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Full Length Article

Characterization of a Strong Constitutive Promoter from Cotton Leaf Curl Kokhran Virus for High Level Gene Expression in Monocotyledonous and Dicotyledonous Plants

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Abstract

The complementary sense rep gene (*C1* rep) promoter of cotton leaf curl Kokhran virus (CLCuKoV) was cloned to assess its constitutive activity by expressing GUS gene both in dicotyledonous and monocotyledonous plants through agro-infiltration or by biolistic methods. Results showed higher GUS expression both in dicotyledonous (tobacco) and monocotyledonous (sugarcane and maize) plants mediated by the *C1* rep promoter as compared to cauliflower mosaic virus promoter (CaMV 35 S). This study further indicated that *C1* rep promoter beside dicotyledonous plants could be used to achieve higher constitutive expression in monocotyledonous plants as well. © 2014 Friends Science Publishers

Keywords: *CI* rep; GUS; Monocots; Dicots; Promoter; *Agrobacterium*; CaMV35S

Introduction

Plant genetic engineering has been widely used to engineer novel traits in plants. Plants have been increasingly used for the expression of therapeutic and other industrial proteins. These engineered plants often express transgenes constitutively by the use of strong promoters. The 35S promoter isolated from *cauliflower mosaic virus* (CaMV) is the most common promoter used in plant genetic engineering. In certain applications, promoters stronger than CaMV35S are desirable. Although CaMV 35S promoter has been extensively used in dicotyledonous plants, studies performed earlier reported low expression of CaMV35S in monocotyledonous plants, especially in sugarcane (Chowdhury and Vasil, 1992; Elliott *et al.*, 1998; Liu *et al.*, 2003). In certain applications it has been observed that the gene expression driven under CaMV35S is poor in phloem cells. Many examples are there where strong promoters may help in better expression like RNA interference based technologies, where the suppression of gene expression depends on levels of short interfering RNAs (siRNAs). Monocotyledonous plants have different physiological systems as compared to dicotyledonous plants, therefore most of the time they respond differently from dicotyledonous plants. Such promoter system must be available with ability to express transgene in both kinds of crop plants. Recent trend in plant engineering is to express multiple genes for manipulation of several agronomic traits. In the first generation transgenic technology, a single gene is expressed, often driven by a strong promoter. In the second and third generation transgenic plants, two or more genes are being expressed for stacking multiple agronomic traits in transgenic crops (Halpin, 2005) which necessitate use of promoters with potentially higher activity.

Geminiviruses are small single-stranded DNA viruses with circular genome and infect both monocotyledonous and dicotyledonous plants. The genome size ranges from 2.6-2.8 kb with geminate capsid morphology (Hanley-Bowdoin *et al.*, 1999). The four genera of geminiviruses including Mastrevirus, Topocuvirus, Curtovirus and Begomovirus are well characterized and are divided on the basis of host range, genome organization and insect vector (Van Regenmortel *et al.*, 2000). Viruses of the genus Begomovirus mediate their transmission by whitefly (*Bemisia tabaci*) and infect only dicotyledonous plants**.** DNA A and DNA B are the two essential components of bipartite begomoviruses have their role in virus proliferation. Monopartite begomoviruses have only a single component equivalent to DNA A and are often associated with DNA satellites (Mansoor *et al.*, 2003a). Previous studies demonstrated that both monopartite and bipartite begomoviruses have regions termed as large intergenic regions (LIR) and common regions, which contain DNA sequences essential for gene regulation (Hanley-Bowdoin *et al.*, 1999).

Transcription of the genomes of geminiviruses is bidirectional, initiating in the intergenic region. Transcription can be initiated in both directions, for the

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replication associated protein (rep) expression and the coat protein expression, both of which are controlled by geminivirus promoter. Rep sequences are associated with rolling circle amplification, being involved in viral replication as well as acting as a transcriptional repressor of its own expression by binding to the iterated elements located in the large intergenic region (Sunter *et al.*, 1993; Hanley-Bowdoin *et al.*, 1999). Intergenic region of geminiviruses located between 5' ends of complementary and virion sense Open Reading Frame (ORFs) is essential for bidirectional transcription of these genes (of complementary and virion sense) and significantly possesses promoter activity (Xie *et al.*, 2003).

