Cotton leaf curl virus resistance transgenics with antisense coat protein gene (*AV1*)

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Cotton leaf curl virus disease is one of the major threats for cotton production and has emerged as a serious disease of cotton in North India. Cotton leaf curl disease (CLCuD) is caused by a geminivirus, transmitted by whitefly (*Bemisia tabaci*) vector. Genetic engineering of cotton transgenics resistant to CLCuD through antisense RNA approach is a potential technique to tackle the disease in cotton. Cotton transgenics resistant to CLCuD using antisense coat protein (ACP) gene was developed via *Agrobacterium*-mediated transformation. A binary vector carrying the ACP gene along with the *npt*II (neomycin phospho transferase) gene driven by CaMV-35S promoter and *nos* (nopaline synthase) terminator was used for transformation. Molecular confirmations of the transformants were carried by PCR and Southern analysis. The individual transgenics were raised in the greenhouse and screened for virus resistance by inoculating with viruliferous whiteflies. Following the challenge with the viruliferous whiteflies, transgenic plants remained symptomless. The T_2 plants were screened using specific primers for ACP gene and primers for *npt*II gene; they showed classical Mendelian pattern of inheritance.

Keywords: *Agrobacterium*-mediated transformation, coat protein gene, cotton leaf curl virus, *Gossypium hirsutum*.

THE most important renewable natural textile fibre worldwide and the world's sixth largest source of vegetable oil is cotton, cultivated in more than 100 countries, and providing fibre and oil products. India is the world's second largest cotton producer. It provides more than 90% of the raw material to textile mills, shares substantially in the total edible-oil production and contributes more than 30% in total foreign exchange earnings. Pests and diseases are a major threat to the crop yield. Cotton leaf curl disease (CLCuD) is one of the major problems in cotton production and has emerged as a serious disease in North India[1–3]. The disease leads to yield losses up to 58% (ref. 4). CLCuD is transmitted by the whitefly, *Bemisia tabaci* and is associated with members of the family Geminiviridae and genus *Begomovirus*, collectively referred to as cotton leaf curl virus (CLCuV)[5]. Geminiviruses occur mainly in tropical and warm climatic areas, where the viruses have unleashed important diseases and significant agricultural losses in dicotyledonous hosts. The genome of geminiviruses is either monopartite or bipartite, and DNA β of monopartite viruses is generally required for viral infection[5]. CLCuV-infected cotton plants contain CLCuV DNA A and a single-stranded satellite DNA molecule called DNA β (1350 nt) which together induce symptoms typical of CLCuV, both in cotton and tobacco[6]. DNA β requires CLCuV DNA A for replication and encapsidation, and encodes putative proteins that share no homology with the DNA β of other *Begomoviruses.* The strategy used by geminiviruses to replicate their single-stranded DNA (ssDNA) genome consists of the conversion of ssDNA into double-stranded DNA (dsDNA) intermediates, and then using dsDNA as a template to produce mature ssDNA genomes by a rolling circle replication mechanism. In addition, the accumulating evidence indicates that viral DNA replication is somehow coupled to the cell-cycle regulatory network of the infected cell. For these reasons geminiviruses are excellent model systems to understand the regulation of DNA replication and cell cycle in plant cells[7].

Virus-resistant transgenic plant development is environmentally safe for the management of viral disease[8]. There are mainly two approaches for developing genetically engineered resistance depending on the source of the genes used. The genes can be either from the pathogenic virus itself or from any other source. The former approach is based on the concept of pathogen-derived resistance (PDR). For PDR, a part or a complete viral gene is introduced into the plant, which subsequently interferes with one or more essential steps in the life cycle of the virus. The mechanism behind the viral resistance in plants was clearly explained as small RNA species of ~25 nt in plants undergoing post-transcriptional gene silencing (PTGS), but not in plants that are not being silenced. It had been initially proposed that these small RNAs might be involved in the production of the double-stranded RNA (dsRNA) to serve as a target for PTGS, since they were shown to be complementary to the targeted mRNAs. The small RNAs might be the result of

