Final Report of the work done on the UGC Major Research Project

1. Project report : Final

2. UGC Reference No. : F. No. 43-478/2014 (SR)-18.11. 2015
3. Period of report : 01.7. 2015 to 30.06. 2018

4. Title of research project : Designing, development and studying the role of Cotton leaf curl virus-AC2 gene derived RNAi constructs

5. (a) Name of the Principal Investigator: Prof. Jawaid Ahmad Khan

(b) Deptt. : Department of Biosciences

(c) University/College where work has progressed: Jamia Millia Islamia,
Jamia Nagar, New Delhi 110025

6. Effective date of starting of the project: 01.4. 2016

7. Grant approved and expenditure incurred during the period of the report:

a. Total amount approved : Rs. 15, 20, 000/-

b. Total expenditure : Rs. 13, 65, 788/-

c. Report of the work done : (Please attach a separate sheet)

i. Brief objective of the project

Cotton, one of the most important crops of India, ranks first in terms of its area under cultivation in the world. For the last two decades, cotton leaf curl disease (CLCuD) has greatly hampered the cultivation and productivity of cotton crop in India. CLCuD has been reported to cause approximately 70% losses in cotton production. The disease is caused by one of the whitefly-transmitted Cotton leaf curl virus species (CLCuV, genus *Begomovirus,* family *Geminiviridae*) in association with ssDNA satellite molecules. Till now, control of CLCuD as well as its whitefly insect vector (*Bemisia tabaci*) through cultural approaches and chemicals, have not yielded desired results. Furthermore, sources of natural resistance against begomoviruses are limited, and often difficult to introgress into crop varieties with desirable agronomic characters due to various compatibility barriers. There is an urgent need to introduce advance

and more potent strategies, such as the one driven by an RNAi-based approach, to safeguard cotton crop against CLCuD led devastation. As a first step to induce RNAi-mediated resistance against CLCuD infection, it was proposed to transform tobacco model plant with RNAi constructs carrying silencing suppressor gene viz. AC2 of CLCuV genome for the development of effective and durable resistance against CLCuD. This will reduce or prevent replication of viral DNA and the appearance of CLCuD symptoms. Therefore, RNAi-based DNA constructs targeting AC2 gene (suppressor of host defense) of CLCuV was designed and developed. And, its role against CLCuD infection was studied in tobacco model plant. Furthermore, this RNAi-based strategy is being extended to cotton to combat CLCuD.

ii.Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication)

Please see final Report-Annexure-I for details.

1. Alam SF, Ara H, Khan JA (2019). RNAi-mediated resistance against cotton leaf curl disease in tobacco and cotton plants (MS under preparation).
2. Alam SF, Khan JA (2018). Development of transgenic cotton (Gossypium hirsutum) plants against *Cotton leaf curl Multan virus*. Abstract 151, paper presented in National Conference on "Bio-Intensive Approaches in Plant Protection and their Socio-economicImpacts held at Department of Plant Protection, A.M.U, Aligarh (29-30 October, 2018) (Oral presentation).

iii. Has the progress been according to original plan of work and towards achieving the objective. if not, state reasons

Progress was made as per the original plan of work.

iv. Please indicate the difficulties, if any, experienced in implementing the project:
First grant of the project was received late. Therefore, the effective date of implementing the project was 01.4. 2016.

V. If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet.

N.A.

VI. If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission.

Summary

Cotton leaf curl disease (CLCuD) is a devastating disease of cotton (*Gossypium hirsutum*) causes enormous losses to cotton. It has become a major challenge for the cultivation of cotton crop in Indian subcontinent. It is induced by complexes of five distinct cotton-infecting monopatite begomoviruses in association with satellite molecules called as beta–, and alpha-satellite molecules. RNA interference (RNAi) is a potential approach that has been successfully tested to develop resistance against begomoviruses. In this research project, RNAi which acts as a natural anti-viral defense system, was applied to combat CLCuD infection. In plants, RNAi is achieved by the introduction of a transgene that expresses a part of the viral genome leading to the development of resistance in the plant against that viral infection. As a first step to generate resistance against CLCuD infection, an RNAi construct based on *AC2* gene of Cotton leaf curl virus (CLCuV) was designed and developed. Leaves from diseased cotton plants showing CLCuD characteristic symptoms (curling of leaves, darkening and thickening of veins, enations on the abaxial side of leaves) were collected. Total genomic DNA was isolated from the infected leaves served as DNA template for amplification of the AC2 gene through PCR. The AC2 gene was cloned, sequenced and characterized at the molecular level. The nucleotide sequence of the AC2 gene was aligned with those of other cotton leaf curl virus sequences archived in GenBank using BLASTn search program. It shared the highest homology of 99% with the AC2 gene of *Cotton leaf curl Rajasthan* virus. AC2 gene both in sense and antisense orientations having intron sequence were finally cloned into the binary vector (pBI121). The RNAi construct thus developed in binary vector pBI121 containing CaMV 35S promoter was named as pBI121-*AC*2. Having confirmed the positions and sequences of the AC2 gene in RNAi construct (pBI121-*AC*2), its potential role against CLCuD infection was studied following transformation in model *Nicotiana tabacum* plants. A protocol was standardized for the transformation of *N. tabacum* with RNAi vector (pBI121-*AC*2). The competent *Agrobacterium tumefaciens* (strain LBA 4404) cells were transformed by the RNAi vector. Transformation of *N. tabacum* was accomplished through *in vitro* culture. A total of five *N. tabacum* lines were generated. The integration and expression of the *AC2* gene in the transformed *N. tabacum* plants was confirmed by molecular analyses. Total genomic DNA was isolated from the leaves of regenerated *N. tabacum* plants transformed with RNAi construct pBI121-AC2. Four putative transformed *N. tabacum*

revealed the presence of *AC2* and *npt*II genes. Further, presence of *AC*2 gene transcript in transformed *N. tabacum* plants was analysed through reverse transcriptase PCR following isolation of total RNA from the transformed and non-transformed *N. tabacum* plants. Approximately, equal level of *AC*2 gene transcript expression was observed in three transgenic *N. tabacum* plants that were analysed, while there was no expression in the non-transformed plants. The level of CLCuD resistance in the transformed tobacco plants was monitored after inoculation of the transgenic lines with viruliferous whitefly (*Bemisia tabaci*) insect vector. Four lines showed no symptoms even after 2 months of virus inoculation. Having successfully developed RNAi-mediated CLCuD resistance in *N. tabacum* plants, this RNAi based approach has already been extended to *G. hirsutum* cultivar HS6.

One bound copy of the final report of the work work done is being sent to UGC.

VII. Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as

(a) Manpower trained One JRF/SRF (DBT)
(b) Ph. D. awarded Pursuing PhD
(c) Publication of results

1. Alam SF, Ara H, Khan JA (2019). RNAi-mediated resistance against cotton leaf curl disease in tobacco and cotton plants (MS under preparation).
2. Alam SF, Khan JA (2018). Development of transgenic cotton (Gossypium hirsutum) plants against *Cotton leaf curl Multan virus*. Abstract 151, paper presented in National Conference on "Bio-Intensive Approaches in Plant Protection and their Socio-economicImpacts held at Department of Plant Protection, A.M.U, Aligarh (29-30 October, 2018) (Oral presentation).