Cotton leaf curl Kokhran virus (CLCuKoV) is a monopartite begomovirus that infects cotton and other dicotyledonous plants (Mansoor *et al.*, 2003b). CLCuKoV is one of the several begomoviruses that are associated with the cotton leaf curl disease (Amrao *et al.*, 2010). The activity of *C1* rep gene promoter of CLCuKoV in dicotyledonous (tobacco) and monocotyledonous (maize and sugarcane) plants with *GUS* reporter gene was investigated. Quantitative *GUS* activity and comparison with *cauliflower mosaic virus* (CaMV) 35S promoter, the most commonly used gene promoter for plant genetic engineering was performed.

Materials and Methods

Promoter Isolation and Sequence Analysis

To isolate the promoter region from viral genome, CLCuKoV genomic DNA was extracted from infected plant leaves using CTAB method (Doyle and Doyle, 1990; Mansoor *et al.*, 2003a). Sequence specific primers were designed and custom synthesized. Oligonucleotides used to amplify CLCuKoV promoter were P1 (5′ GTTGACTAAAATTGAATCACC-3′) as forward with the addition of *Sac*I restriction site and P2 (5′-CAAACGCATACTTAGCAACG-3′) as reverse primer with the addition of *Sal*I restriction site. The product of 288 base pairs was amplified through PCR and cloned into pTZ57R/T (Fermentas, USA). The clone was custom sequenced to verify the DNA sequence of CLCuKoV *C1* Rep promoter.

Vector Construction

Two expression vectors pGNCI Rep (CLCuKoV *C1* Rep cloned in pGreen0029) and pGN35S (CaMV 35S promoter cloned in pGreen0029) for expression studies were constructed. For vector construction, a product of 288 bps of *C1* Rep lifted from *Sac*I and *Sal*I restriction sites and cloned in pJIT166 vector. The expression cassette (3kb approx) containing CLCuKoV *C1* Rep sequence, *GUS* reported gene and CaMV terminator lifted with *Sac*I and *Xho*I restriction sites and cloned into pGreen0029 binary plant expression vector.

Particle Bombardment

Particle bombardment to transfer DNA coated particles using Particle Delivery system (PDS-1000/He BioRad, USA) was performed. Before bombardment, rupture discs, micro-carriers, macro-carries holders and stopping screens were sterilized with 100% ethanol for 10 min followed by drying in contamination-free environment. For micro-carrier preparation, 3 mg of gold particles (1.0 μm) for 6 bombardments were used and freshly prepared gold particles were coated with plasmid DNA and were delivered through PDS-1000/He. Absolute ethanol for coating gold particles with plasmid DNA was used. The bombarded tissues (callus) were kept in dark at 25°C in growth room for 24 h before being processed for *GUS* staining.

Agro-infiltration

Two plant expression vectors, pGNC1-Rep and pGN35S were electroporated into *Agrobacterium tumefaciens* strain GV3101 (Fiedle and Wirth, 1988). For Agro-inoculation, glycerol stocks of *Agrobacterium* harboring gene constructs were streaked on solid LB minimal medium plates containing antibiotics (50 μg/μL kanamycin, 25 μg/μL rifampicin, 50 μg/μL tetracycline) and incubated at 28°C for 48 h. A single bacterial colony was picked with a sterile wire loop and incubated into 50 mL LB medium containing the required antibiotics and placed in a shaker (160 rpm) at 28ºC until the OD_{600} of culture reached 1.0 (Hanley-Bowdoin *et al.*, 1999). The cells were harvested by centrifugation at 6000 rpm for 8 min and resuspended in LB medium containing acetosyringone (final concentration 100 μM). For agro-inoculation, *Nicotiana benthamiana* plants at 4 to 5 leaf stage were selected and were not watered for 24 h before infiltration. Young leaves were infiltrated by gently pressing a 5 mL sterile syringe to the abaxial surface and depressing the plunger until water-soaked appearance was achieved.