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the dsRNA degradation, and their role would be to act as a silencing signal[9]. In case of *Begomoviruses*, expression of viral coat protein (CP)[10], replicase[11] and movement proteins has proved more promising. The viral CP gene was the first and one of the most widely used genes to confer PDR against plant viruses[12]. CP-mediated resistance has been successfully applied to numerous crop species[8,13,14]. Resistance was mediated by the RNAs of the CP transgene, rather than the protein, as an inverse correlation between resistance and the accumulation levels of mRNAs of CP transgene were observed, indicating that a PTGS mechanism is likely to be involved in the CP RNA-mediated protection.

The antisense RNA molecules (complementary RNA sequence) that bind to the naturally occurring (sense) mRNAs, can inhibit target gene expression in a variety of ways. The best-known antisense mechanism that triggers mRNA degradation is translational repression by ribosome interference in a common natural antisense mechanism. The production of antisense RNAs in transgenic plants has been widely used for decreasing the expression of the endogenous genes by silencing the expression of the corresponding genes as a tool for introducing the desired traits to a given crop[15,16]. This technology has been successfully applied for engineering resistance against geminiviruses. RNA silencing in plants is an antiviral response triggered against most or all viruses[17,18]. Based on the analyses of virus-encoded silencing suppressors, RNA silencing limits the extent of virus multiplication and long-distance movement of viruses. dsRNA induces sequence-specific PTGS or homology-dependent gene silencing (HDGS) in many organisms by a process known as RNA interference (RNAi), and is thought to protect cells against invasive nucleic acids, such as viruses and transposons[19]. Evidence suggests that transgene loci and RNA viruses can generate dsRNAs similar in sequence to the transcribed region of the target genes, which then undergo endonucleolytic cleavage to generate small interfering RNAs (siRNA) that promote degradation of cognate RNAs[17,19–22]. In plants, dsRNA expressed from a transgene can induce not only PTGS, but also transcriptional gene silencing (TGS) correlated with *de novo* methylation of cognate DNA in the genome[15] by a hypothetical RNA-dependent DNA methylase (RdDM). The association of gene silencing and viral resistance has been achieved in plants that have accumulated low levels of the transgene mRNA[17,23].

Geminivirus replicates inside the host nucleus, where sense mRNAs (translatable RNA) are transcribed, as antisense transcripts are also produced in the host nucleus, so that there are chances for duplex formation between the sense and the antisense mRNA. dsRNA duplex is prone to degradation. Therefore, it is possible that there would be greater likelihood of success in using antisense RNA technology for engineering virus-resistant plants against geminiviruses[12,18]. Antisense RNA expression against the CLCuV DNA A-specific genes could provide tolerance and/or resistance to CLCuV in elite cultivars of cotton. A number of CLCuV genomic DNA A and DNA β fragments can be exploited to repress the expression of viral genes. The present study was designed with the following objectives for the development of transgenic plants with viral resistance against CLCuV through antisense CP (ACP) gene-mediated resistance.

