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Full Length Research Paper

Characterization of the replication-associated protein (*Rep*) promoter of an alpha-satellite associated with *Tobacco curly shoot virus*

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Alpha-satellites, nanovirus-like DNA components associated with begomoviruses (Family: *Geminiviridae*), encode a replication-associated protein (Rep) and depend on their helper viruses for spread within and between plants. In this study, using the *Agrobacterium*-mediated transient expression and stable transgenic systems, respectively we demonstrated that a 420 nt fragment located upstream of the *Rep* gene of Tobacco curly shoot alpha-satellite (TbCSA) was capable of initiating transcription of the *β-glucuronidase* (*GUS*) gene in tobacco plants, and the promoter activity was about 18% that of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Using *GUS* and the green fluorescent protein (*GFP*) as the reporter gene, respectively deletion analysis indicated that 3'-terminus deletion of the 420 nt fragment was not functional, while the 5'-terminus deletions exhibited varying promoter activities. Furthermore, the 227 nt fragment located upstream of the *Rep* gene showed the same promoter activity as the longest 420 nt fragment and this should be considered as the minimal promoter sequence. To our knowledge, this is the first report on the promoter of an alpha-satellite associated with begomoviruses.

Key words: Promoter, alpha-satellite, *Tobacco curly shoot virus*, begomoviruses.

INTRODUCTION

Members of the genus *Begomovirus* in the family Geminiviridae have either bipartite or monopartite single-stranded DNA genomes (Hanley-Bowdoin et al., 1999; Saunders et al., 2000). Recently, most monopartite begomoviruses have been shown to be associated with beta-satellites (formerly called DNA β) and in many cases, alpha-satellites (formerly called DNA 1) were also identified in the Old World (Briddon et al., 2003, 2004; Bull et al., 2004; Jiang and Zhou, 2005; Wu and Zhou, 2005; Guo et al., 2007; Xiong et al., 2007). Both of the satellites are approximately half the sizes of the viral genomic DNA (~1.4kb) and share little, if any, sequence identity with their helper viruses (Briddon et al., 2003, 2004; Xie et al., 2010). Beta-satellites contain a single gene called *βC1*, which encodes a pathogenicity protein (Cui et al., 2004; Saunders et al., 2004). It is probably believed that alpha-satellites had evolved from nano-viruses (*Nanoviridae*; another family of circular ssDNA viruses) that became associated with begomoviruses during mixed infections (Nawaz-ul-Rehman and Fauquet, 2009). All characterized alpha-satellites share three conserved features: a gene encoding a replication-associated protein (Rep) similar to nanoviruses (Mansoor et al., 1999; Saunders and Stanley, 1999), a conserved intergenic region (IR), and an adenine-rich (A-rich) region (Briddon et al., 2004; Xie et al., 2010). Alpha-satellites can replicate autonomously in suitable host cells, but require helper begomoviruses for systemic spread in plants and transmission by insects (Briddon et al., 2004;

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Abbreviations: Rep, Replication-associated protein; **TbCSA,** Tobacco curly shoot alpha-satellite; **CaMV,** *Cauliflower mosaic virus*; **GUS,** *β-glucuronidase*; **GFP,** green fluorescent protein; **IR,** intergenic region; **A-rich,** adenine-rich.

Table 1. Primers used to amplify promoter fragments.

Primer name	Sequence (5′ - 3′)	Underlined restriction site	Position on TbCSA (nt)
pRep420F	GGAATTCAAGCTTATACGAGTATAAATACGTTAATT	*Eco*RI/*Hind*III	1017-1039
pRep383F	GGAATTCAAGCTTAAAGAAGGAATGAAATG	*Eco*RI/*Hind*III	1054-1070
pRep293F	GGAATTCAAGCTTTGATGCAGGCCCAAAT	*Eco*RI/*Hind*III	1144-1159
pRep227F	GGAATTCAAGCTTTATAAAAACGACGTCGTATGA	*Eco*RI/*Hind*III	1209-1229
pRep215F	GGAATTCAAGCTTGTCGTATGAGTAAGTGGG	*Eco*RI/*Hind*III	1221-1238
pRep115F	GGAATTCAAGCTTGGGTTCCTAGGATATAAAT	*Eco*RI/*Hind*III	1321-1339
pRep420R	CGAGCTCGGATCCTTCTGTGAAGAAGAGAGAG	*Sac*I/*Bam*HI	50-68
pRep364RR	CGAGCTCGGATCCCCGAGGCTGGGTAAT	*Sac*I/*Bam*HI	1365-12

