RESEARCH

Engineering resistance against cotton leaf curl disease with the application of antisense RNA technology targeting complementary sense transcript βC1

Abhinav Kumar[1,4]*, Javed Ahmad[2], Jawaid A. Khan[1,3]

[1.]Molecular Virology Lab, National Botanical Research Institute, Rana Pratap Marg Lucknow-226001, India
[2.]College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia
[3.]Department of Biosciences, Jamia Millia Islamia (a Central University), New Delhi, India (Present address)
[4.]Dept of Biotechnology, IILM-College of Engineering and Technology, Greater Noida, UP-201306

*Corresponding author E-mail: abhibiot2k4@gmail.com.

ABSTRACT

Begomoviruses (family *Geminiviridae*) are widely distributed plant viruses and number of infected plants by this group of viruses is rapidly increasing due to adaptation of its vector in different geographical conditions and ability to recombine with other Geminiviruses. Begomovirus associated betasatellite DNA has a complementary sense transcript β*C1*, which is responsible for symptom induction. Antisense construct targeting β*C1* gene was developed in plant expression vector pBI121 and transgenic *Nicotiana tabacum* plants were raised using this construct. No visible alteration was observed in the transgenic plants. Upon inoculation with viruliferous whiteflies (*Bemisia tabaci*) the β*C1*-anti transgenic plants showed resistance against cotton leaf curl disease and no visible symptom was observed even after 7 weeks post inoculation. Furthermore, the key component (small RNA) was isolated from the tested plants and hybridized with suitable probe. Dark band appeared on the membrane which indicates the production of small RNAs from resistant plants in response to virus attack which was absent from control plants. This study highlighted antisense technology as an effective tool to develop resistance transgenic plants against cotton leaf curl disease (CLCuD).

KEYWORDS: Cotton leaf curl Virus. Antisense. siRNA. Whitefly. Resistance.

Citation: Kumar A et al (2020) Engineering resistance against cotton leaf curl disease with the application of antisense RNA technology targeting complementary sense transcript βC1. Research Reports. doi:10.9777/rr.2020.10004

INTRODUCTION

Plants viruses are important pathogens and may cause severe loss to agriculture crops thus directly affecting the economy of a country. The virus species which comes under family *Geminiviridae* is among the most important plant viruses. This class of virus is characterized by single-stranded DNA genome, encapsidated within twinned quasi-icosahedral particles (Mansoor et al., 2003). Geminiviruses consists of nine sub groups, *Begomovirus*, *Curtovirus*, *Topocuvirus*, *Mastrevirus*, *Turncurtovirus*, *Capulavirus*, *Eragrovirus*, *Grablovirus* and *Becurtovirus*, based on genome organization, insect vector and host range (Zerbini et al., 2017). Among them Begomovirus is geographically most distributed worldwide and is transmitted by whitefly *Bemisia tabaci* (*B. tabaci*). Begomovirus have either monopartite or bipartite (DNA-A and DNA-B; genome size of about 2.5-3.0 kb) genome organization, encapsidated within twinned quasi-icosahedral particles (Mansoor et. al., 2003; Kumar et al., 2010) and some species of this virus are reported to be associated with satellite molecules viz betasatellite and alphasatellite DNA. The betasatellite and alphasatellite molecules are having size approximately 1.3 kb and the former is required for symptom induction (Briddon et al. 2001).

