number (line 7). The signals were absent in the non-transformed control plants.

PCR Based Detection of β*C1* Gene in Transformed (T_1) *N. tabacum* Plants

Total genomic DNA was isolated from T_1 transformed *N. tabacum* plants and subjected to βC1 amplification by PCR as discussed in the previous section. All the five T_1 progenies showed the presence of DNA fragment of expected size (~350 bp) corresponding to that of positive DNA (Fig. 5). There was, however, no amplification of the DNA isolated from the non-transformed control plants.

Evaluation of CLCuD Resistance

Level of resistance in seven T_0 and three T_1 *N. tabacum* transgenic lines was evaluated by inoculating the transgenic plants with viruliferous *B. tabaci* . Following virus inoculation, transgenic plants were observed for the appearance of disease symptoms for 3 months. No symptoms could be developed on the five transgenic (T_0) lines, while two lines (2 & 4) showed delayed symptoms (Fig. 6) as

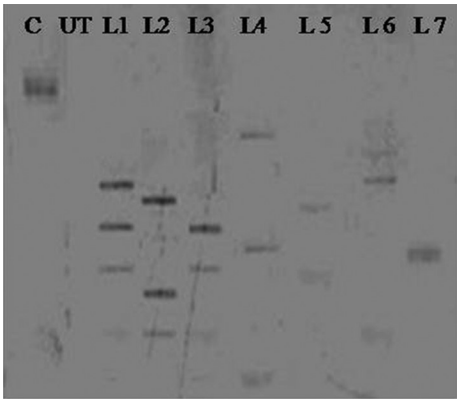

Fig. 4 — Southern hybridization of total DNA isolated from T_0 transformed tobacco (*N. tabacum*) plants for stable integration of the β*C1* based RNAi construct. 1-3 copies of the construct can be seen. [UT, Untransformed; C, Positive; L1-L7, Test samples from T_0 tobacco plants].

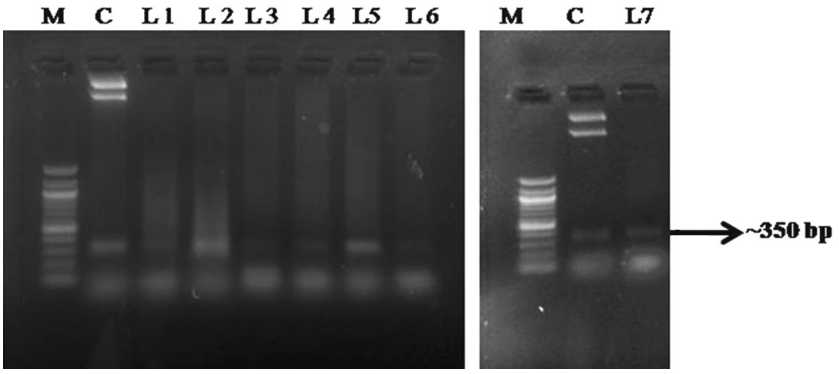

Fig. 3 — PCR amplification of β*C1* gene fragment (~350 bp) from total DNA isolated from transformed T_0 tobacco (*N. tabacum*) plants: M, 100 bp Gene ruler; C, Control, β*C1* RNAi plasmid DNA; Lanes L1-L7, PCR amplified DNA fragments derived from transgenic tobacco transformed with β*C1* RNAi construct.

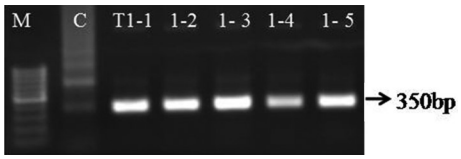

Fig. 5 — PCR amplification of β*C1* gene fragment (~350 bp) from total DNA isolated from transformed T_1 tobacco (*N. tabacum*) plants: M, 100 bp Gene ruler; C, Control, βC 1 RNAi plasmid DNA; Lanes T_1-1 to $_1$-5, Showing PCR amplified DNA fragments derived from transgenic T_1 tobacco transformed with β*C1* RNAi construct.