(d) other impact, if any:

(PRINCIPAL INVESTIGATOR)
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(CO-INVESTIGATOR) Nil

(REGISTRAR/PRINCIPAL)
(Seal)
कुलसचिव / Registrar
जामिया मिल्लिया इस्लामिया / Jamia Millia Islamia
केन्द्रिय विश्वविद्यालय / Central University
नई दिल्ली / New Delhi - 110025

Annexure-I

FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project: Designing, development and studying the role of Cotton leaf curl virus-AC2 gene derived RNAi constructs

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR:

Prof. Jawaid Ahmad Khan

Department of Biosciences

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Jamia Nagar

New Delhi 110025

3. NAME AND ADDRESS OF THE INSTITUTION:

Jamia Millia Islamia

Jamia Nagar

New Delhi 110025

4. UGC APPROVAL LETTER NO. AND DATE

F No. 43-478/2014 (SR) dated 18.11. 2015

5. DATE OF IMPLEMENTATION

April 01, 2016

6. TENURE OF THE PROJECT

Three Years w.e.f. 01.7.2015

7. TOTAL GRANT ALLOCATED

Rs. 15,20, 000/-

8. TOTAL GRANT RECEIVED

Rs. 13,95, 000/-

9. FINAL EXPENDITURE

Rs. 13,65,788/-

10. TITLE OF THE PROJECT

Designing, development and studying the role of Cotton leaf curl virus-AC2 gene derived RNAi constructs

11. OBJECTIVES OF THE PROJECT

1. Molecular characterization of CLCuV-AC2 gene
2. Designing of RNAi constructs targeting CLCuV genome component
3. Introduction of RNAi expression cassettes into binary vector
4. Transformation of *Nicotiana tabacum* with RNAi vector cassettes
5. Biological and molecular analysis of *Nicotiana tabacum* carrying AC2 gene

12. WHETHER OBJECTIVES WERE ACHIEVED:

All objectives have been completed successfully as per details given below:

Objective 1: Molecular characterization of CLCuV- *AC*2 gene

For developing AC2 gene-derived RNAi constructs, it was characterized following cloning and sequencing of the AC2 gene as described below.

Sample collection: Cotton plants (*Gossypium hirsutum*) showing characteristic symptoms of cotton leaf curl disease (CLCuD), such as curling of leaves, vein darkening and thickening, enation on the abaxial side were collected from ICAR-Central Institute for Cotton Research (CICR), Sirsa, Haryana.

Isolation of total genomic DNA from CLCuD-infected leaves of *Gossypium hirsutum* plants: It was attempted to isolate total genomic DNA from the CLCuD-infected leaves of cotton (*G. hirsutum*) plants using DNAeasy plant mini kit as per supplier's protocol (QIAGEN, Germany). Leaf tissue (100 mg) was grounded in liquid nitrogen using autoclaved mortar and pestle. Buffer AP1 (400 µl) and RNaseA (4 µl) were added, incubated at 65˚C for 10 min followed by addition of Buffer AP (130 µl) and incubation on ice for 5 min. The lysate was centrifuged (5 min/ 14000 rpm/room temperature), transferred into QIAshredder spin column, which was placed in a collection tube (2 ml) and centrifuged for 2 min at 14000 rpm. The flow-through was transferred into a fresh Eppendorf tube, Buffer AP3/E (1.5 volume) was added and gently mixed. The obtained mixture (~ 650 µl) was transferred into a DNeasy Mini spin column placed in a

collection tube (2 ml). It was centrifuged for 1 min at 8000 rpm and the resulting flow-through was discarded. In a fresh collection tube, spin column was placed and Buffer AW (500 µl) was added. Following centrifugation (8000 rpm/1min), flow-through was discarded. Buffer AW (500 µl) was added again and centrifuged (2 min/14000 rpm). The spin column was transferred to a new microcentrifuge tube (1.5 ml) and Buffer AE (100 µl) was added for eluting the genomic DNA, incubated for 5 min at room temperature (RT) and centrifuged (8000 rpm/1 min). The elutant containing genomic DNA was stored at -20ºC.

Amplification of *AC2* gene of CLCuV: An oligo primer pair viz. P1 and P2, incorporating restriction sites *Pac*I/*Swa*I and *Asc*I/*Spe*I (5′→3′), was designed to amplify *AC*2 gene of CLCuV through PCR.

P1 5′-TTAATTAA/ATTTAAATT/GTTCAGATATTTGAGGACTTGGGT-3′

P2 5′-ACTAGT/GGCGCGCC/TGCGATCTTCATCACACTTGA-3′

For PCR-based amplification of *AC*2 gene, isolated total genomic DNA and oligo primers P1/P2 were used. PCR was conducted in a reaction mixture having template DNA (0.5 µg), dNTPs (0.2 mM), oligo primers (20 pmol of each forward and reverse), *Taq* DNA polymerase (1 U) and *Taq* buffer (1×) (GeNei, India). The PCR was done in BIO-RAD T100^{TM} Thermo Cycler following incubation of the reaction mixture (94°C/5 min/1 cycle). The first PCR cycle was followed by another 32 cycles with incubation at 94°C for 1 min, annealing at 55°C (1 min) and extension at 72°C (1 min). To extend premature DNA synthesis, if any, an additional final extension (72°C for 5 min) was given at the end of 32 cycles. The PCR product was analysed on agarose gel (1.0%) and photograph was taken over transilluminator in Gel Documentation System (BIO-RAD, USA). PCR yielded a DNA fragment of ca. 450 bp from the genomic DNA isolated from CLCuD-infected cotton (*G. hirsutum*) plants (Fig. 1).

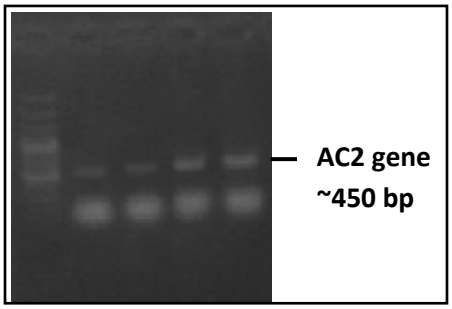


Fig. 1: PCR-based amplification of AC2 gene of Cotton leaf curl virus (l. 1-4).

Molecular cloning and nucleotide sequence determination of *AC2* gene: The PCR amplicon (~450 bp) was excised from the agarose gel and DNA was extracted using QIAEX II gel extraction kit as per manufacturer's instructions (QIAGEN, Germany). The eluted DNA presumably containing *AC*2 gene was cloned into *Eco*RI-digested pDRIVE vector and transformed into *E.coli* DH5α cells.

The cloning of *AC2* gene was confirmed following restriction digestion of the cloned pDRIVE vector by *SpeI/PacI* restriction enzymes yielding a DNA fragment of *Ca*. 450 bp, thus confirming cloning of *AC2* gene in the pDRIVE vector (Fig. 2A).

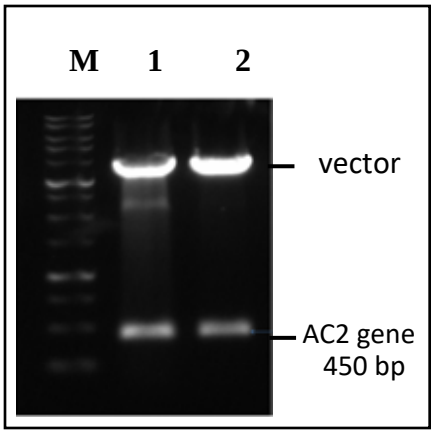


Fig. 2A: Restriction digestion of pDRIVE-AC2 vector carrying AC2 gene with *Spe*I and *Pac*I restriction enzymes yielding DNA fragment of *ca*. 450 bp.