Genetic engineering offers a direct method that selectively targets one or few traits for introduction into the crop plants. The most commonly used methods for the transformation of cotton plants is *Agrobacterium*-mediated gene transfer. The first transgenic plants were produced exploiting the natural capacity of the bacterium, *Agrobacterium tumefaciens* to transfer genetic material to plant cells[24]. In cotton, *Agrobacterium*-mediated transformation was successfully carried out[25–27] with a standardized protocol for rapid genotype-independent transformation and regeneration. Various explants like hypocotyls and cotyledons of *Gossypium hirsutum* were inoculated with a suspension of *A. tumefaciens* and differentiated somatic embryos, which eventually germinated and developed into mature plants over a period of 8–10 month distinct stages[28,29]. Somatic embryogenesis and regeneration of Australian cotton cultivars, Siokra 1–3 via *A. tumefaciens* has been used to efficiently produce fertile transgenic plants to express the novel genes such as neomycin phospho transferase (*nptII*) and *gus*[30]. Genetic transformation of cotyledon and hypocotyl explants with reporter gene *gus* and selection marker *npt*II, and individual embryogenic colonies was tested for the presence of transgene by appropriate assays like *npt*II and *gus* histochemical assay and plantlets were regenerated[31]. Transgenic cotton plant of Texas cultivar (UBQHRPIS) obtained using *Agrobacterium*-mediated transformation with shoot apex explants[32]. Various aspects of transformation and regeneration process to improve efficiency in the production of transgenic cotton with green fluorescent protein (GFP) gene by *Agrobacterium* mediation, high frequency stable transformation by particle bombardment[33], and embryogenic cell suspension culture reported transient and stable expression of β-glucuronidase gene which was monitored on cell suspension culture[34]. Transformation protocol was reported for Indian varieties through *Agrobacterium*-mediated transfer using shoot tips[35]. The presence of *gus* and *nptII* genes in the transgenic plants was verified by histochemical GUS assay and PCR analysis. Transformation of *cry1A*(*b*) gene into Indian cotton cultivar (Anjali) was reported and confirmed the gene integration by Southern blot and expression of Cry protein by ELISA test[36]. High-frequency shoot recovery from cotton transformation has been obtained by slow desiccation[37]. Factors influencing *Agrobacterium*-mediated transfer[38] and tissue-specific reduction of toxic gossypol by RNAi approach disrupts gossypol biosynthesis in cotton seed tissue and interfere the expression of the δ-cadinene

synthase gene[39]. *Agrobacterium*-mediated genetic transformation of an elite Indian genotype (Bikaneri Nerma) of cotton (*G. hirsutum* L.) was obtained using shoot apical meristems isolated from seedlings as explants and co-cultivating with gene construct having a synthetic gene encoding Cry 1Ac δ-endotoxins of *Bacillus thuringiensis*[40]. In our earlier report on the development of CLCuV transgenics with *rep* gene using *Agrobacterium*-mediated transformation, we could achieve 0.3% of transformation frequency[41,42].

Materials and methods

Transformation system

Seeds of cotton variety HS 6 were obtained from Central Institute for Cotton Research (Regional Station), Sirsa, Haryana, India.

Bacterial strain and vector

A. tumefaciens strain EHA 105 harbouring a binary plasmid pBIN AR with ACP gene and *nptII* gene as selection marker in the T-DNA driven by Cauliflower mosaic virus (35S CaMV) promoter and NOS-terminator was used as the vector system for transformation (Figure 1). Bacterial culture was maintained on YEMA medium (1.0% w/v yeast extract, 1.0% w/v mannitol, 0.1% w/v sodium chloride, 0.2% w/v magnesium sulphate, pH 7.0) containing 50 mg/l kanamycin and 25 mg/l rifampicin. For inoculation, a single colony was grown overnight on liquid YEMA at 28°C with appropriate antibiotics.

Transformation of cotton plants

Cotton variety HS 6 seedlings were raised aseptically on half-Murashige and Skoog (MS) medium. The embryonic axes were excised and trimmed from both the sides and used for co-cultivation with *A. tumefaciens*. The explants were co-cultivated in the half-MS liquid medium with actively growing culture of *A. tumefaciens* at 1.0 OD and 100 mM acetosyringone. After overnight co-cultivation, shoots were decontaminated in the half-MS medium containing 250 mg/l cefotaxime. The explants were then transferred to the selection medium containing 0.1 mg/l kinetin, 0.1 mg/1 BAP and 50 mg/l kanamycin. The kanamycin-resistant shoots were sub-cultured in a medium containing 0.1 mg/l BAP for root induction. Rooted plants were rinsed well and transferred to pots containing peat, soil and sand in the ratio 1 : 1 : 1. Plants were covered with plastic bags and then transferred to a pot with soil for hardening for 15 days before shifting them to the greenhouse under natural condition. T_1 plants were raised in the greenhouse.