GUS Assays and Histochemical *GUS* Staining

Histochemical *GUS* staining assays were performed for both constructs according to the Jefferson *et al.* (1987) method. Transient *GUS* expression studies were made 24 h after calli bombardment. Tissues were incubated at 37ºC overnight in dark in *GUS* substrate solution containing 1 mM X-Gluc, 50 mM sodium phosphate pH 7.0, 0.05% Triton X-100 and 0.5 mM potassium ferricyanide. After incubation with *GUS* substrate, tissues were destained (in case of plant leaves) with 70% and eventually with 100% ethanol. Tissues were examined for *GUS* expression and photographed under stereomicroscope.

Results

Sequence Homology of CLCuKoV *C1* Rep

In present study, the 288 bps sequence of *C1* Rep of

CLCuKoV promoter was isolated, which showed potential promoter activity with *GUS* reporter gene. The PCR amplified DNA product was cloned in T/A cloning vector, sequenced and was 100% identical to CLCuKoV cloned from Faisalabad (Mansoor *et al.*, 2003b).

Transient Expression Assay in Dicotyledonous Plant (*Nicotiana benthamiana*)

Transient expression assays were performed first in *Nicotiana benthamiana* plants to investigate the potential promoter activity of CLCuKoV *C1* Rep in dicotyledonous plants. The viral promoter constructs pGN*C1*-Rep in comparison with pGN35S was agro-infiltrated into young leaves of *Nicotiana benthamiana* plants. Young and newly emerging leaves were selected. Three days after infiltration plant leaves were detached and were subjected to histochemical *GUS* staining. Histochemical *GUS* staining of infiltrated leaves showed higher *GUS* activity in leaves with pGN*C1*-Rep as compared to *GUS* expression with pGN35S (positive control), while no *GUS* expression was observed in leaves inoculated with culture without any construct as negative control (Fig. 1). Stereomicroscopic studies of agro-inoculated leaves further confirmed that pGNC1-Rep has stronger constitutive *GUS* expression as compared to PGN35S.

Transient Expression Assay in Monocotyledonous Plant (*Zea mays*)

The purpose of present study was to characterize a super strong promoter for monocotyledonous plant system. Transient *GUS* expression studies were performed in *Zea mays* to check the activity of CLCuKoV *C1* Rep promoter in monocotyledonous plants. Transient assay was performed with two constructs pGNC1-Rep and pGN35S by agro-infiltration in leaves of *Zea mays*. Young and soft tissues were selected to ensure better infiltration of *Agrobacterium* culture. Infiltrated leaves were subjected to *GUS* staining assay 48 h after inoculation. Infiltration of *Zea mays* leaves with pGNC1-Rep and PGN35S (positive control) resulted in stronger *GUS* expression in leaves with pGNC1-Rep as number of blue foci were more as compared to mild and diffused *GUS* expression with pGN35S (Fig. 2). These results confirmed CLCuKoV *C1* Rep promoter as a strong promoter, which can be used in monocotyledonous plants for higher gene expression.