Wu and Zhou, 2005; Xie et al., 2010). Infectious assay indicated alpha-satellites were apparently dispensable for symptomatic induction in host plants (Saunders and Stanley, 1999; Briddon et al., 2004; Wu and Zhou, 2005; Xie et al., 2010).

Cis-elements involved in transcriptional regulation have been widely studied by *Agrobacterium*-mediated transient expression and stable transformation in begomoviruses and their beta-satellites (Dry et al., 2000; Eagle and Hanley-Bowdoin, 1997; Eini et al., 2009; Shivaprasad et al., 2005; Usharani et al., 2006). Previous studies indicated that among all these characterized begomoviruses promoters, some are able to drive constitutive gene expression in transgenic plants (Ding et al., 2009; Sunter and Bisaro, 1997; Xie et al., 2003), while others are more specific (Dinant et al., 2004; Guan and Zhou, 2005; Ramos et al., 2004). Additionally, a few promoters from nanoviruses like *Banana bunchy top virus, Subterranean clover stunt virus* and *Milk vetch dwarf virus* had been characterized (Hermann et al., 2001; Schünmann et al., 2003a, b; Shirasawa-Seo et al., 2005a, b).

Tobacco curly shoot virus isolate Y35 (TbCSV-Y35) was isolated in Yunnan, China, and was found to be associated with a beta-satellite and an alpha-satellite (Zhou et al., 2003; Xie et al., 2004). Evidence has shown the Y35 alpha-satellite (subsequently named Tobacco curly shoot alpha-satellite [TbCSA]) could attenuate leaf-curling symptoms induced by TbCSV-Y35 or TbCSV-Y35 plus Y35 DNA β in the early stage of symptom development and induced leaf cluster at a later stage of symptom development in *Nicotiana benthamiana* plants (Li et al., 2005; Wu and Zhou, 2005). To better understand the transcriptional regulation of alpha-satellites, in this study, we have characterized the potential promoter of the *Rep* gene of TbCSA using both transient and stable transgenic expression systems.

MATERIALS AND METHODS

Plant expression vector construction

To obtain the longest and truncated sequences of potential promoter sequence of *Rep* gene from TbCSA (GenBank accession no. AJ579345), a series of primers (Table 1) were designed to amplify fragments by polymerase chain reaction (PCR), with the TbCSA infectious clone as a template (Wu and Zhou, 2005). PCR-amplified fragments covering the putative promoter region were cloned into the pGEM-T easy vector (Promega, Madison, USA), and then digested individually with *Hind*III/*Bam*HI or *Eco*RI/*Sac*I restriction enzymes after sequencing. The resulting fragments were inserted into the corresponding restriction sites in the binary vector pINT121 (Liu et al., 2003) or pCHF3-eGFP (Xiong et al., 2008) to replace the original *Cauliflower mosaic virus* (CaMV) 35S promoter, resulting in expression constructs pRep420, pRep383, pRep293, pRep227, pRep215, pRep115 and pRep364R, respectively (Figure 1). The plasmid pINT121 or pCHF3-eGFP, in which the reporter gene is driven by the CaMV 35S promoter, was used as a positive control. These constructs of the pBINGUS (the *GUS-Nos* fragment was cut from the *Bam*HI/*Eco*RI sites of pINT121 and inserted into the corresponding sites of the binary vector “pBINPLUS”) (van Engelen et al., 1995) and pCHF3 without the *eGFP* gene were used as the negative controls (Figure 1). The expression vectors were introduced individually into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Transient expression assay

Transient expression by *Agrobacterium*-mediated delivery into plants was carried out as described previously (Yang et al., 2000). *A. tumefaciens* EHA105 harboring the constructs was incubated in YEP medium containing 50 μg/ml kanamycin and 50 μg/ml rifampicin until OD_{600}=0.8. After centrifugation, approximately 500 μl of bacterial suspension in MMA solution (10 mM MES, 10 mM $MgCl_2$, 100 μM acetosyringone) was infiltrated into leaves of 4 week-old *N. benthamiana* plants using a 1 ml plastic syringe. Three independent experiments were carried out for each construct.