Cotton is an important cash crop in India and the current export value of cotton and its related products stood at 36.2 billion dollars. The production of cotton is severely affected by several pathogens and pests and among them leaf curl disease ranks very high in terms of destruction. This disease is caused by *Cotton leaf curl virus* (CLCuV), a type member of Begomovirus and is whitefly transmitted *(B. tabaci)*. This disease has a major negative impact on the production of cotton in our neighbor country Pakistan as well, and they registered some 30% loss worth US$5 billion. In India, there is no such monitoring of loss, but every year there are some 20-30% losses in cotton growing areas in Punjab and Rajasthan due to this disease (data collected through field survey conducted by us and conversation with farmers). During 2015-2016, an outbreak of this disease in Indian Punjab region witnessed severe loss in the cotton production (70-100%), and several farmers committed suicide, which had caught attention world-wide. Also, in India, some areas particularly in Punjab, excessive amount of insecticides are used to kill whiteflies and it is feared that this might change the texture of soil in near future. Several strategies to control CLCuV have been employed but they account only limited success. However, pathogen derived resistance (PDR) mediated strategies (either protein or RNA mediated) are effective against plant viruses (Baulcombe DC., 1996; Shelly et al., 2005; Hashmi et al., 2011). Among PDR approaches, modern tool such as antisense RNA approach is considered as an ideal approach against this class of virus (Sinha et el., 2017).

Antisense RNA which is complementary to target RNA (say mRNA) has a capability to hybridize the messenger RNA, a tool to natural and artificial gene regulation by silencing the suppression of the corresponding gene (Erickson and Izant, 1992). The targeted gene inactivation by interfering and inhibiting the translation of RNA has now become a prominent tool in applied molecular biology. The duplex RNA formation due to sense- antisense interaction also led to the formation of siRNA (Asad et al. 2003). This strategy has been successfully applied in the development of transgenics against different Geminiviruses (Shepherd et al. 2009, Sinha et al., 2017).

In this study we provide antisense RNA approach against Cotton leaf curl disease targeting betasatellite molecule. This satellite molecule has a

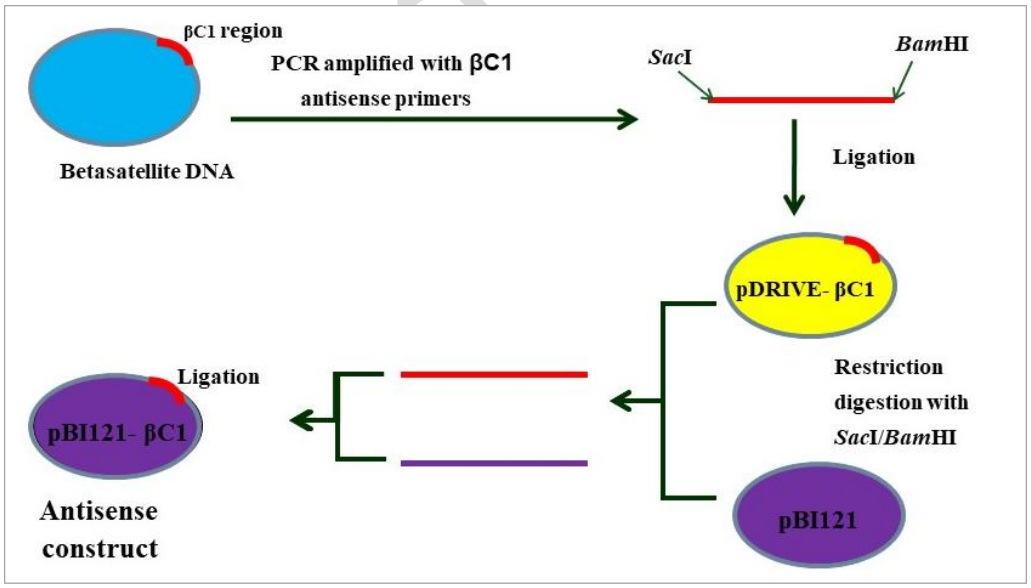
complimentary sense transcript, βC1 which is actually determinant of both pathogenecity and suppression of gene silencing (Briddon and Stanley, 2006). The betasatellite DNA was amplified from total DNA isolated from symptomatic leaves collected from Sri-Ganganagar distt. of Rajasthan, cloned and further analyzed. Antisense construct of DNA beta molecule targeting βC1 gene was developed and transgenic *N. tabacum* plants were raised following standard protocols. In order to check the resistance of antisense plants, these were inoculated with whiteflies (*B.tabaci*) harboring begomovirus, and after 7 weeks the tested plants were symptomless.

