



ORIGINAL ARTICLE

Development of Cotton leaf curl virus resistant transgenic cotton using antisense β C1 gene



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Abstract Cotton leaf curl virus (CLCuV) is a serious pathogen causing leaf curl disease and affecting the cotton production in major growing areas. The transgenic cotton (*Gossypium hirsutum* cv. Coker 310) plants were developed by using β C1 gene in antisense orientation gene driven by Cauliflower mosaic virus-35S promoter and nos (nopaline synthase) terminator and mediated by *Agrobacterium tumefaciens* transformation and somatic embryogenesis system. Molecular confirmation of the transformants was carried out by polymerase chain reaction (PCR) and Southern blot hybridization. The developed transgenic and inoculated plants remained symptomless till their growth period. In conclusion, the plants were observed as resistant to CLCuV.

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1. Introduction

Cotton growth and productivity is severely reduced by various types of stress. Therefore, adequate techniques and management is required to increase its productivity. Cotton leaf curl virus (CLCuV) has emerged as a serious threat to cotton plants by causing leaf curl disease and affecting cotton production by making up to 80% loss in North India and Pakistan (Varma and Malathi, 2003; Mansoor et al., 2003; Sattar et al., 2013). Cotton leaf curl virus belongs to begomovirus group, family, *Geminiviridae*. CLCuV possesses DNA-A and a satellite molecule known as β DNA. The β DNA has only one gene (β C1 gene). It acts as the pathogenicity, a suppressor of post transcriptional gene silencing (Hammond et al., 2001) and

Abbreviations: CLCuV, Cotton leaf curl virus; CP, coat protein; PCR, polymerase chain reaction; PDR, pathogen derived resistance; MS, Murashige and Skoog's; *A. tumefaciens*, *Agrobacterium tumefaciens*

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modulates the level of microRNAs development (Qazi et al., 2007; Saeed et al., 2005; Amin et al., 2011a,b).

Virus resistant plants can be developed by using pathogen derived resistance (PDR). PDR based on cross protection and antisense approach has long been employed in the management of viral diseases in plants. In case of begomoviruses, expression of viral coat protein (CP), replicase and movement proteins has been proved to be more promising (Yang et al., 2004). The CP mediated resistance has been successfully applied to numerous crop species (Prins, 2003; Pang et al., 2000).

Currently, various new technologies are being used like antisense RNA, RNAi, siRNA, miRNA to develop transgenic plants for virus resistance (Agrawal et al., 2003; Shelly et al., 2005; Brodersen et al., 2008; Prins et al., 2008; Ai et al., 2011; Vu et al., 2012; Ali et al., 2013). A number of CLCuV genome and β DNA fragments can be exploited to repress the expression of viral genes. The development of CLCuV transgenic with rep gene using *A. Tumefaciens* mediated transformation has been reported earlier by 0.3% of transformation frequency (Balasubramani et al., 2003; Katageri et al., 2007; Amudha et al., 2011; Hashmi et al., 2011). Recently, transgenic tobacco resistant to Cotton leaf curl Burewala virus has been developed by using artificial microRNA technology (Ali et al., 2013). Various explants like hypocotyl, shoot apex and cotyledons of cotton were inoculated with a suspension of *A. tumefaciens* and differentiated somatic embryos, which eventually germinated and developed into mature plants (Kumar and Tuli 2004; Tenllado et al., 2004; Amudha et al., 2011). The present study was designed for the development of transgenic cotton plant resistant to CLCuV through expression of antisense β C1 genes by *A. tumefaciens* mediated transformation using a standardized protocol for rapid genotype-independent transformation and regeneration (Kumar and Tuli, 2004). *A. tumefaciens* mediated transformation protocol has been reported for Indian varieties using shoot tips as explants. In this study, the developed cotton plants showed better resistance against CLCuV as compared to non transgenic plants.

2. Materials and methods

2.1. Cotton seed material

Cotton (*Gossypium hirsutum* L.) cv. Coker 312 seeds were washed with tap and double distilled water. The seeds were sterilized with HgCl_2 (0.1%) followed by rinsing with sterile water. The seeds were inoculated in 1/2 MS medium (Murashighe and Skoog's) and incubated for 2 weeks at 16 h photo period, 28 °C temperature for germination on culture racks.