Screening for transformed plants using PCR

Genomic DNA was isolated as described by Paterson *et al.*[43] from the young leaves of T_0 plants grown in the polyhouse. The template DNA was used for PCR amplification with ACP gene-specific primer (5′–3′) F-CATGAATTCATGTCGAAGCGAGC and R-TTAAAGCTTTAATCCAACAAA and *nptII* specific primer F-GAGGCTAATTCGGCTATGACTG and R-ATCGGGAGAGGCGATACCGTA. PCR was performed in 20 µl (total volume) reaction mixture containing 1.0 µl of 100 ng DNA, 2.0 µl 10× reaction buffer, 2.0 µl of 10 mM dNTPs, 1.0 µl of 100 nM of each forward and reverse primer, 3.0 µl of 25 mM $MgCl_2$ and 0.5 µl of 1U *Taq* DNA polymerase. The following PCR conditions of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min and 5 min of final extension at 72°C were maintained in a thermo cycler (BIOMETRA). The amplified products were electrophoresed on 1% w/v agarose gel and documented.

RT–PCR

The mRNA was isolated using mRNA capture kit (Roche, Germany) from young leaves of T_0 plants. The mRNA was used for cDNA synthesis by Transcriptor high fidelity cDNA kit (Roche, Germany).

Southern hybridization of transformed plants

To confirm the gene integration in the transgenic plants (T_0 plants) Southern blotting method was used[44]. Genomic DNA was isolated from the leaves of the T_0 plants. For Southern hybridization, 10 µg of total genomic DNA

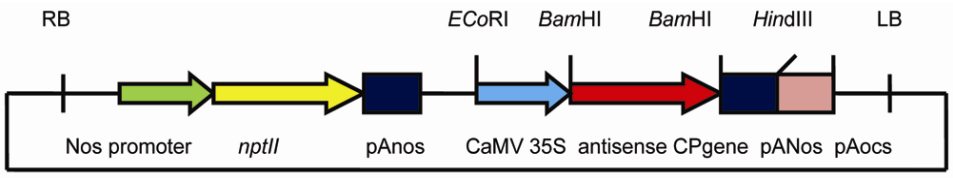

Figure 1. Schematic representation of the binary vector pBin AR carrying full length of 35S CaMV promoter sequence and 770 bp of coat protein (CP) gene in antisense orientation. LB, Left border; RB, Right border.

from the putative transgenics was digested with *Eco*RI and *Hin*dIII and visualized in 0.8% agarose. The probe ACP gene was labelled with non-radioactive DIG labelling kit (Roche, Germany).

Results

Plant transformation and regeneration

Embryonic axes measuring about 5–10 mm from 2 to 3-day-old cotyledons were trimmed and used for co-cultivation (Figure 2 *a*). After co-cultivation they were selected in MS medium with 50 mg/l kanamycin as selection marker, which allows only the transformants to grow. Shoot induction was observed after 10–15 days in the shoot induction medium (Figure 2 *b*). The putatively transformed shoots were sub-cultured twice in the shooting medium and then transferred to rooting medium after the shoots attained a height of 5–6 cm (Figure 2 *c*). Rooted plants were rinsed well and transferred to pots containing peat, soil and sand in the ratio 1 : 1 : 1 (Figure 2 *d*). The number of embryonic axes used for the experiment and the transformation frequency are given in Table 1. T_1 plants were grown in the greenhouse under controlled conditions.