METHODS

Designing of βC1 antisense constructs

Based on the sequence submitted into the GenBank (#GQ249185) primer pair βC1 anti-F (5'-CGAGCTCGATGACAACGAGCGGAAC-3') and βC1anti-R (5'-CGGGATCCCGGGAATTTAAACGGTGAAC-3') were designed and employed to amplify antisense strand of βC1. Forward and reverse primers contain *Sac*I and *Bam*HI restriction sites respectively (underlined).The PCR conditions used: 94°C/3min for one cycle as initial denaturation, 94°C/45 seconds 50°C/30 seconds 72°C/90 seconds for 30 cycles and a final amplification 72°C/5min. Amplified product was first cloned into pDRIVE vector (Qiagen), following manufacturers instruction then released using restriction enzyme pair (*Sac*I/*Bam*HI) and further introduced into similar site of the binary vector pBI121 (Fig 1) to develop antisense construct. The antisense construct was further sequenced to identify possible mutation. Sequence analysis showed that there is no mutation introduced into the clone to be used to transform *Nicotiana tabacum* (*N. tabacum*).

Fig 1. A scheme for construction of expression module using pBI121 vector for the stable transformation of *Nicotiana tabacum*. The gene βC1 was amplified using specific primers, ligated into pDRIVE (Qiagen, Gmb) to make pDRIVE- βC1. The βC1 was further digested with enzymes *Sac*I and *Bam*HI, and introduced into the same restriction site of pBI121 to become pBI- βC1 construct.



Transformation of *N. tabacum*

Antisense construct was mobilized into *Agrobacterium tumefaciens* strain GV3101 following improved freeze-thaw method (Hofgen and Willmitzer, 1988). The transconjugants were selected on gentamycin (30μg/ml) and kanamycin (50μg/ml). Transgenic *Nicotiana* plants were developed by tobacco leaf disc using co-cultivation method (Horsh et al., 1985). Callus tissues were allowed to grow on MS- medium supplemented with antibiotic Kanamycin (50μg/ml). After 2 weeks, the callus tissue was differentiated by inoculating them into differentiating medium (shooting medium) with supplementary light corresponding to 16h of day length.

Analysis of transgenic plants.

Total DNA isolated from transgenic tobacco plants developed with antisense constructs, were subjected to PCR using β*C1*anti primers mentioned in materials section. The amplified products (*Ca*. 350 bp) were run on agarose gel and transferred by capillary action to Hybond N+ membrane (Amersham Bioscience), and then UV cross linked for 7 min. The membranes were prehybridized at 42°C (1 h) followed by hybridization with the radiolabeled probe prepared from β*C1* antisense genes respectively at 65°C overnight. It was washed twice with 2X SSC, 0.1% SDS and 1X SSC, 0.1% SDS for 5 and 15 min respectively, at room temperature followed by another wash with 1X SSC, 0.1% SDS at 65°C for 15 min. The blot was exposed to Fuji film for autoradiography.

Segregation analysis

T_0 transgenic plants were taken up to T1 generation. Approximately 20–25 seeds from each progeny line were germinated under controlled conditions, and were tested by PCR for the presence of β*C1*-antisense gene.

Whitefly inoculation to antisense plant.

Resistance capacity of developed β*C1*-anti transgenic tobacco plants was tested by inoculating viruliferous whiteflies (*B. tabaci*). The whiteflies were also fed on to healthy tobacco plant. A total of nine independently transformed plants were obtained and five lines were analyzed for the generation of resistance against CLCuV. Approximately 30-40 flies were allowed to feed on each leaf, and were kept in controlled conditions (in culture room at 25°C). After three weeks (21 days), insecticides were applied on plants (both control and transgenic) and kept in strict supervision to check the possibility of symptom induction.