Transient Expression Assay of *C1*-Rep in *Saccharum officinarum*

To assess the transient expression of *GUS* gene under CLCuKoV *C1* Rep promoter in embryogenic calli of *S. officinarum*, particle bombardment was performed. The deliveries of transgenes (in this case pGNC1-Rep and pGN35S) into embryogenic calli tissues were mediated by

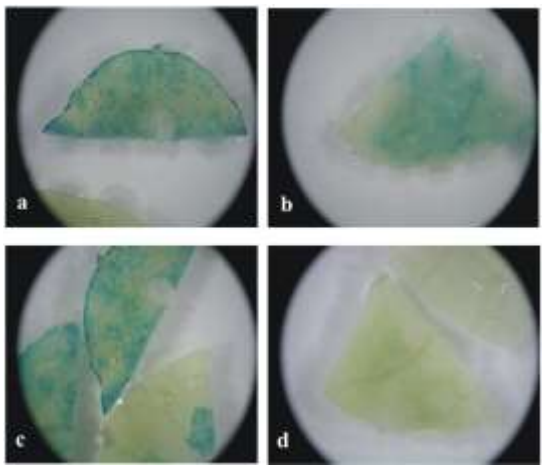

Fig. 1: Represents transient expression of *gus* gene driven by CLCuKoV *C1* Rep promoter in *Nicotiana benthamaiana* leaves after *GUS* staining. Panel a and c represents strong *gus* expression driven by CLCuKoV *C1* Rep in leaves. Panel b with CaMV promoter showing low *gus* expression as compare to *CI* Rep promoter of CLCuKoV with no *gus* expression in control (Panel d) *Nicotiana benthamaiana* leaves

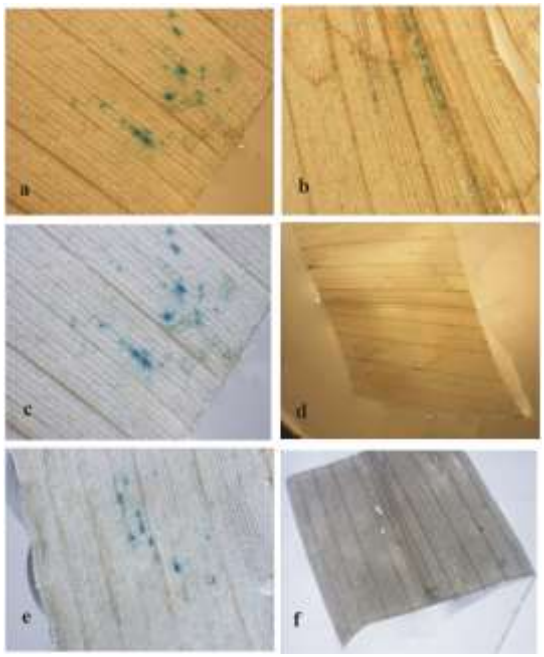

Fig. 2: Represents transient expression of *gus* gene driven by CLCuKoV *C1* Rep promoter in *Zea mays* leaves after *GUS* staining. Panel a, c and e represents strong *gus* expression as number of blue foci observed 3 days after infiltration into *Zea mays* driven by CLCuKoV *C1* Rep in leaves. Panel b and d with CaMV promoter showing low *gus* expression as compared to *CI* Rep promoter of CLCuKoV with no *gus* expression in control (Panel f) *Zea mays* leaves

particle bombardment. Forty days old calli of *S. officinarum* propagated on optimized callus induction medium were selected for particle bombardment. A large number of blue foci were observed on bombarded calli with constructs. Calli bombarded with pGNC1-Rep showed strong *GUS* expression as compared to *GUS* expression in calli with pGN35S (positive control). Overall expression of *GUS* was diffused and scattered (Fig. 3). *GUS* Expression became more diffused when the time for *GUS* substrate studies after bombardment was prolonged.

Discussion

The objective of the study presented here was to identify and characterize a viral promoter for strong expression as compared to CaMV35S promoter in monocotyledonous and dicotyledonous plants. The activity of *C1* Rep gene promoter of CLCuKoV was investigated in dicotyledonous (tobacco) and monocotyledonous (maize and sugarcane) plants with *GUS* reporter gene. Histochemical *GUS* staining assays and comparison with *cauliflower mosaic virus* 35S promoter was performed. The expression of *C1* Rep in tobacco was 4 to 5 fold higher than 35S promoter and is consistent with an earlier report by Xie *at al*. (2003), where CLCuMV *C1* promoter was evaluated in dicots.