Molecular analysis of transformants

The genomic DNA of the T_0 plants was tested for the presence of the *nptII* gene by using specific primers and PCR products yielded a 700 bp amplicon (Figure 3). Presence of ACP gene was also confirmed by amplifying the gene with the CP gene-specific primer and the expected 770 bp segment was observed (Figure 4). PCR-positive T_0 plants were further analysed for integration of the gene into the plant genome by Southern blot hybridization. Southern hybridization was carried out to confirm the integration of the transgene in the T_2 generation with the ACP gene probe. The non-radioactive dig-labelled probe was hybridized with the blot. After washing, the blot showed integration of the gene in the transgenic plants (Figure 5) and no band was seen in the non-transformed plant. PCR analysis of 36 T_2 plants revealed 12 plants for the presence of gene integration, which fits in the Mendelian ratio 3 : 1 of segregation and λ^2 value at 1 df on 5% level of significance was 0.12 (Table 2).

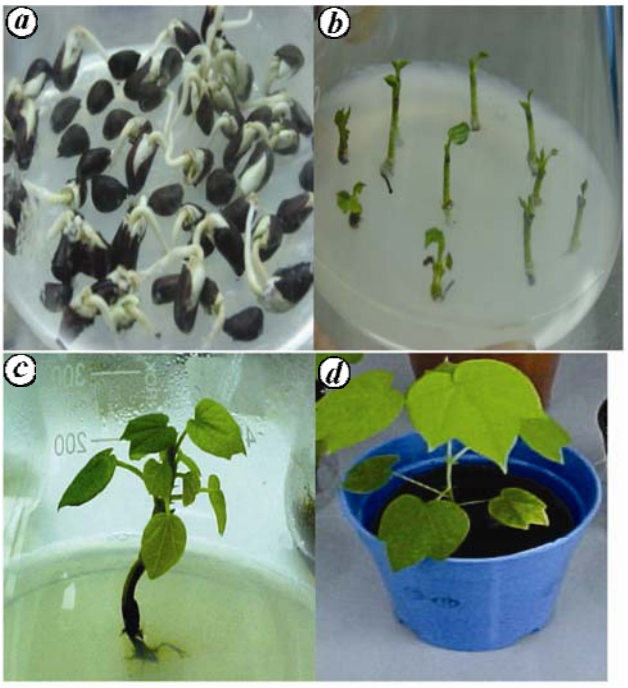

Figure 2. ***a***, Three-day-old embryonic axes. ***b***, Transformed shoots in the selection medium; ***c***, Rooting of the shoots, and ***d***, Hardening of the rooted plants.

RT–PCR

The mRNA isolated from the young leaves and cDNA was synthesized. The cDNA was amplified with the CP gene-specific primer with the PCR conditions as above, which could produce the specific product of 770 bp (Figure 6).

Viruliferous whitefly screening

Individual transgenic events were screened with viruliferous whiteflies (24 h after the acquisition period) in the polyhouse. The transgenic plants were under screening for one month and they were observed for the disease symptom (Figure 7). The resistant transgenics did not show any symptoms and were maintained in the greenhouse.

Discussion

Rapid advances in the techniques of molecular biology have resulted in the cloning and sequence analysis of the genomic components of a number of plant viruses. A majority of plant viruses have a single-stranded positive-sense

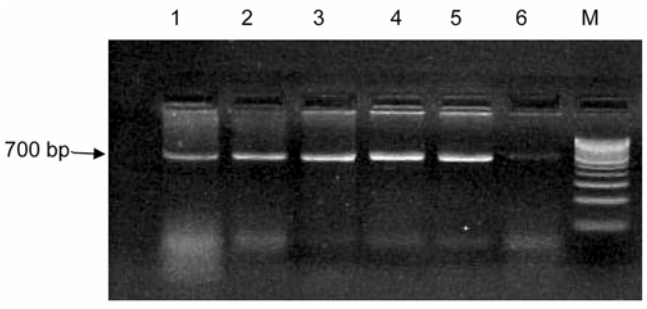

Figure 3. Electrophoresis analysis of PCR products of transgenic cotton plants showing the presence of the expected 700 bp fragment of *nptII* gene. Lanes 1–6, Transformed plant DNA; lane M, 100 bp marker.