Small RNA Northern blot.

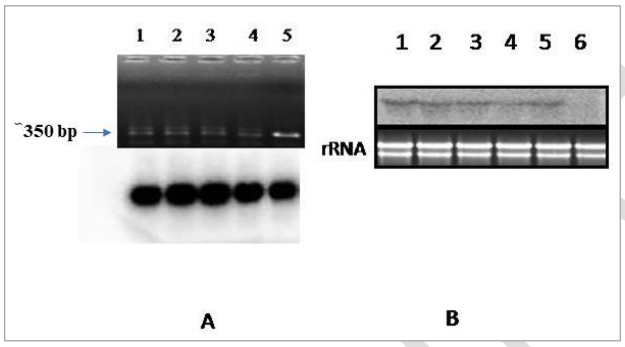
Small RNA was isolated from resistant transgenic plants using mirVana™ microRNA (miRNA) isolation kit (USA) following manufacturers instruction. 15 microgram of low molecular weight RNA was separated in 15% Tris-borate-EDTA Urea acrylamide gel and transferred onto nylon membrane (Amersham Biosciences). Blotted RNA was hybridized at 42 °C using αP-dCTP labeled with β*C1*-antisense probes (Sambrook and Russsel, 2001).

RESULTS AND DISCUSSION

Verification of transgene (βC1-antisense) integration in putative transgenic plants

Transgenic tobacco plants (representing β*C1* antisense construct), were tested for the potential presence of the gene. Total DNA was isolated from transgenic tobacco leaves and subjected to PCR using same primer pairs for antisense. Sharp bands of PCR amplicon having band size of ~350 nts appeared which was hybridized with β*C1* gene (anti) as a probe. Dark bands appeared which confirms the presence of antisense gene in the transgenic tobacco plants (Fig 2A).

Fig 2A, B. Polymerase Chain Reaction (PCR) and Southern blot analysis of transgenic *Nicotiana tabacum*. Total DNA was isolated from the transgenic plants and PCR was performed employing primers viz. βC1 anti for and βC1 anti rev (lane, 1-4). Lane 5 is positive control. Expected size of DNA fragments (*Ca*. 350 bp) were visualized on the gel (upper portion of fig 2 A), which was hybridized with α-P^{32}-labelled DNA probe representing βC1anti. The dark bands on the blot indicates strong hybridization signal between the PCR products and radiolabeled probe (Lower portion of fig 2B). Fig. B Northern blot analysis of transgenic plants transformed with βC1 antisense. Lane 1-5 are small RNAs from positive plants confirmed through Southern and lane 6 is negative control (RNA from non-transgenic *N.tabacum*).



To access the resistance capacity of transgenic plants, all the nine T_0 lines were challenged with whiteflies harboring *cotton leaf curl virus*. The flies were allowed to feed for 21 days inside the chamber under controlled conditions of light and temperature. Further, the flies were killed by applying 1% Confidor (insecticide) and the plants were kept inside culture room maintained at temperature 25^0 C. The plants were consistently kept under supervision for several weeks to check the possibility of symptom induction. None of the plants showed any kind of observable phenotypic change. The control plant showed typical geminiviral symptoms (Fig 3). Further, transgenic plants were tested for the possible presence/replication of begomovirus. To this end, total DNA was isolated from the test plants and control plant following CTAB (cetyltrimeth ylammonium bromide) method and amplified using primer pair as described (Kumar et al., 2011). Desired bands of size 1.2 and 1.6 kb were obtained from control plant, while no amplification has been observed from transgenic plants (data not given). The results here suggested that Begomovirus did not replicated inside test plant while it replicated in control plant under similar conditions. The results here thus demonstrated that anti β*C1* gene targets and selectively suppress the expression of geminiviral gene.