After confirming strong promoter activity of *C1* rep in dicotyledonous plants, further expression studies were performed using monocotyledonous plant system. Results for all the experiments confirmed strong constitutive promoter activity of *C1* rep in monocotyledons plants as well. In comparison to CaMV35S, overall GUS expression was higher in maize leaves in all replicates (Fig. 2). Furthermore histochemical *GUS* staining assay studies of bombarded calli revealed *C1* Rep as strong promoter in sugarcane calli as the number of blue foci of *GUS* with *C1* Rep were higher than number of foci with CaMV35S promoter under stereomicroscopic studies (Fig. 3).

Most reported plant viral promoters showed low activity in comparison to CaMV35S. Zhan *et al*. (1991) reported *C1* promoter superior to *V1* promoter in the absence of trans-activator in *African cassava mosaic virus* (ACMV) and *Wheat dwarf virus* (WDV) [Hofer *et al.*, 1992]. CLCuKoV *C1* promoter superiority over the CaMV35S has special significance. *Figwart mosaic virus* (FMV) 34S and *Tobacco golden mosaic virus* (*TGMV)* coat protein promoters have 2-fold activity than that of CaMV35S (Verdaguer *et al.*, 1996). Complementary-sense promoter of *Tobacco yellow dwarf Mastrevirus* of family *Geminiviridae*, was reported to show only 15-20% of the CaMV 35S promoter activity (Xu *et al.*, 1998). As the efficient promoter activity of CaMV35S promoter is well established, for those plant systems with its reduced expression need to substitute with comparatively efficient transcription initiation sequences. For monocotyledonous plant system *C1* Rep promoter superiority over the CaMV35S validates its broader application for enhanced gene expression.

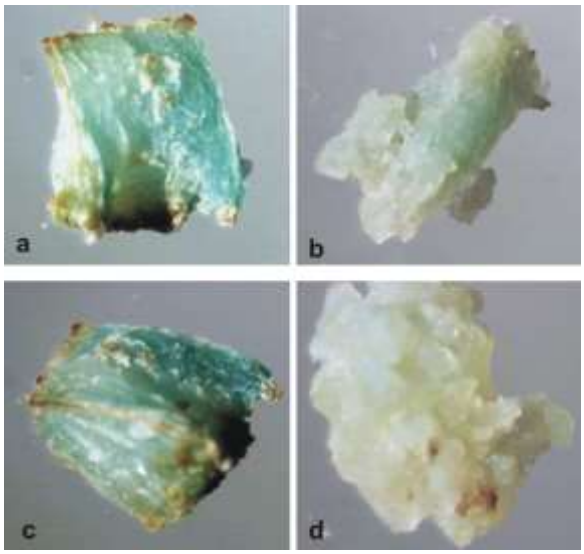

Fig. 3: Represents transient expression of *gus* gene driven by CLCuKoV *C1* Rep promoter in *Saccharum officinarum* calli after *GUS* staining. Particle bombardment was done to transformed promoter constructs into 40 days old *Saccharum officinarum* calli. Embryogenic calli was used for transient expression studies of bombard calli. Panel a and c represents strong *gus* expression driven by CLCuKoV *C1* Rep in leaves. Panel b CaMV promoter showing low *gus* expression as compare to *CI* Rep promoter of CLCuKoV with no *gus* expression in control (Panel d) non-transformed *Saccharum officinarum* calli

In conclusion, the present study demonstrates that *C1* Rep is a constitutive, super strong promoter for high level gene expression in monocotyledonous and dicotyledonous crop plants. The strength of *C1* Rep in driving gene expression significantly dominates over *Cauliflower mosaic virus* CaMV 35S promoter. This promoter has many potential uses including its important application for RNAi constructs to produce high copy number of short interfering (siRNA) for efficient gene silencing.

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