TTAATTAAATTTAAATTGTTCAGATATTTGAGGACTTGGGTTTTGAATACCCTTAAGAAAAGAC
CAGTCTGAGGCTGTAAGGTCGTCCAGATTCGGAATGTTAGAAAACACTTGTGCAGTCCCAGAGC
TTTCCGAGTGTTGTAGTTGAACTGGATCCTGATCGTGAATATGTCCATATTCGTCGTGAATGGA
CGGTTGACGTGGCTGATTATCTTGAAATAAAGGGGATTTGGAACCGCCCAATATATGCGCCATT
CCCTGCTTGAGCTGCAGTGATGGGTTCCCCTGTGCGTGAATCCATGGTTGTGGCAGTTGATTGA
CAGATAATAAGAACACCCGCATTCAAGATCTACTCTCCTCCTCCTGTTGCGCCTCTTCGCTTCC
CTGTGCTGTACTTTGATTGGTACCTGAGTACATGGGTCTATCAAGTGTGATGAAGATCGCAGGC
GCGCCACTAGT

Figure 2B: Nucleotide Sequences of AC2 gene.Positions of primers is shown by underlines

Plasmid DNA was isolated from the pDRIVE-AC2 vector carrying *AC*2 gene and subjected to nucleotide sequence determination (Fig. 2B). The sequences were aligned with those of other cotton leaf curl virus sequences archived in GenBank using BLASTn search program. The *AC2* gene (under study) shared the highest homology of 99% with that of *Cotton leaf curl Rajasthan virus* (Accession ID: KJ959628). The sequence data further confirmed that the *AC2* gene was cloned in the antisense orientation in the pDRIVE-AC2 vector.

Objective 2: Designing of RNAi constructs targeting CLCuV genome component

The RNAi cassette namely pAC2-GUS-AC2 was designed and developed in pDRIVE-AC2 vector after sub-cloning of GUS sequence as an intron and the *AC2* gene in sense orientation. It was accomplished through the following steps.

Deletion of GUS intron: pFGC1008 plasmid harbouring GUS (~400 bp) as an intron was released following restriction digestion by restriction enzymes *Asc*I and *Spe*I (Fig. 3). The GUS fragment thus obtained was ligated into pDRIVE-AC2 vector and transformed into competent *E. coli* DH5α cells.

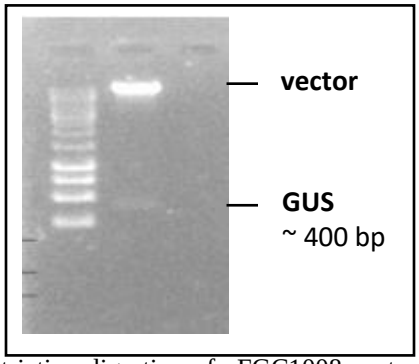


Fig. 3: Restriction digestion of pFGC1008 vector with restriction enzymes *Asc*I and *Spe*I showing release of GUS fragment

Cloning of AC2 gene (sense orientation): For cloning of *AC2* gene (sense orientation) in pDRIVE based RNAi cassette (pDRIVE-AC2), it was amplified through PCR employing oligo

primers P1/P2 (P1 5´GCATGC/CCCGGG/ TGTTCAGATATTTGAGGACTTGGGT 3´) and P2 (5´ ACTAGT/ TGCGATCTTCATCACACTTGA 3´) as described above. A DNA fragment, presumably representing AC2 gene (*Ca.* 450 bp) was obtained through PCR based amplification (Fig. 4).

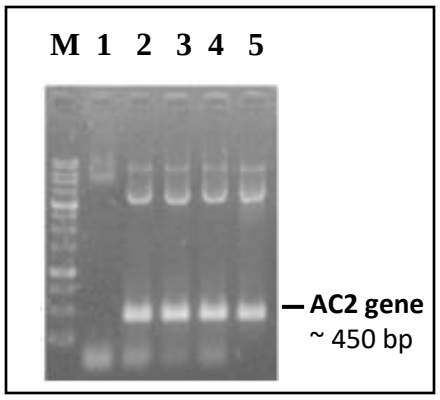


Fig. 4: PCR-based amplification of AC2 gene of Cotton leaf curl virus (Lanes 1-5); M-100 bp DNA ladder

The amplified fragments of *AC2* gene (~450 bp) was excised from the gel (Fig. 5), purified using QIAEX II gel extraction kit (QIAGEN, Gremany) and cloned in pDRIVE based RNAi vector (p-AC2) carrying *AC2* (antisense orientation) and GUS genes.

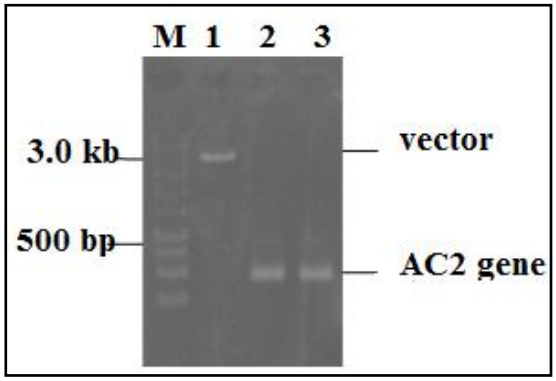


Fig. 5: Agarose gel showing linearised vector pAC2-GUS-AC2 (Lane 1); AC2 gene (Lane 2-3,); Lane M-DNA ladder marker

The presence of *AC*2 gene (sense orientation) was confirmed following restriction digestion of the RNAi cassette viz. pAC2-GUS-AC2 by restriction enzymes *Sph*I and *Spe*I, yielding a DNA fragment representing AC2 gene of ~ 450 bp (Fig. 6, Fig. 7).

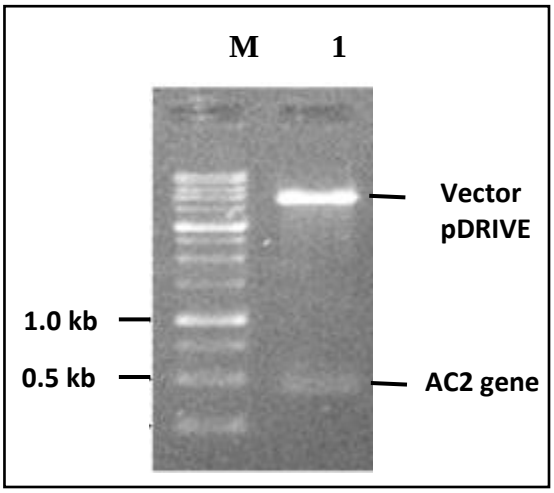


Fig. 6: Restriction digestion of pDRIVE vector-based RNAi cassette (pAC2-GUS-AC2) by restriction enzymes *Sph*I and *Spe*I.

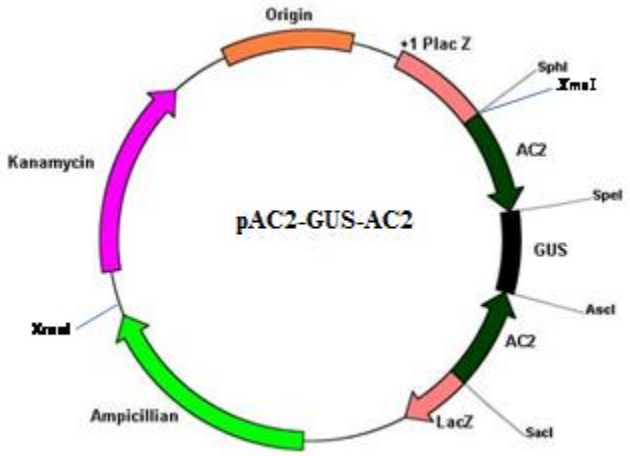


Fig. 7: Schematic diagram of pDRIVE-based RNAi cassette (pAC2-GUS-AC2) carrying AC2 gene both in sense as well as antisense orientations with GUS as an intron.

Sequential restriction digestion and nucleotide sequence determination of complete RNAi cassette (pAC2-GUS-AC2) in pDRIVE vector: Cloning of the complete RNAi cassette (pAC2-GUS-AC2) was confirmed following sequential restriction digestion by endonucleases *Xma*I,

*Sac*I and its nucleotide determination. Restriction digestion yielded DNA fragment of expected size (1.4 kb) representing the RNAi cassette (pAC2-GUS-AC2, Fig. 8).