2.2. Callus induction and somatic embryo proliferation

The hypocotyls were excised from germinated cotton seedlings and used as explants. The callus induction and somatic embryo proliferation were obtained after culturing on modified MS medium (Kumar and Tuli, 2004).

2.3. Bacterial strain and vector

The β C1 gene was amplified by PCR and cloned into pGEMT-Easy vector in antisense orientation. The resulting clones were sequenced and further sub-cloned into binary vector-PBI 121 and electroporation was used to mobilize into *A. Tumefaciens* (strain LBA 4404). The confirmation of correct clones were done by colony PCR and sequencing. Bacterial culture was maintained on luria broth medium containing kanamycin (50 mg/l) and Rifampicin (25 mg/l). The Agrobacterium culture was developed by inoculating and overnight culture of a single colony in the Agrobacterium minimal medium. The design of gene construct is presented in Fig. 1.

2.4. Cotton transformation

The hypocotyl explants were co-cultivated with bacterial culture prepared in Agrobacterium minimal medium supplemented with 100 μM Acetosyringone. Half-MS medium and Cefotaxime (300 mg/ml) was used for washing of co-cultivated explants and further cultured on MS medium containing growth hormone 2,4-D (0.5 mg/l) and BAP (0.1 mg/l). After 4 weeks, the explants were further sub-cultured on MS medium for somatic embryo induction till 6 weeks. The resistant somatic embryos were sub-cultured in a MS medium for elongation. The elongated somatic embryos were cultured on MS with BAP and GA3 medium for germination. The germinated seedling were hardened well and transferred to bigger pots and finally shifted to greenhouse under natural condition.

2.5. Screening for transformed plants using PCR

DNeasy plant mini kit (Qiagen) was used to isolate DNA from newly emerged cotton leaves. The template DNA was used for PCR amplification with β C1 gene (antisense) primer F-T CACCATCGCTAATCAAGTATG and R-CATTGCTGGT TTGTGTTTGGAA. The PCR reaction was performed in a mixture, containing 1.0 μl DNA (50 ng) 2.0 μl buffer (10 \times), 2.0 μl dNTPs (10 Mm), 0.5 μl of 100 ng forward and reverse primer, 0.5 μl of 2.5U *Taq* DNA polymerase. The following

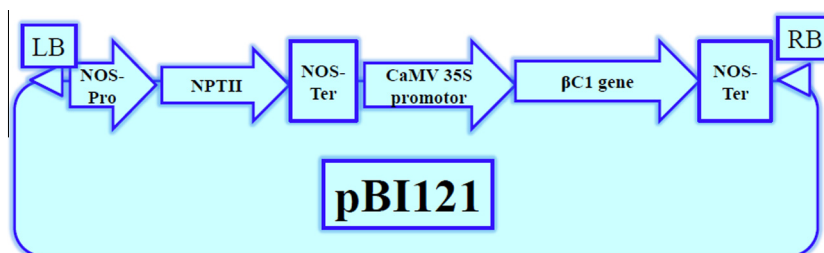


Figure 1 Schematic representation of the binary vector pBI 121 carrying full length of 35S CaMV promoter, NPTII, NOS promoter and terminator and β C1 gene in antisense orientation. LB, Left border; RB, Right border.

PCR conditions of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min followed by extension for 5 min at 72 °C was used. Southern blotting was performed by using PCR amplified products. The blot was hybridized with random labeled β C1 gene with P^{32} dCTP probe following the standard Southern hybridization protocol (Sambrook et al., 1989).

2.6. Inoculation of whiteflies

The virus inoculation was done by using whitefly (*Bemisia tabaci*) on individually raised cotton seedlings. Healthy whiteflies were reared and maintained on tobacco plants under insect proof greenhouse. The acquisition access was given for 24 h on infected cotton plants and thereafter a group of 15–20 whiteflies were released to putative transgenic cotton plants and after 2 days, the whiteflies were killed by insecticide spraying. The developed plants were further tested at T2 stage for resistance in the greenhouse and natural field. The inoculated plants were kept under observation till their growth period for symptom development. The virus infection was confirmed by PCR in both transgenic and non transgenic plants (data not shown).