Plant transformation and histochemical staining for *β-glucuronidase* (GUS) activity

Agrobacterium-mediated tobacco leaf disc transformation was carried out according to the procedure described by Horsch et al. (1985). Regenerated kanamycin-resistant plants were grown on a rooting medium, and then transferred to soil after confirmation of the presence of the transgene by PCR using oligonucleotide primers GUS-F (5’-ATGTTACGTCCTGTAGAAACC-3’) and GUS-R (5’-TCATTGTTTGCCTCCCTGC-3’) which amplify the *GUS* gene. GUS activity was histochemically assayed in a staining solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in 50 mM sodium phosphate, pH7.0) at 37°C for 3 to 12 h as described by Jefferson et al. (1987).

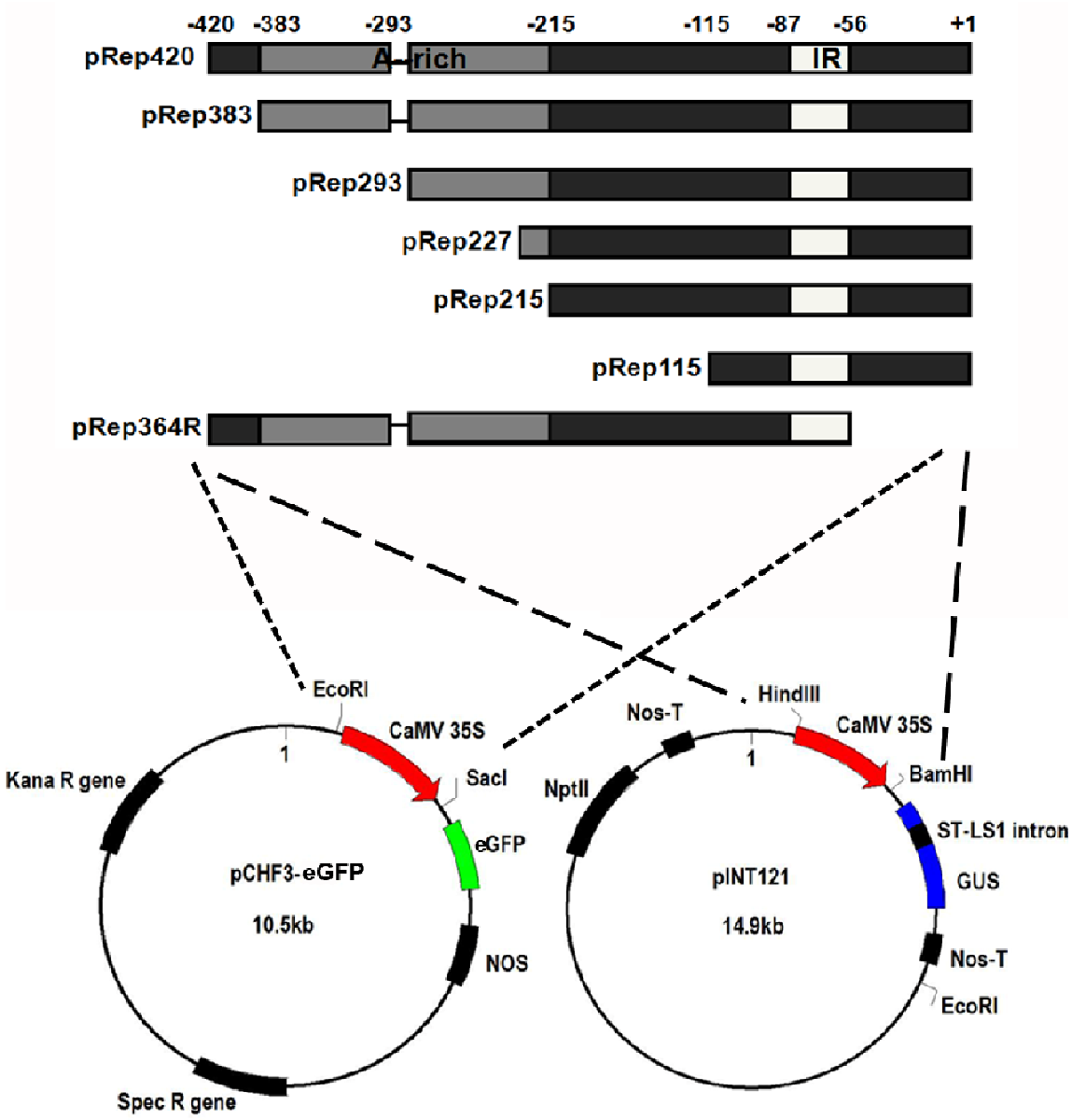

Figure 1. Diagram of the production of *Rep* promoter-reporter contructs. The A-rich region and intergenic region (IR) are shown in gray. All of the constructs were cloned into the pINT121 or pCHF3-eGFP binary vector for the analysis of GUS and GFP activity, respectively. The presumed translation start site of *Rep* gene is assigned coordinate +1.

Fluorometric GUS assay

Quantitative determination of GUS activity was performed by a previously described protocol (Jefferson et al., 1987). The protein content of the samples was determined using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). The resulting data, obtained in three separate experiments was analyzed by analysis of variance (ANOVA) using the PASW Statistics 18 packed program for windows (SPSS Inc., Chicago, IL, USA).

GFP visualized detection

Leaves of 4 week-old *N. benthamiana* plants were infiltrated with the *A. tumefaciens* cultures harboring the constructs as described (Liu et al., 2009). About 48 h after infiltration, 1 cm^2 leaf fragments were excised and green fluorescent protein (GFP) fluorescence was examined in epidermal cells using a Confocal Laser Scanning Microscope (CLSM, Leica TCS SP5, Mannheim, Germany).