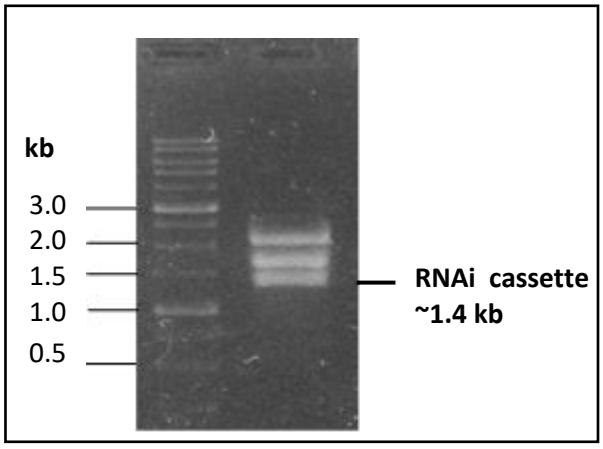


Fig. 8: Restriction digestion of RNAi vector (pAC2-GUS-AC2) yielding DNA fragment of 1.4 kb representing RNAi cassette

Further confirmation of complete cassette was done after its nucleotide sequence determination by automated sequencer (ABI 3500xL Genetic Analyzer) at Chromous Biotech (Bangalore, India).

Cloning of pDRIVE RNAi cassette into Binary vector (pBI121): The RNAi cassette (pAC2-GUS-AC2) was cloned into the binary vector (pBI121). Restriction digestion of binary vector (pBI121) resulted into the deletion of DNA fragment (GUS) of about 2 kb. The resulting digested DNA fragment representing the RNAi cassette (AC2-GUS-AC2) of 1.4 kb was cloned into binary vector pBI121 and transformed into *E.coli* DH5α cells [Sambrook et al.(1989). Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, New York].

The cloning of the RNAi cassette (AC2-GUS-AC2) into pBI121 binary vector was confirmed following restriction digestion by *Sac*I and *Xma*I yielding a DNA insert of desired size (~ 1.4 kb, Fig. 9).

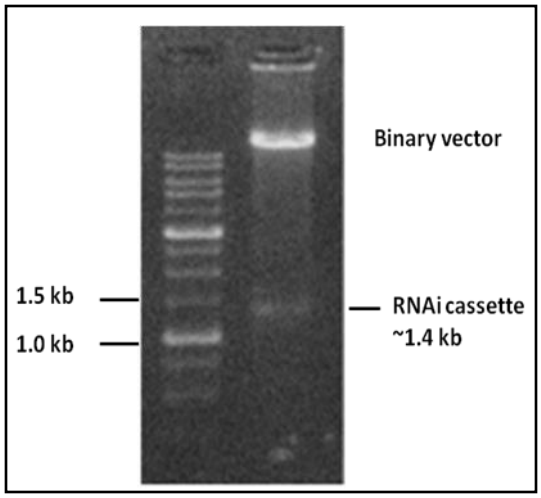
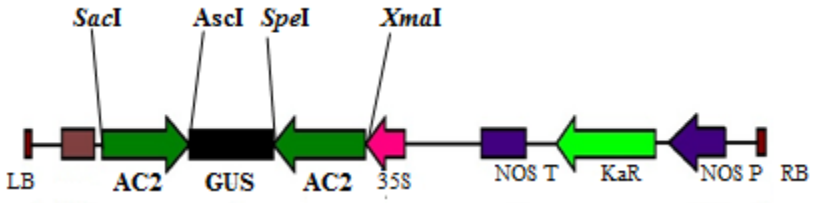
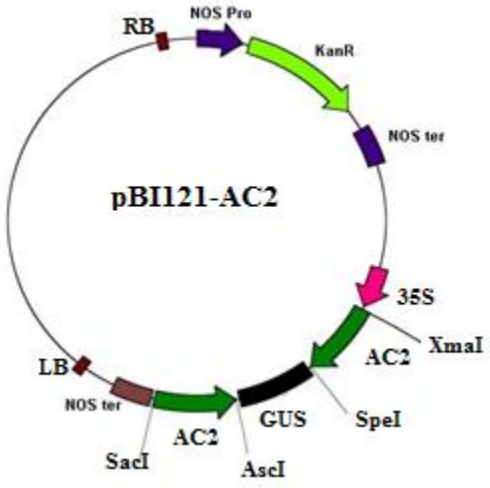


Fig. 9: Restriction digestion of RNAi vector (pBI121-AC2) by *Xma*I and *Sac*I yielding linearized binary vector and RNAi cassette (Lane 1)DNA fragments (RNAi cassette)

The RNAi construct thus developed in binary vector pBI121 containing CaMV 35S promoter was named as pBI121-*AC*2 (Fig. 10 A, B).



A.



B.

Fig. 10: Schematic representation of (A) RNAi cassette (AC2-GUS-AC2); (B) in binary vector pBI121-AC2

Objective 3: Introduction of RNAi cassette into binary vector

The RNAi cassette developed in binary vector (pBI121-*AC*2) was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw method.

Preparation of competent cells of *A. tumefaciens*: The strain LBA4404 of *A. tumefaciens* was selected for the preparation of competent cells. A fresh colony of *A. tumefaciens* (LBA4404) was picked, inoculated into 5 ml sterilized YEM (Yeast Mannitol) broth medium containing antibiotic rifampicin (Himedia, 50 µg/ml) and incubated at 28ºC (48 hrs/200 rpm).

Freshly grown bacterial culture (200 µl) was reinoculated into YEM medium (50 ml) containing the same antibiotic and allowed to grow as the secondary culture at 28ºC for 16 hr at 200 rpm, until OD_{600} of the cells was achieved as 0.6-1.0. The secondary culture was transferred aseptically into an Oakridge centrifuge tube and kept on ice (30 min). It was centrifuged at 4000 rpm (5 min/4ºC). While the supernatant was poured off, bacterial cells were re-suspended in chilled 100 mM $CaCl_2$ (20 ml). The same step was repeated and the cells were finally dissolved in 1 ml solution containing 100 mM $CaCl_2$ and 15% glycerol. The competent cells were aliquoted (100 µl) into Eppendorf tube (1.5 ml, pre-chilled sterilized) and stored at -80ºC.

Transformation of *A. tumefaciens* strain LBA4404 with the RNAi vector viz. pBI121-*AC*2: The competent *A. tumefaciens* (strain LBA 4404) cells were transformed by the RNAi vector (pBI121-*AC*2) following freeze-thaw method [Höfgen and Willmitzer, (1988). Nucleic Acids Res. 1988 Oct 25; 16(20): 9877]. The cells were allowed to thaw on ice for 5 min, 10 µl of recombinant plasmid (~10 µg) was mixed with the cells, kept on ice for 5 min, transferred into liquid nitrogen for 5 min and immediately given heat shock at 37ºC (5 min). YEM broth medium (4x volume) was added to the cells and were incubated at 28ºC (3hr/200 rpm). Following a brief spin, pellet was dissolved in 100 µl of remaining supernatant and plated on YEM agar plate containing antibiotics rifampicin (50 mg/ml) and kanamycin (HiMedia, 50mg/ml). The plate was incubated at 28ºC for 24-36 hours. Few colonies were appeared and they were screened by colony PCR.

Colony PCR: The *A. tumefaciens* (strain LBA4404) colonies developed on antibiotics containing YEM medium were randomly picked up and homogenized in distilled water as a

template and subjected to colony PCR employing oligo primers P1/P2 (described in previous section). The colony PCR yielded a DNA fragment of *ca*. 450 bp, thus confirming the transformation of *A. tumefaciens* (strain LBA4404) with the RNAi construct pBI121-AC2 (Fig. 11).