Fig 3. To check the efficacy of raised transgenic plants, they were inoculated with the whiteflies harboring begomovirus. The photograph of A, B (antisense) transgenic and C non- transgenic tobacco plant (control) after whitefly inoculation. After 4 weeks non-transgenic plant shows leaf deformation (F), while transgenic plant did not show any notable symptom (D, E).

Antisense RNA strategies have been successfully used since 1991 to target and degrade geminiviral genes (Day et al., 1991). After then several transcripts have been targeted such as replicase gene of TGMV, TYLCV, TGMV and BCTV (Bendahmane and Gronenborn, 1997) etc. In case

of *cotton leaf curl virus* several genes such as Rep, TrAP, and REn have been targeted (Kumar and Khan, 2019). The transcript βC1 was also targeted successfully in one of the studies by Sohrab et al., 2016, but small RNA isolation was not demonstrated. Recently this strategy was used against *Croton leaf curl mosaic virus* by Sinha et al., 2017.

Northern Blotting

The isolated siRNA from tested transgenic lines (antisense) was tested through Northern blot by using the probe corresponding to βC1. The appearance of a hybridization signal after radiography indicated the presence of transgene-derived siRNA accumulation in symptomless plants (Fig 2B). The results demonstrated that engineered βC1 gene targets/selectively suppress the expression of the geminiviral gene.

As the antisense RNA molecule is complementary to a particular mRNA, base pairing occurs and this duplex RNA is supposed to induce Post transcriptional gene silencing (PTGS) by the formation of siRNAs. This duplex RNA is supposed to be diced by plant Dicers and production of siRNA takes place.

To strengthen this study, we further analysed the potential of the resistant transgenic lines (T1 and T2) developed using antisense constructs in natural conditions. We kept few plants in the experimental fields of CSIR-National Botanical Research Institute, Lucknow, which were used to grow cotton plants (Table 1). The field had a significant presence of whiteflies. Even after 2 months, the transgenic plants were symptomless; however, only one antisense plant of T2 generation showed loss of resistance. This may be due to the high inoculum pressure of the virus titre in natural conditions.

Table 1.

Event No. of lines and Plants generated		number of plants tested (only 5 lines tested in glass house)		Field testing - number of plants	
	T_0 (9) lines, 5 plants/line)	T_0 (1 plant/line)	T_1 (1 plant/line)	T_1	T_2
Antisense	45	05	05	5	5

The betasatellite DNA is essentially associated with CLCuD. Our data showed that the gene silencing approach using βC1 gene is a more favorable approach to combat CLCuD. This resistance is mediated by RNA silencing. Only one tobacco plant developed using the antisense construct showed loss of resistance due to a high inoculum pressure. The results of this study can be used by cotton farmers in India to grow cotton resistance against whitefly-transmitted diseases.

ACKNOWLEDGEMENT

Authors are thankful to Director, National Botanical Research Institute (CSIR-NBRI, Lucknow) for providing necessary facilities. First author is thankful to CSIR-UGC for fellowship and Dr. Aminuddin for his support and Dr. B.D. Ajmera for culture collection.

Conflicts of interest

The authors declare no conflict of interest.

Authors' contributions

Author AK conducted experiment and Manuscript writing, JA helped in experiment and author JAK contributed in designing of experiment.

REFERENCES

Asad, S., Haris, W.A.A., Bashir, A., Zafar, Y., Malik, K.A.A., Malik, N.N., and Lichtenstein, C.P. (2003). Transgenic tobacco expressing Gemini viral RNAs are resistant to the serious viral pathogen causing cotton leaf curl disease. Arch. Virol. *148*, 2341–2352

Bendahmane,M., and Gronenborn, B. (1997). Engineering resistance against Tomato yellow leaf curl virus (TYLCV) using antisense RNA. Plant Mol. Biol. *33*, 351–357.

Briddon, R.W., and Stanley, J. (2006). Sub-viral agents associated with plant-infecting single-stranded DNA viruses. Virology *344* (1), 198–210.