RESULTS

Analysis of the putative promoter sequence of TbCSA *Rep* gene

The putative promoter sequence of the *Rep* gene of

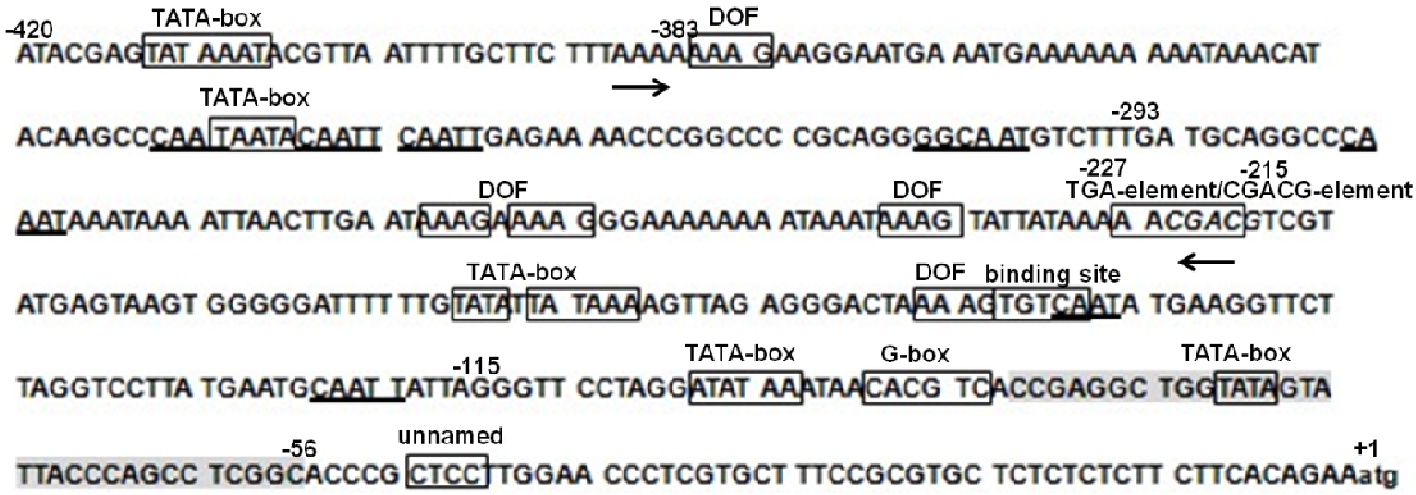

Figure 2. Putative promoter sequence of TbCSA *Rep* gene. The *Rep* start codon is indicated in lower case at the end of the sequence, and putative motifs including some DOFs, a TGA-element, a binding site, some TATA-box, a G-box and an unnamed motif are shown in frame, CAAT-box are underlined, while a CGACG-element partially overlapping the TGA-element is shown in italics. IR is shown by shading and the A-rich region is shown between two arrows. The positions of deletion sites used to generate various constructs are indicated by negative numbers.

TbCSA was analyzed using the PlantCARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002) and PLACE database (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999). The entire non-coding region (420 nt, including all possible promoter regulatory elements) upstream of the *Rep* gene was studied, and the first nucleotide of the translation start site of the *Rep* gene was designated as +1. Some typical *cis*-elements and regulatory motifs, including TATA-box (-77, -104, -182, -187, -340, -413), CAAT-box (-124, -155, -282, -304, -330, -335, -413) and a G-box (-94), were identified within the sequence. Additionally, a TGA-element (-221), a CGACG-element (-219), a binding site (-158), an unnamed motif (-50) and some DOF motifs (-162, -234, -253, -258, -383) were predicted (Figure 2).

Identification of the *Rep* promoter sequence in both transient and transgenic assays

To analyze TbCSA *Rep* promoter activity, the 420 nt fragment was introduced into a promoter-less pINT121 vector to produce pRep420. As a positive control, the pINT121 vector containing the GUS gene driven by the CaMV 35S promoter was used. Sixty-four hours after infiltration into leaves of *N. benthamiana* plants, GUS activity driven by