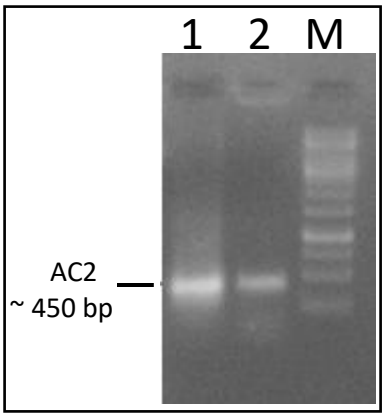


Fig. 11. Colony PCR based amplification of AC2 gene in *Agrobacterium tumifaciens* (strain LBA4404) cells transformed with the RNAi vector pBI121 (L. 1, 2).

Objective 4: Transformation of *Nicotiana tabacum* with RNAi vector cassettes

Transformation of *N. tabacum* with the RNAi vector was accomplished through following steps.

Surface sterilization of seeds: Seeds of healthy *Nicotiana tabacum* were given $HgCl_2$ (0.1%) treatment following shaking gently (2-3 min). They were then rinsed with sterile double distilled water (5 times). Around, 10-15 seeds were allowed to germinate in culture tubes containing half strength of MS basal medium (Murashige and Skoog (1962). Physiol. Plant. 15:473-97] for 3 weeks (28 ±2 °C; 16 hour light and 8 hour dark photoperiods).

Preparation of *Agrobacterium* culture: *Agrobacterium tumefaciens* (strain LBA4404) harboring RNAi construct pBI121-AC2 was inoculated in YEM broth (10 ml) supplemented with antibiotics kanamycin (50 µg/ml) and rifampicin (25 µg/ml). The culture was incubated in dark at 28°C for 36-40 hour with shaking at 150 rpm as primary culture. Primary culture (1%) was taken to setup secondary inoculum and incubated in dark at 28°C with shaking at 200 rpm until OD_{600} reached 0.4 – 0.6.

Explant preparation: *N. tabacum* leaves were excised from *in vitro* grown seedlings using autoclaved forceps and sterilized surgical blades under aseptic conditions. Leaves were sliced into small pieces and pe-cultured on basal MS medium (Murashige and Skoog, 1962) for 24 hour at 28±2°C inside the tissue culture room under 16 hour light and 8 hour dark periods.

Co-cultivation: Pre-cultured leaf pieces were co-cultivated in liquid co-cultivation medium (MS medium, 3% w/v Sucrose, 10 mM MES, 10 mM $MgCl_2$, 10 µM Acetosyringone, pH-5.7), having actively growing *A. tumefaciens* (LBA4404) cells harboring RNAi construct pBI121-AC2, for 15-20 minutes at 28°C with gentle shaking. Pieces of the infected leaves pieces were dried on autoclaved filter paper and incubated on solid co-cultivation medium (MS medium, 1.5% w/v Sucrose, 10 µM acetosyringone, 0.8% agar, pH-5.7) for 48 hour at 28±2°C under dark condition.

Regeneration of transformed *N. tabacum* plants

Selection of explants: Co-cultivated leaf pieces of *N. tabacum* were sub-cultured on a callus induction medium (full-strength MS medium, 1.5% w/v Sucrose, 100 mg/L m-inositol, 200 mg/L PVP, NAA 1.0 mg/L, BAP 1.0 mg/L, 0.8% agar, pH-5.7) containing cefotaxime (300 mg/L) and kanamycin (50 mg/L) antibiotics. Healthy kanamycin-resistant calli were sub-cultured in a shoot induction medium containing (MS medium, 1.5% w/v sucrose, 100 mg/L, m-inositol, 200 mg/L PVP, 0.8% agar, BAP 1.0 mg/L, pH-5.7) antibiotics viz. kanamycin (50 mg/L) and cefotaxime (300 mg/L). The cultures were allowed to establish in Petri plates containing 10 sections per plate. Explants were incubated at 26 ±2°C under 16 h of diffused cool white-light (25-30 µmol m-2s-1). With a view to compensate the degradation of hygromycin in the media and to maintain a constant selection pressure, all the explants were subcultured onto a fresh regeneration/selection medium every 10 days.

Root induction: Elongated shoots of tobacco (*N. tobacum*) were transferred into rooting medium (full-strength MS medium) containing Indolebutyric acid (IBA) and Naphthalene acetic acid (NAA) for the induction of roots. The regenerated plantlets were incubated at 28±2°C (16-hour light and 8-hour dark photoperiods), and maintained on the rooting medium until roots were developed. They were then placed individually in glass jars containing the same medium under the same environmental conditions for further development of the shoots and rooting system (Fig. 12)**.**

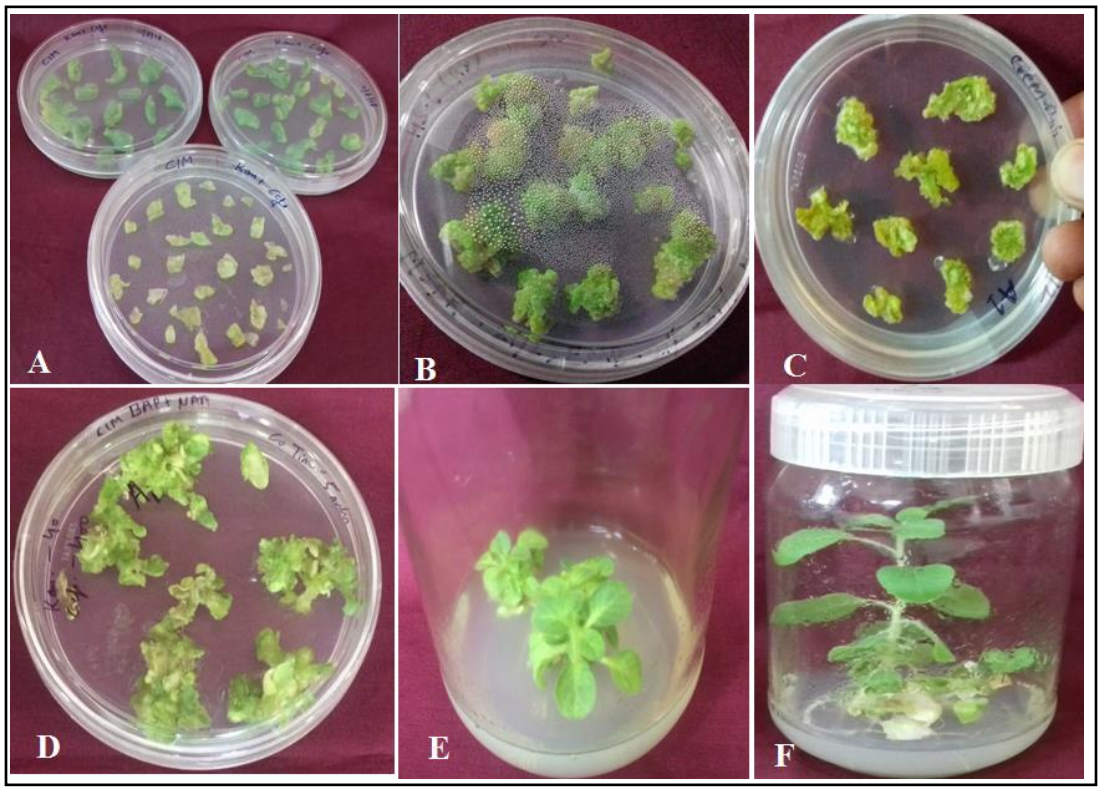


Fig. 12: Different stages of *N. tabacum* plant from leaf disc explants to young plantlet following *Agrobacterium*-mediated transformation. (A) explants preparation and co-cultivation, (B) and (C) leaf discs on selection medium, (D) selected leaf discs on callus induction medium, (E) shoot elongation medium and (F) regenerated plantlets on rooting medium.

Hardening and establishment of plants: Plantlets with intensive root systems (several roots, 24-45 mm in length) were carefully removed from the culture glass jars and the roots were carefully rinsed with sterilized water to remove tissue culture residues. Plants were transferred to pots filled with sterilized soil and agropeat (1:2; v/v). Before shifting to the greenhouse, regenerated *N. tabacum* plants were covered with plastic bags for maintaining humidity and kept for 15-20 days at 26 ±2°C under 16 h of diffused cool-white light (Fig. 13).