Table 1. The number of embryonic axes used and transformation frequency

Variety HS 6	Gene construct (EHA 105)	No. of explants (EA)	Putative transformants	*nptII* positive	Transformation frequency (%)
Experiment I	ACP	890	9	3	0.33
Experiment II	ACP	990	13	4	0.40
Experiment III	ACP	945	10	3	0.32

ACP, Antisense coat protein.

Table 2. Segregation analysis of T_2 plants derived from selfed T_0 plants

Variety	No. of plants tested	*nptII* and gene-specific positive	Segregation ratio	λ^2 value at 1 d*f*	*P*-value
Experiment I HS 6	36	11	3 : 1	0.12	0.729

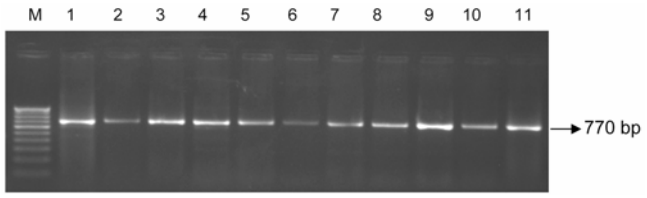

Figure 4. Electrophoresis analysis of PCR products of transgenic cotton plants showing the presence of the expected 770 bp fragment of CP gene. Lanes 1–11, Transformed plant DNA; lane 12, Plasmid DNA (control), and lane M, 100 bp marker.

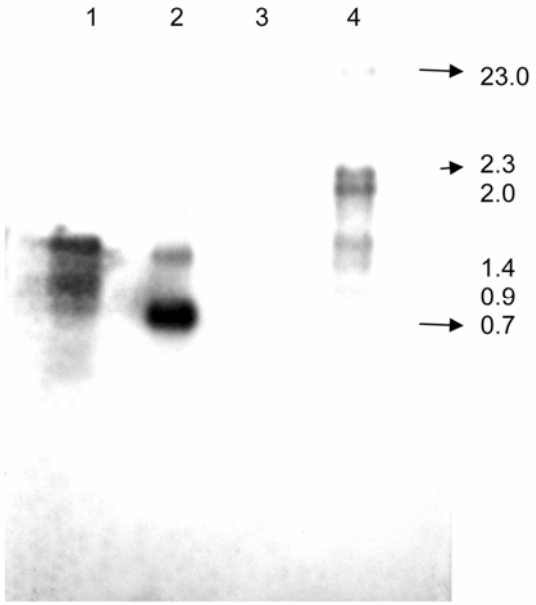

Figure 5. Southern blot analysis of T_0 transgenic plants. Genomic DNA (10 μg) was digested with *Hin*dIII and hybridized with antisense CP gene probe labelled with non-radioactive dig-labelling method. Lanes 1, 2, Individual transgenic plant genomic DNA; lane 3, Non-transformed plant DNA and lane 4, Lamda double digest (*Eco*RI and *Hin*dIII).

RNA as the genome. However, some of the most important viruses in tropical countries like India have ssDNA and dsDNA genomes and RNA genomes of ambience polarity, i.e. genes oriented in both directions. Concomitantly, tremendous advances have taken place in our understanding of plant–virus interaction in the process of pathogenesis and resistance. These, along with associated advances in the genetic transformation of a number of crop plants, have opened up the possibility of an entirely new approach of genetic engineering towards controlling plant virus diseases.

The efforts by Mubin *et al.*[45] to engineer resistance against the Geminiviruses were mainly focused on silencing of complementary-sense virus genes involved in virus replication. They selected movement protein (*AV2*) gene to develop resistance against Tomato leaf curl virus (ToLCV). We have focused on silencing the CP gene using ACP gene (*AV1*). Cotton transgenics developed by Sanjaya *et al.*[46] in an Indian variety (F846) was resistant against CLCuD. They introduced the antisense movement protein gene (*AV2*) via *Agrobacterium*-mediated transformation using the shoot tips from two-day-old embryo as explants. In the present study the genotype HS 6 was used for transformation and transformed the antisense gene for CP (*AV1*) cloned into the vector, pBin AR. We have chosen explants from embryonic axes of two-day-old cotyledons and co-cultivated with late log-phase culture overnight.