pRep420 exhibited about 18% of that observed in leaf tissues infiltrated with the CaMV 35S promoter (Figure 3A). To further visualize the whole plant expression of GUS driven by the 420 nt fragment, the pRep420 construct was stably transformed into tobacco plants by *Agrobacterium*-mediated transformation. Nine independent transgenic lines were selected after confirmation of the presence of the transgene by PCR (data not shown). Of these, two representative plant lines showing the best GUS activity were analyzed by histochemical staining for GUS. As shown in Figure 3B, the *GUS* gene was stably expressed in the tobacco plants.

Identification of promoter elements regulating *Rep* promoter activity

To define the promoter region controlling TbCSA *Rep* expression, the longest promoter construct (pRep420) and a series of deletion mutants of the sequence were constructed and fused to the *GUS* or *eGFP* reporter gene, respectively (Figure 1). Fluorometric assays were conducted to compare GUS activity driven by the promoter constructs. The results showed that no significant difference was found among the GUS expression levels driven by pRep420, pRep293 and pRep227 ($P>0.05$), though the promoter activity of pRep383 was less than that observed in leaf tissue infiltrated with pRep293 ($P=0.03$) (Figure 4A). It is worth noting that 5'-terminus deletion including a TGA-element and a CGACG-element between 215 and 227 nt upstream of the *Rep* gene (pRep215), resulted in a marked reduction in GUS expression level to just 7% of that provided by CaMV 35S promoter. The 5'-terminus deletion of pRep115 and 3'-terminus deletion of pRep364R reduced GUS expression levels closer to that of promoter-less pBINGUS construct ($P>0.05$), which was used as negative control (Figure 4A).

In addition, significant differences in the intensity of green fluorescence were observed among the various constructs as illustrated in Figure 4B. Using the CaMV