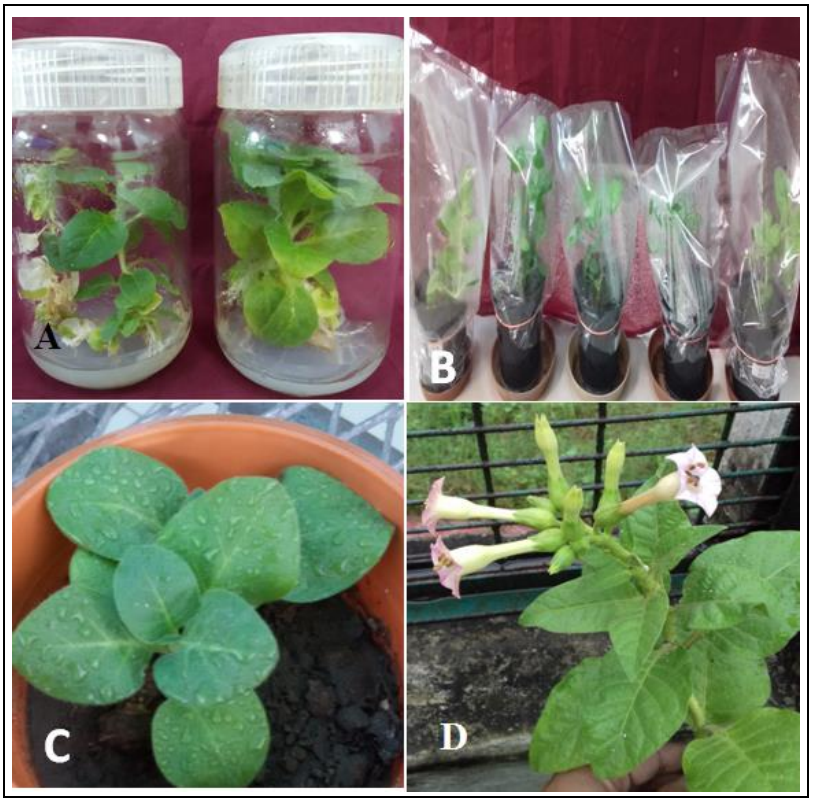


Fig. 13: Different stages of hardening of regenerated *N. tabacum* plant from young plantlet to mature plant (A to D).

Objective 5: Biological and molecular analysis of *Nicotiana tabacum* carrying AC2 gene

Selection of *Nicotiana tabacum* transformed plants

Detection of *AC*2 and *npt*II (Neomycin phosphotransferase) genes: Total genomic DNA from the leaves of regenerated *N. tabacum* plants transformed with RNAi construct pBI121-AC2 was isolated using DNeasy plant mini kit as per supplier's recommendation (Qiagen, Germany). Fresh leaf tissues (100 mg) were grounded in liquid nitrogen with the help of autoclaved mortar and pestle. Grounded leaf tissue powder was immediately taken in microcentrifuge tubes (2 ml) containing AP1 buffer (400 µl) and RNase A (4 µl). These tubes were placed at 60°C for 20 min and vortexed regularly after 2-3 min for proper cells lysis. To the tubes, 130 µl of Buffer P3 was added and kept on ice for 10 min. The lysate was centrifuged at 14000 rpm (5 min/room temperature), transferred into QIAshredder spin column, placed in a collection (2 ml) tube and followed by centrifugation at 14000 rpm (2 min). The supernatant was taken in a fresh Eppendorf tube and Buffer AP3/E (1.5 volume) was added to it and mixed gently by pipetting. The supernatant (650 µl) was transferred into a DNeasy Mini spin column, placed in a 2 ml-collection tube, centrifuged for 1min at 8000 rpm and the flow-through was discarded. Spin column was placed in a new collection tube, Buffer AW (500 µl) was added and centrifuged for

1 min at 8000 rpm, and again discarded flow-through. Buffer AW (500 µl) was added and centrifuged (2 min/ 14000 rpm). The spin column was transferred to a fresh microcentrifuge tube (1.5 ml) and Buffer AE (100 µl) was added for eluting the genomic DNA, incubated for 5 min at RT and centrifuged for 1 min at 8000 rpm.

Concentration and integrity of the isolated total genomic DNA was checked by agarose gel electrophoresis and visualized under UV light of Gel Doc system (Fig. 14).

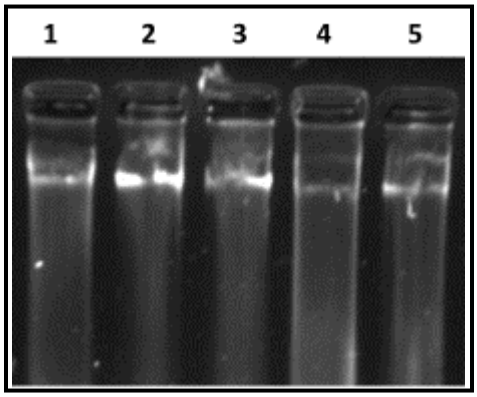


Fig. 14: Total genomic DNA isolated from putative transformed *Nicotiana tabacum* plants.

Detection of *AC*2 gene in the putative transformed *N. tabacum* plants: Presence of AC2 gene in the transformed plants was confirmed using total genomic DNA as the template isolated from the transformed plants in PCR. It was performed in a reaction mixture consisting of dNTPs (0.2 mM), AC2 gene specific primers viz. P1 and P2 (20 pmol of each primer), genomic DNA template (100 ng), *Taq* DNA polymerase (1 U, GeNei, India). The reaction mixture was subjected to PCR amplification following incubation at 94°C (5 min/1 cycle). It was followed by another 32 cycles at 94°C for 1 min, annealing at 55°C (1 min.) and extension at 72°C (1 min). At the end of 32 cycles, an additional final extension (72°C/5 min) was performed to extend any premature DNA synthesis. The PCR amplicon was analysed on agarose gel and photographed over transilluminator in Gel Documentation System (BIO-RAD, USA). PCR yielded a DNA fragment of Ca. 450 bp corresponding to the AC2 gene. A total of four putative transgenic *N. tabacum* revealed the presence of AC2 gene (Figure 15).

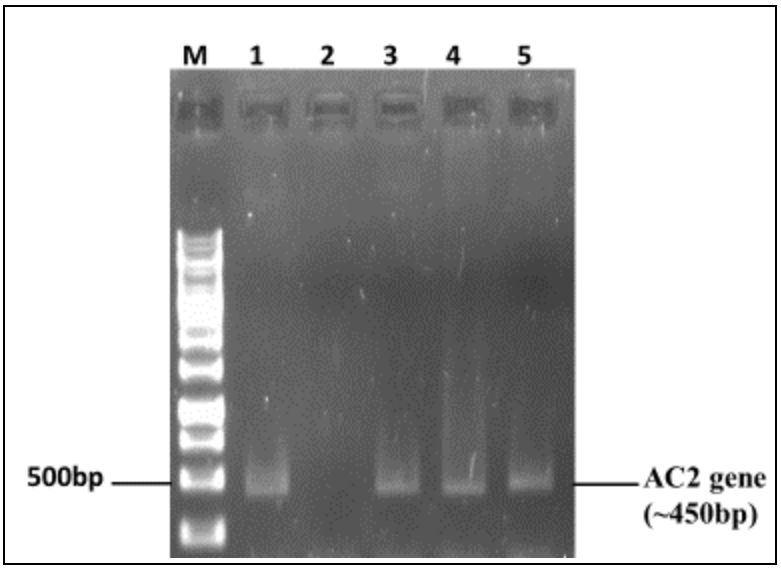


Fig. 15: PCR based amplification of AC2 gene of Cotton leaf curl virus in transformed *Nicotiana tobacum* plants (Lanes 1, 3-5).