3. Results and discussion

3.1. Cotton transformation and regeneration

A total of 2346 hypocotyl explants were co-cultivated with *Agrobacterium* culture in 4 independent experiments and 475 transformed explants were further selected by using kanamycin 100 mg/l. The callus formation was observed after 20 days culture on callus induction medium. The embryogenic callus was observed after 3–4 subculture and embryo formation were observed just after five sub-cultures. The developed embryos were germinated elongated in the elongation medium. After two weeks proper root formation took place upon culture in the rooting medium. Total, nineteen PCR positive cotton plants were regenerated from transformed tissue and hardened into big pots (Fig. 2, Table 1).

3.2. Molecular confirmation of transformants

The developed plants were shifted to bigger pots and maintained under greenhouse. The presence of β C1 gene was confirmed by PCR amplification of developed plants (Fig. 3).

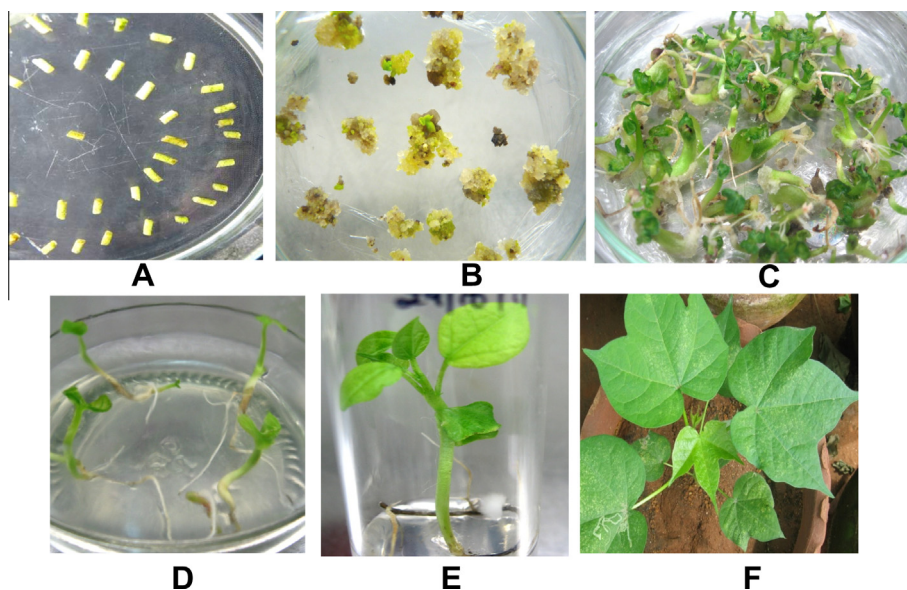


Figure 2 Stage of transformed cotton regeneration and plant development. (A) Hypocotyls. (B) Somatic embryos. (C) Emerged cotton plants. (D) Elongated plants. (E) Rooted plants. (F) Hardened cotton plant.

Table 1 Number of hypocotyls (Coker 312) used and plants developed.

S. No.	Number of experiments	Gene constructs used	Total No. of explants used	Total number of plants developed (PCR positive)
1	1	β C1 gene	580	5
2	2	β C1 gene	576	2
3	3	β C1 gene	598	6
4	4	β C1 gene	592	6
Total			2346	19

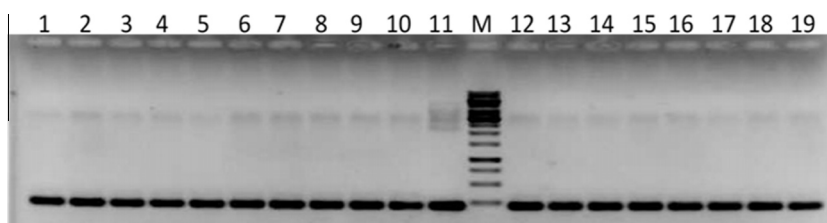


Figure 3 Electrophoretic analysis of putative cotton transgenic showing PCR amplification of β C1 gene using specific primers. Lane 1–19. Putative plants; M: 1 kb ladder.