Regeneration of cotton is genotype-specific nature and our methodology is genotype-independent. Coker genotypes, which are amenable for regeneration by somatic embryogenesis, are widely used in genetic transformation experiments[25,33,37,47]. Limited success has been obtained with shoot meristems till recently by low transformation efficiency protocol[26,35]. Genotype-independent procedure to transform non-coker genotypes have been reported by Gizant and Weintraub[16]. Cotton transgenics with antisense *AV2* gene for resistance against CLCuV have been reported by Sanjaya *et al.*[46]. In this study successful introduction of ACP gene through *Agrobacterium*-mediated transformation method was made. Geminiviruses can cause significant yield losses and accumulate in the plant cell nuclei where they replicate and develop disease. The antisense RNA pairs with the complementary target mRNA and will inhibit the expression of homologous

genes by degrading the target mRNA and thus prevent translation. The rationale of antisense RNA technology leading to gene silencing is the formation of dsRNA from sense/antisense counterparts of endogenous/transgene segments, which initiates the surveillance system within the plant, for degradation of transgene mRNA and target RNA[18,48]. Antisense RNA is actually a part of the complex natural pathways for gene regulation by HDGS mechanisms, where sense transcripts are able to silence gene expression[49,50].

Transgenic plants with ACP gene will arrest the replication of the invading viral genome by targeting the complementary mRNA produced by the plant. Cotton transgenics obtained in the present study pave the way to develop virus resistance in cotton. PTGS results in the degradation of RNA from host genes and homologous transgene after transcription in the nucleus. Several models have been postulated to explain the observed gene silencing phenomenon, which includes the involvement of antisense RNA[17], cosuppression[51] and RNA interference[52].

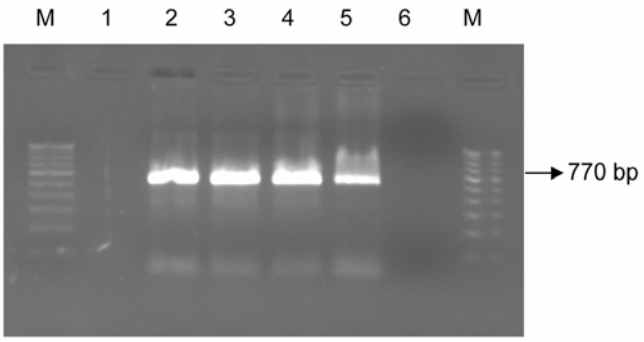

Figure 6. RT–PCR products of transgenic cotton plants showing the presence of the expected 770 bp fragment of CP gene. Lanes 1 and 6, Non-transformed plant DNA; lanes 2–5, Transformed DNA, and lanes M, 100 bp marker.

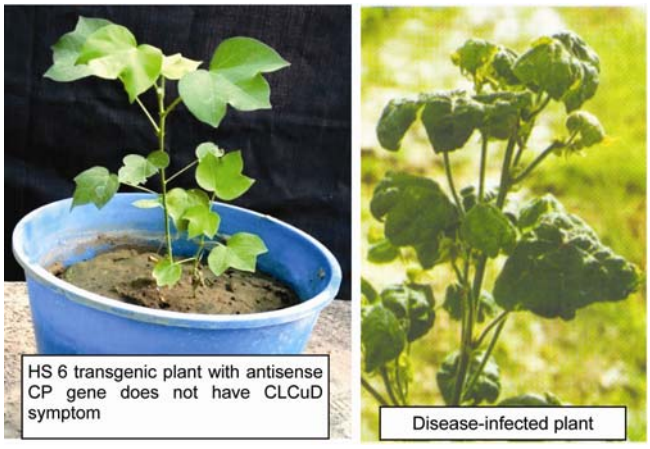

Figure 7. Green house plants were screened for cotton leaf curl disease (CLCuD) by inoculating the transgenics with viruliferous whitefly (24 h after acquisition period).