Detection of *npt*II gene in the transformed *N. tabacum* plants: An oligo primer pair (viz. Fwd and Rev) was designed to amplify *npt*II marker gene present in the RNAi vector pBI121-AC2.

Forward primer (Fwd) 5′- GGAGCGGCGATACCGTAAAGC-3′

Reverse primer (Rev) 5′- GAGGCTATTCGGCTATGACTG-3′

Total genomic DNA extracted from the transformed *N. tabacum* served as template in PCR for the detection of RNAi vector pBI121 specific-*npt*II marker gene employing primer pair viz. Fwd and Rev). The PCR amplification was performed in a reaction mixture consisting of template DNA (500 ng), dNTPs (0.2 mM), oligo primers (20 pmol each), *Taq* polymerase (1U, GeNei, India). For the first PCR cycle, reaction mixture was incubated at 95°C for 5 min . It was followed by 25 cycles at 94°C for 30 sec, annealing at 57°C (30 sec) and extension at 72°C for 1 min. At the end of 25 cycles, last extension was done at 72°C for 10 min.

The PCR yielded a DNA fragment of expected size (~ 700 bp) from the four transformed *N. tabacum* plants as observed following electrophoresis of PCR amplicon in agarose gel and visualization under UV light of GelDoc^{TM}EZ Imager (Figure 16).

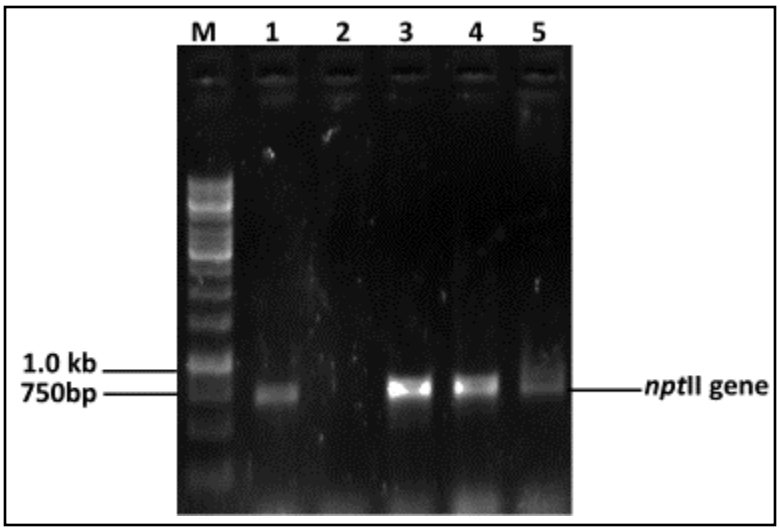


Fig. 16: PCR based detection of neomycin phosphotransferase (nptII) marker gene in transformed *Nicotiana tobacum* plants (Lanes 1-5), M: DNA ladder marker.

Analysis of *AC*2 gene transcript in transformed *N. tabacum* plants

Isolation of total RNA: Total RNA was isolated from the transformed and non-transformed *N. tabacum* plants using RNeasy Plant Mini kit as per supplier's protocol (Qiagen, Germany). Fresh leaf tissues (100 mg) were crushed to powder in liquid nitrogen using autoclaved mortar and pestle (pre-cooled). The leaf powder was transferred into Eppendorf tube (pre-cooled, RNase-free) containing Buffer RLC (450 µl) and β-mercaptoethanol (10 µl), incubated for 30 min at 50° C while vortexing at every 5 min. Lysate was transferred into QIAshedder spin column and centrifuged (3 min at 14000x g at RT). The flow through was gently poured into a microcentrifuge tube, absolute ethanol was added to it, mixed gently by pipetting and subjected to centrifugation for 2 min at 14000 rpm. The RNase mini spin column was washed with buffer RW1 (700 µl) by centrifugation (14000 rpm, 2min). This step was repeated with buffer RPE (500 µl) at the same centrifugation in order to remove other entities. Spin column was allowed (3-5 min) to dry the membrane. Dried RNase column was placed in a fresh Eppendorf tube, nuclease-free water (25µl) was added to it and centrifuged for 2 min (12000 rpm) for eluting the total RNA. The eluted RNA was used for cDNA synthesis as described below.

Synthesis of cDNA: The cDNA was prepared using M-MuLV RT-PCR kit (Merck, Germany).The reaction was done in a reaction mixture (20 µl) containing 3µl of template RNA(~2µg), RT assay buffer (4 µl), 40U/µl RNasin, 20 µM Oligo dT (1µl), 100 mM DTT (1µl), dNTPs mix (1µl), reverse transcriptase M-MuLV (1µl) and remaining nuclease free water (9 µl).

Reaction mixture was mixed gently and incubated at 37°C for 1 hour. The reaction was terminated following incubating the reaction mixture at 94°C for 2 min and immediately transferring on ice for 5 min.

Reverse transcriptase (RT) - PCR based amplification of *AC*2 gene transcript: RT-PCR based amplification of the *AC*2 gene using cDNA as the template was conducted in a reaction mixture employing the designed *AC*2 gene-specific primers (fwd and rev) as discussed in the above sections. The reaction conditions remained same as described earlier. The PCR amplicon was resolved on 1% agarose gel and visualized under VU light of Gel Doc system. Approximately, equal level of *AC*2 gene transcript expression was observed in three transgenic *N. tabacum* plants that were analysed (Figure 17, lanes 1-3), while there were no expression in the non-transformed plants (Fig 17, lanes 5-7).

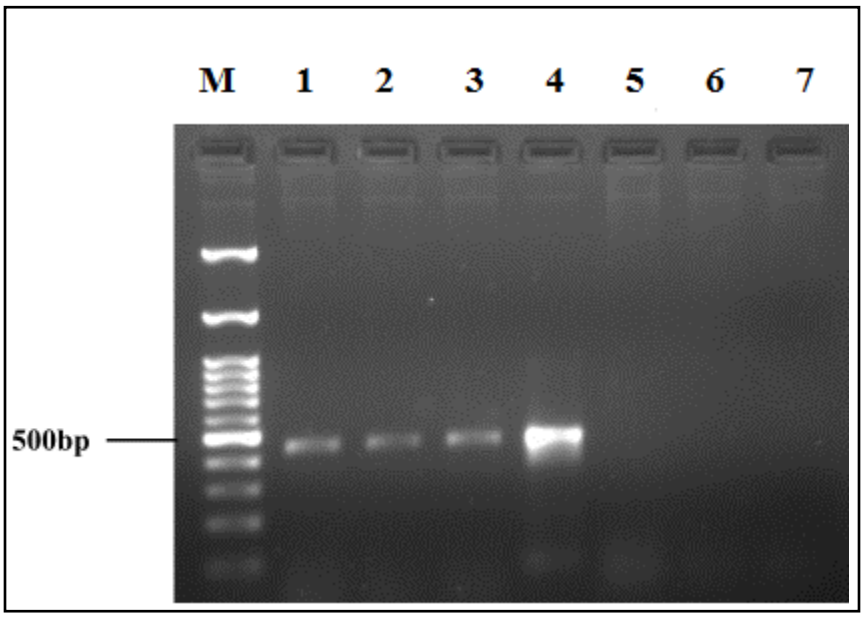


Figure 17: Semi-quantitative Reverse transcriptase based amplification of expressed AC2 gene transcripts in transgenic *Nicotiana tabacum* plants (Lanes 1-3), non-transformed plants (lanes 5-7).