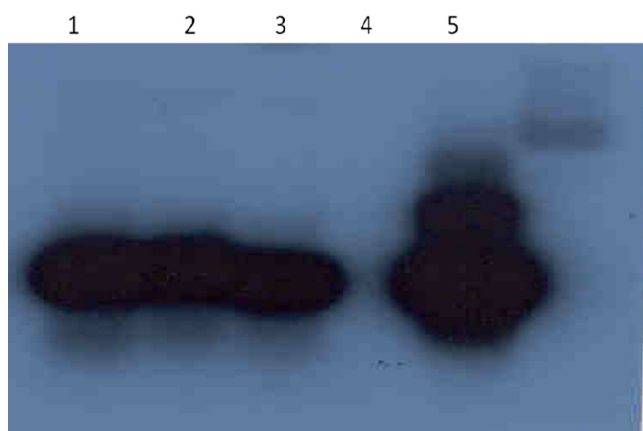


Figure 4 Southern blot analysis of transgenic cotton plants. Lane 1–3; Independent transgenic cotton lines Lane 4; Non-transformed cotton. 5: Positive control (Cloned DNA).

The Southern blot results confirm the gene integration in developed transgenic plants (Fig. 4).

3.3. Whitefly screening and evaluation of resistance

Resistance evaluation was done for individual plants by inoculation with Cotton leaf curl virus by a whiteflies vector (*B. tabaci*). After whiteflies inoculation, leaf curling was observed in non transgenic plants. All T1 plants showed 60–70% resistance as compared to control. The few lines remained free from symptoms throughout the growth period. Based on the resistance, three plants were finally selected showed good resistance.

We found that entire β C1 gene integrated with antisense orientation in susceptible cotton plants were very much promising to combat viral infection in outdoor condition. Similar report showed that begomovirus gene has been utilized for developing pathogen derived resistance against plant disease which involved the use of entire, partial, sense, antisense, truncated viral genomes or artificial microRNA (Yang et al., 2004; Shelly et al., 2005; Amudha et al., 2011; Hashmi et al., 2011; Ali et al., 2013). Numerous models have been proposed for the gene silencing induction and operation associated with antisense, MicroRNA, SiRNA and RNAi strategies (Meister and Tusch, 2004; Brodersen et al., 2008; Ali et al., 2013). The antisense approach has also been utilized, based on homology dependent for development of resistant transgenic plants against Tomato yellow leaf curl virus resistance (Yang et al., 2004), Tomato leaf curl virus (Praveen et al., 2005), Cotton leaf curl virus (Asad et al., 2003) and Bean golden mosaic

virus (Aragao et al., 2013). Cotton transgenic has been developed earlier by using antisense movement protein gene an Indian variety (F846) and was observed to be resistant against cotton leaf curl disease (Sanjaya et al., 2005). The genetic transformation and regeneration of cotton (*G. hirsutum* cv. Coker 310) plants have been developed earlier by using somatic embryogenesis (Chaudhary et al., 2004; Kumar and Tuli 2004). However, in our study, the β C1 gene has been used to transform the hypocotyls of cotton (Coker 312). The developed plants were confirmed by PCR and Southern blot hybridization. Southern blot hybridization is the excellent techniques to determine the virus load in the plant tissue and has a direct correlation with disease resistance (Taylor et al., 2004). The developed cotton lines showed a variable resistance pattern against Cotton leaf curl virus after inoculation, not only in the greenhouse, but also in open field conditions. It is observed that the level of virus accumulation in transgenic plants was low as it produced delayed or less severe symptoms. Finally, we were able to select some resistant plants with significant or delayed symptom appearance. The developed plants have single copy integration in the cotton genome and their inheritance pattern showed the stable integration, but their stability will be further evaluated in the breeding program.

Conflict of interest

The authors confirm that there is no conflict of interest for the information presented in this manuscript.

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