Saudi Journal of Biological Sciences (2016) 23, 358–362

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Development of Cotton leaf curl virus resistant transgenic cotton using antisense ßC1 gene

Sayed Sartaj Sohrab [a,*], Mohammad A. Kamal [a], Abdul Ilah [c], Azamal Husen [d], P.S. Bhattacharya [b], D. Rana [b]

[a] King Fahd Medical Research Center, King Abdulaziz University, Post Box No. 80216, Jeddah 21589, Saudi Arabia
[b] Division of Biotechnology, JK-AgriGenetics Ltd., Hyderabad, A.P., India
[c] Faculty of Medical Technology, Omar Al Mukhtar University, Tobruk, Libya
[d] Department of Biology, College of Natural and Computational Sciences, University of Gondar, Post Box No. 196, Gondar, Ethiopia

Received 4 November 2014; revised 10 November 2014; accepted 10 November 2014
Available online 21 November 2014

KEYWORDS

Transgenic cotton plants;
CLCuV resistance;
Gene transfer;
Molecular verification;
Virus resistance

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 βC1 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.

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, *Geminivirideae*. CLCuV possesses DNA-A and a satellite molecule known as β DNA. The β DNA has only one gene (βC1gene). 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*

* Corresponding author at: Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Post Box No. 80216, Jeddah 21589, Saudi Arabia. Tel.: +966 554627872, +966 64001000x73530; fax: +966 6952521.
E-mail addresses: ssohrab@kau.edu.sa, sohrab_sartaj2@rediffmail.com (S.S. Sohrab).

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2014.11.013
1319-562X

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 βC1genes 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 βC1gene 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 βC1gene (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×), 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

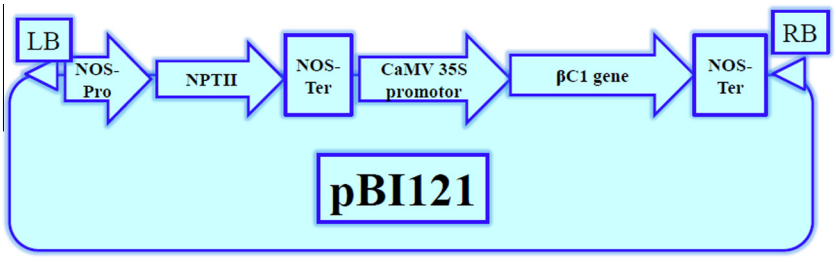

Figure 1 Schematic representation of the binary vector pBI 121 carrying full length of 35S CaMV promoter, NPTII, NOS promotor and terminator and βC1 gene in antisense orientation. LB, Left border; RB, Right border.