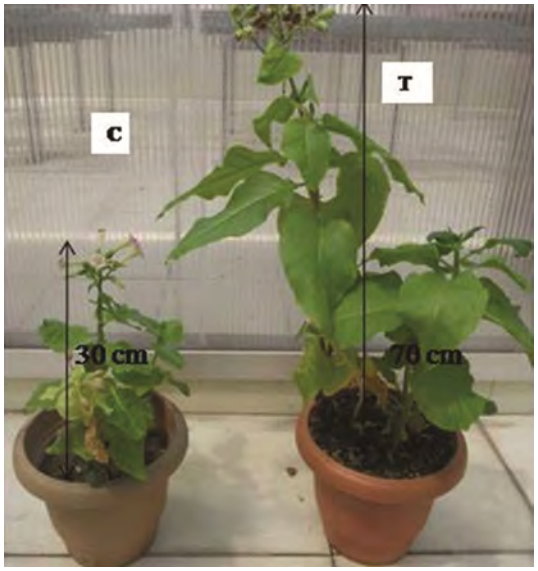

Fig. 6 — Whitefly mediated inoculation on non-transformed control (C) and transgenic tobacco (*N. tabacum*) (T) plants. Plants were transformed with β*C1* derived RNAi construct. Symptom development on leaves of control tobacco plants (C); No symptoms developed on leaves of transgenic tobacco plants (T).

compared to the control plants. In the T_1 progenies, lines 3 and 4 remained free of CLCuD symptoms, and line 5 showed delayed symptoms (Fig. 6) as compared to the control plants.

Discussion

CLCuD is the most important biotic constraint to the cotton cultivation in the Indian subcontinent. It is induced by one of five presently known distinct begomoviruses in association with CLCuMB. The associated betasatellite induces disease symptoms in cotton and other susceptible hosts. It encodes a multifunctional protein designated as C1, which is responsible for cell-to-cell moment, induction of disease symptoms in its primary host and inhibits defense pathway of the host, and thereby suppresses PTGS[25-27].The suppression of such a pathogenicity determinant betasatellite molecule can effectively provide resistance to the devastating cotton disease.

Control of CLCuD *via* conventional methods has not been met with significant success. Lack of natural genetic resistance sources in cotton, frequent recombination among geminiviruses and breakdown of CLCuD due to emergence of resistance breaking recombinant strain of CLCuV have prompted plant virologists to apply RNAi based strategy against CLCuD. RNAi constructs encoding self complementary ihpRNA has shown to be capable of generating post-transcriptional silence to undetectable levels of the targeted mRNA transcript. The application of siRNAs has become a powerful tool to specifically down regulate the expression of genes[28]. The RNAi appears to hold greater potential as an effective approach to prevent the losses incurred by CLCuD in cotton[20].

In this study, it was hypothesized that down regulating the pathogenecity determinant silencing suppressor β*C1* gene may lead to the suppression of symptoms in plants transformed with β*C1* gene[29]. The ihpRNAi construct derived from β*C1* gene was transferred into *N. tabacum* plants by *Agrobacterium*-mediated transformation. Then the transgenic *N. tabacum* plants were tested for the integration, copy number and expression of the RNAi gene as well as the CLCuD symptoms. *N. tabacum* plant was chosen because it can be easily cultured *in vitro*, has high regeneration potential and efficient transformation frequency. Binary vector pFGC1008 was designed and developed that carried β*C1* gene in sense and antisense orientations with sequence of GUS as an intron. The β*C1* gene with GUS was driven by 35S CaMV promoter for continuous stable expression. pFGC 1008 also contains a bacterial selection marker gene that provides resistance against chloramphanicol and a plant selection marker for hygromycin resistance.

The ihpRNAi construct was transferred into *N. tabacum* plants by *Agrobacterium*-mediated transformation. CLCuD resistance was evaluated following challenge inoculation of transgenic *N. tabacum* plants. A total of ten transgenic (T_0=7, T_1=3) lines were tested for resistance to CLCuD infection. Seven lines showed no disease symptoms, while two lines exhibited delayed symptoms.

The β*C1*-derived RNAi resistance strategy developed in tobacco plants in the present study could be extended to achieve CLCuD resistance in cotton and other important agricultural crops susceptible to CLCuMB. It has the potential to generate

'Make in India' *beta cotton* against the disastrous CLCuD infection in the Indian subcontinent. Though RNAi-mediated resistance being the sequence based strategy, a high degree of sequence specificity between small RNA (si/mi RNA) and viral target gene and frequent recombination in geminiviruses remain serious drawbacks to this transgenic approach.

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