Our experiment agrees with most of the strategies for genetically engineered resistance to begomoviruses involving ACPs, and studies have focused on using partial, entire, sense/antisense or mutated begomovirus *rep* genes[11,53]. There have been a number of models proposed for the induction and operation of gene silencing involved with antisense strategies. In most models, it was demonstrated that by pairing with a complementary targeted RNA, the antisense RNA would inhibit expression of the homologous gene by preventing translation or promoting degradation of the targeting RNA[7,22]. ACP gene of CLCuV would in principle block the viral CP gene expression either by preventing translation or through homology-dependent degradation of target viral RNA. The resistance to leaf curl disease demonstrated in this study, with ACP gene construct has antisense RNA that inhibits the expression of the sense gene by pairing and leading to the degradation of targeting RNA, which is in agreement with the studies of Yang *et al.*[11]. Shelly *et al.*[54,55] showed that transforming ToLCV plants with the homologous replicase gene constructs that produce RNAs capable of duplex formation, confers gene silencing and results in the recovery of the infected plants. They proposed that the antisense suppression in the virus-infected plants provides a threshold level of dsRNA, triggers the degradation mechanism and high amounts of siRNAs get accumulated. These siRNAs induce gene silencing leading to viral suppression, which is in agreement with our study. There was delay in development of the disease in the transgenic plants after screening for the virus, similar to that reported with CP gene in tobacco[56].

Transgenic plants expressing chimeric transgene from ToLCV and cucumber mosaic virus (CMV), carrying the genes *CMV*-cp and *ToLCV-cons-rep* from isolates of CMV and ToLCV, which are transcriptionally fused under the control of CaMV 35S promoter showed that after infection with transforming ToLCV and CMV that produced RNAs, capable of duplex formation, confers gene silencing. It was also proposed that the antisense suppression in ToLCV-infected plants provides a threshold level of dsRNA needed to induce gene silencing, whereas sense suppression in CMV-infected plants may be operating through cosuppression, leading to delayed and attenuated symptoms[50,57]. Mubin *et al.*[45], had reported engineering resistance against the geminiviruses by silencing of complementary-sense virus genes involved in virus replication by selecting *AV2* gene to develop resistance against ToLCV. The tomato plants transformed with an antisense construct targeting the *AV2* gene were resistant to the virus following the challenge with the virus, transgenic plants remained symptomless, although viral DNA could be detected in some plants by PCR.

To explain our results, the cornerstone of our model is gene silencing, induced by the formation of duplex RNA with sense (viral origin) and antisense (transgene), resulting in the recovery of virus-infected plants. For CLCuD,

an antisense approach seems to have better potential to get specific resistance. CLCuD has only one molecule of a single-stranded DNA, DNA-A, which codes for two proteins, AV1 or CP, AV2 or movement protein in the sense orientation and four proteins, viz. AC1, AC2, AC3 and AC4 in antisense orientation, and lacks the DNA-B component[58]. Thus the function of DNA-B-coded proteins, viz. BV1 (involved in the transport of viral DNA into/out of the nucleus) and BC1 (involved in cell-to-cell movement) is performed by the AV1 and AV2 respectively, in these monopartite begomoviruses. Thus antisense RNA of AV1 will form a duplex RNA with sense (viral origin) and would disrupt the function the gene. Therefore viral replication, movement and encapsidation will be affected. Transgenic plants expressing ACP gene (*AV1*) developed in the present study could result in the arrest of infection by interference with viral replication, movement and encapsidation.

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