Briddon, R.W., Mansoor, S., Bedford, I.D., Pinner, M.S., Saunders, K., Stanley, J., Zafar, Y., Malik, K.A., and Markham, P.G. (2001). Identification of DNA components required for induction of cotton leaf curl disease. Virology *285*, 234-243.

Day, A.G., Bejarano, E.R., Buck, K.W., Burrell, M., and Lichtenstein, C.P. (1991). Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus Tomato golden mosaic virus. Proc. Natl. Acad. Sci. U.S.A. *88*, 6721–6725.

Erickson, R. P., and Izant, J. G. (eds.) (1992). Gene Regulation: Biology of Antisense RNA and DNA (New York, Raven Press) 1-10.

Hashmi, J.A., Zafar, Y., Arshad, M., Mansoor, S., and Asad, S. (2011). Engineering cotton (Gossypium hirsutum L.) for resistance tocotton leaf curl disease using viral truncated AC1 DNA sequences.Virus Genes *42*, 286–296.

Hofgen, R., and Willmitzer, L. (1988). Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res.*16*,9877.

Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R.T. (1985). A simple and general method of transferring genes into plants. Science *227*, 1229-1231.

Kumar, A., Kumar J., and Khan, J.A. (2010). Sequence characterization of Cotton leaf curl virus from Rajasthan: Phylogenetic relationship with other members of Geminiviruses and detection of recombination. Virus Genes *40*, 282-289.

Kumar, A., Snehi, S.K., Raj, S.K., Kumar, J., and Khan, J.A. (2011). Association of *Cotton leaf curl Burewala virus* and an alphasatellite with cotton leaf curl disease in India. New Disease Reports*24*,18.

Kumar, A., and Khan, J.A. (2019). Geminivirus resistance strategies (in Geminiviruses: Impact Challenges and Approaches). DOI 10.1007/978-3-030-18248-9.

Mansoor, S., Briddon, R. W., Zafar, Y., and Stanley, J. (2003). Geminivirus disease complexes: an emerging threat. Trends in Plant Science *8*, 128-134.

Sambrook, J., Russel, D.W., (2001). Molecular cloning: A laboratory manual, 3rd edn (Cold Spring Harbour Laboratory, New York).

Saunders, K., Bedford, I.D., Briddon, R.W., Markham, P.G., Wong, S.M., and Stanley, J. (2000). A novel virus complex causes Ageratum yellow vein disease. Proc. Natl. Acad. Sci. USA *97*,6890-6895.

Shepherd, D.N., Martin, D.P., and Thomson, J.A. (2009). Transgenic strategies for developing crops resistant to Geminiviruses. Plant Science *176*,1-11.

Shelly, P., Kushwaha, C.M., Mishra, A.K., Singh, V., Jain, R.K.,and Varma, A. (2005). Engineering tomato for resistance to tomato leafcurl disease using viral rep gene sequences. Plant Cell Tissue OrganCult. *83*, 311–318.

Sinha, V., Sarin, N.B., Bhatnagar, D. (2017). The efficacy of antisense-based construct for inducing resistance against Croton yellow vein mosaic virus in Nicotiana tabacum. Virus Genes*53*(6), 906–912.

Sohrab, S.S., Kamal, M.A., Ilah, A., Husen, A., Bhattacharya, P.S., and Rana D (2016). Development of Cotton leaf curl virus resistant transgenic cotton using antisense ßC1 gene. Saudi J Biol Sci. *23*(3), 358-362.

Zerbini, F.M., Briddon, R.W., Idris, A., Martin, D.P., Moriones, E., Navas-Castillo J., Rivera-Bustamante, R., Roumagnac, P., Varsani, A. (2017). ICTV virus taxonomy profile: Geminiviridae. J Gen Virol *98*,131–133

Baulcombe, D.C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. Plant Cell *8*, 1833–1844.