Inoculation of transgenic *N. tabacum* plants with Cotton leaf curl virus (CLCuV):

Four transgenic tobacco (*N. tabacum*) lines were selected for evaluating their resistance against CLCuV infection. Resistance in the transgenic *N. tabacum* plants against CLCuV was assayed following their inoculation with viruliferous whitefly (*B. tabaci*) insect vector carrying CLCuV as essentially described by Khan & Ahmed, 2005 (Current Science 88 (11): 10 JUNE 2005). For

inoculating the putative transgenic plants with CLCuV, healthy *B. tabaci* were reared on *G. hirsutum* plants grown in insect-proof cages. Healthy *B. tabaci* were allowed a 48-h acquisition access period on *G. hirsutum* plants showing cotton leaf curl disease (CLCuD) symptoms. Around 100 viruliferous whiteflies were allowed a 72-h inoculation access period on each transgenic *N. tabacum* plant in insect-proof cages. After inoculation, plants were sprayed with insecticide and kept in an insect-proof glasshouse. Plants were regularly monitored for the development of CLCuD symptoms at 2-weeks post inoculation of the virus, and compared with non-transformed *N. tabacum* plants.

CLCuD symptoms such as curling of leaves, thickening and darkening of veins and stunted growth were observed in all the non-transformed *N. tabacum* plants at 3-weeks post-inoculation. After 4 weeks of inoculation, the non-transformed plants remained stunted. However, all the four transgenic *N. tabacum* lines exhibited no virus symptoms (Figure 18).

Fig. 18: No symptoms development in transgenic *Nicotiana tabacum* plant (T) 4-weeks post inoculation with viruliferous whitefly (*Bemisia tabaci*) carrying CLCuV. Symptoms were appeared in non-transformed (NT) *N. tabacum* plants.

13. ACHIEVEMENTS FROM THE PROJECT

For developing resistance against cotton leaf curl disease, an RNAi construct based on *AC2* gene of Cotton leaf curl virus (CLCuV) was designed and developed. And, its role against CLCuD infection was studied following transformation of AC2 gene construct in model *Nicotiana tabacum* plants. The integration and expression of the *AC2* gene in the transgenic *N. tabacum*

plants was confirmed by molecular analyses. The intensity of CLCuD resistance in the transgenic plants was monitored after their inoculation with viruliferous whitefly (*Bemisia tabaci*) insect vector. A total of five transgenic lines of *N. tabacum* were generated. Among them, four lines showed no symptoms even after 2 months of virus inoculation. Having successfully developed CLCuD resistance in transgenic *N. tabacum* plants, this RNAi based approach has already been extended to *G. hirsutum* cultivar HS6.

14. SUMMARY OF THE FINDINGS:

(IN 500 WORDS**)**

Cotton leaf curl disease (CLCuD) is a devastating disease of cotton (*Gossypium hirsutum*) causes enormous losses to cotton. It has become a major challenge for the cultivation of cotton crop in Indian subcontinent. It is induced by complexes of five distinct cotton-infecting monopatite begomoviruses in association with satellite molecules called as beta–, and alpha-satellite molecules. RNA interference (RNAi) is a potential approach that has been successfully tested to develop resistance against begomoviruses. In this research project, RNAi which acts as a natural anti-viral defense system, was applied to combat CLCuD infection. In plants, RNAi is achieved by the introduction of a transgene that expresses a part of the viral genome leading to the development of resistance in the plant against that viral infection. As a first step to generate resistance against CLCuD infection, an RNAi construct based on *AC2* gene of Cotton leaf curl virus (CLCuV) was designed and developed. Leaves from diseased cotton plants showing CLCuD characteristic symptoms (curling of leaves, darkening and thickening of veins, enations on the abaxial side of leaves) were collected. Total genomic DNA was isolated from the infected leaves served as DNA template for amplification of the AC2 gene through PCR. The AC2 gene was cloned, sequenced and characterized at the molecular level. The nucleotide sequence of the AC2 gene was aligned with those of other cotton leaf curl virus sequences archived in GenBank using BLASTn search program. It shared the highest homology of 99% with the AC2 gene of *Cotton leaf curl Rajasthan* virus. AC2 gene both in sense and antisense orientations having intron sequence were finally cloned into the binary vector (pBI121). The RNAi construct thus developed in binary vector pBI121 containing CaMV 35S promoter was named as pBI121-*AC*2. Having confirmed the positions and sequences of the AC2 gene in RNAi construct (pBI121-*AC*2), its potential role against CLCuD infection was studied following transformation in model *Nicotiana tabacum*

plants. A protocol was standardized for the transformation of *N. tabacum* with RNAi vector (pBI121-*AC*2). The competent *Agrobacterium tumefaciens* (strain LBA 4404) cells were transformed by the RNAi vector. Transformation of *N. tabacum* was accomplished through *in vitro* culture. A total of five *N. tabacum* lines were generated. The integration and expression of the *AC2* gene in the transformed *N. tabacum* plants was confirmed by molecular analyses. Total genomic DNA was isolated from the leaves of regenerated *N. tabacum* plants transformed with RNAi construct pBI121-AC2. Four putative transformed *N. tabacum* revealed the presence of *AC2* and *npt*II genes. Further, presence of *AC*2 gene transcript in transformed *N. tabacum* plants was analysed through reverse transcriptase PCR following isolation of total RNA from the transformed and non-transformed *N. tabacum* plants. Approximately, equal level of *AC*2 gene transcript expression was observed in three transgenic *N. tabacum* plants that were analysed, while there was no expression in the non-transformed plants. The level of CLCuD resistance in the transformed tobacco plants was monitored after inoculation of the transgenic lines with viruliferous whitefly (*Bemisia tabaci*) insect vector. Four lines showed no symptoms even after 2 months of virus inoculation. Having successfully developed RNAi-mediated CLCuD resistance in *N. tabacum* plants, this RNAi based approach has already been extended to *G. hirsutum* cultivar HS6.

15. CONTRIBUTION TO THE SOCIETY:

CLCuD was reported on cotton (*G. hirsutum*) from Sriganganagar in Rajasthan state in 1993, and subsequently it spread to Haryana and Punjab in 1994. During 2009-10 crop seasons, this disease appeared in a severe form in some areas of North zone. Even the resistant cotton varieties showed susceptibility to CLCuD in hot spot areas of Northern India. A large-scale manifestation of whiteflies in cotton fields of Punjab have caused heavy losses worth million 630-670 million US $ due to drastic decline of cotton yield as a result of CLCuD epidemic in the year 2015-16. More than two-thirds of total cotton cultivated area was reported to be completely infected by CLCuD causing drastic losses to cotton production. The incurred heavy losses had serious social implications linking farmers' suicides in the states. A reduction of cotton yield up to 70% has been recorded from other continent. So far, CLCuD resistant variety of cultivated cotton is not available across the globe.

RNAi-derived cotton plants, expressing siRNAs directed against the potential C2 (AC2) gene of CLCuV, offers an attractive way of controlling CLCuD. In this project, RNAi-mediated approach was successfully applied to generate resistance against CLCuD in *N. tabacum* model plants. Few *N. tabacum* lines have shown resistance against CLCuV infection. Having tested RNAi-mediated approach in *N. tabacum* plants, it is being extended to elite cotton (*G. hirsutum*) cultivar. Indeed, development of RNAi-derived cotton variety against CLCuD alleviation will be a valuable addition to the betterment of the cotton crop in India. Moreover, success in this endeavour could extend this technology to other important crops as well.

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT

One DBT JRF has been enrolled for PhD

17. NO. OF PUBLICATIONS OUT OF THE PROJECT

I. Alam SF, Ara H, Khan JA (2019). RNAi-mediated resistance against cotton leaf curl disease in tobacco and cotton plants (MS under preparation).
II. Alam SF, Khan JA (2018). Development of transgenic cotton (Gossypium hirsutum) plants against *Cotton leaf curl Multan virus*. Abstract 151, paper presented in National Conference on "Bio-Intensive Approaches in Plant Protection and their Socio-economic Impacts held at Department of Plant Protection, A.M.U, Aligarh (29-30 October, 2018) (Oral presentation).

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(CO-INVESTIGATOR): NIL

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(Seal)

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