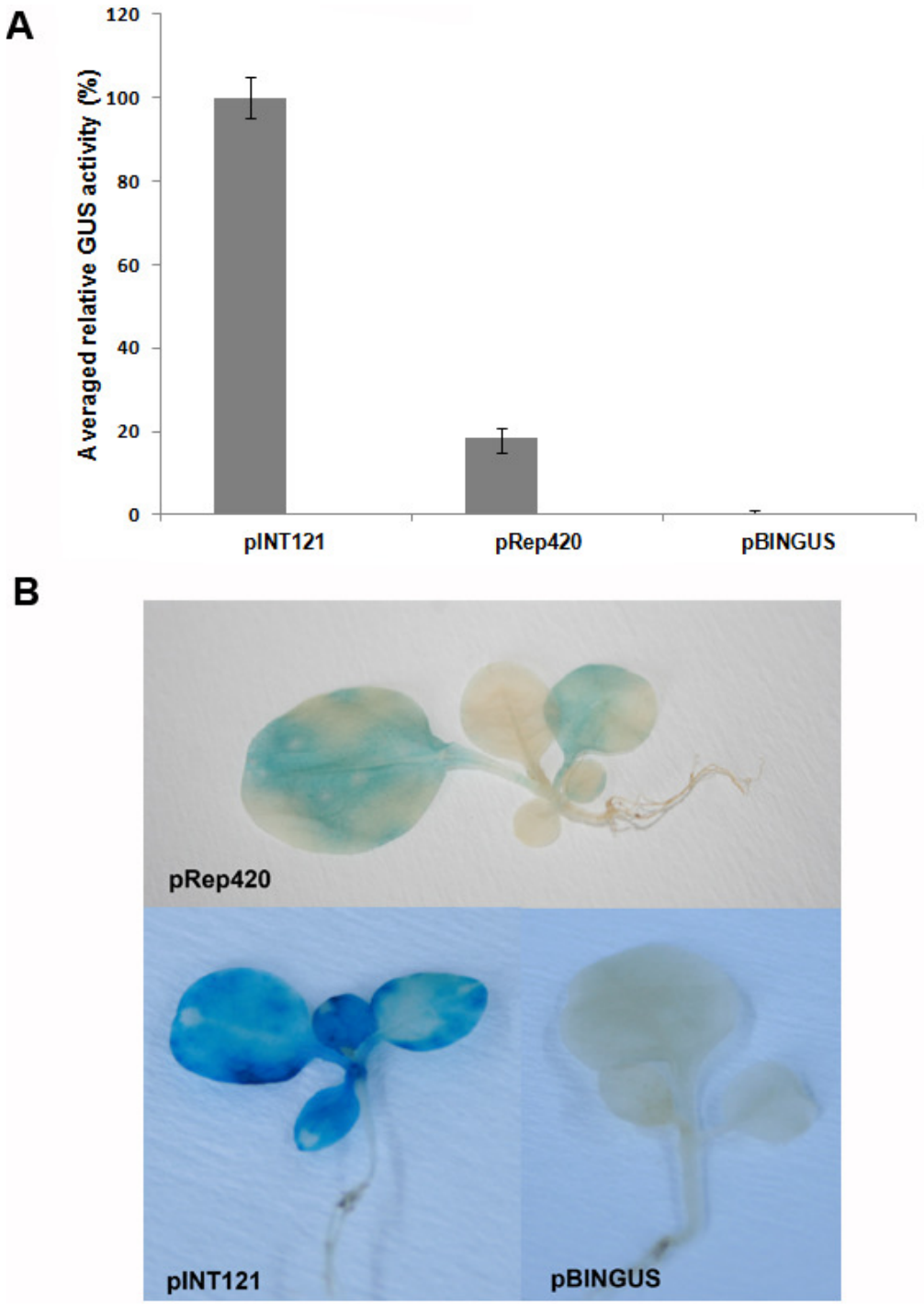

Figure 3. GUS expression of the putative longest promoter sequence in both transient and stable transgenic systems. (A) Fluorometric GUS activity analysis in *Nicotiana benthamiana* leaves after transient expression. The mean GUS activity from the CaMV 35S promoter of pINT121 was taken as 100%. The standard deviation in each case is shown by the error bars. Nine replicates were used for each construct. (B) Histochemical staining for GUS expression in transgenic tobacco plants. pINT121 and pBINGUS were used as positive and negative control, respectively.

35S-GFP from pCHF3-eGFP as positive control, pRep420, pRep383, pRep293 and pRep227 produced relatively higher levels of fluorescence than that provided by negative control pCHF3, though markedly weaker than the pCHF3-eGFP. Meanwhile, fluorescence activity of pRep115 and pRep364R was nearly same as the

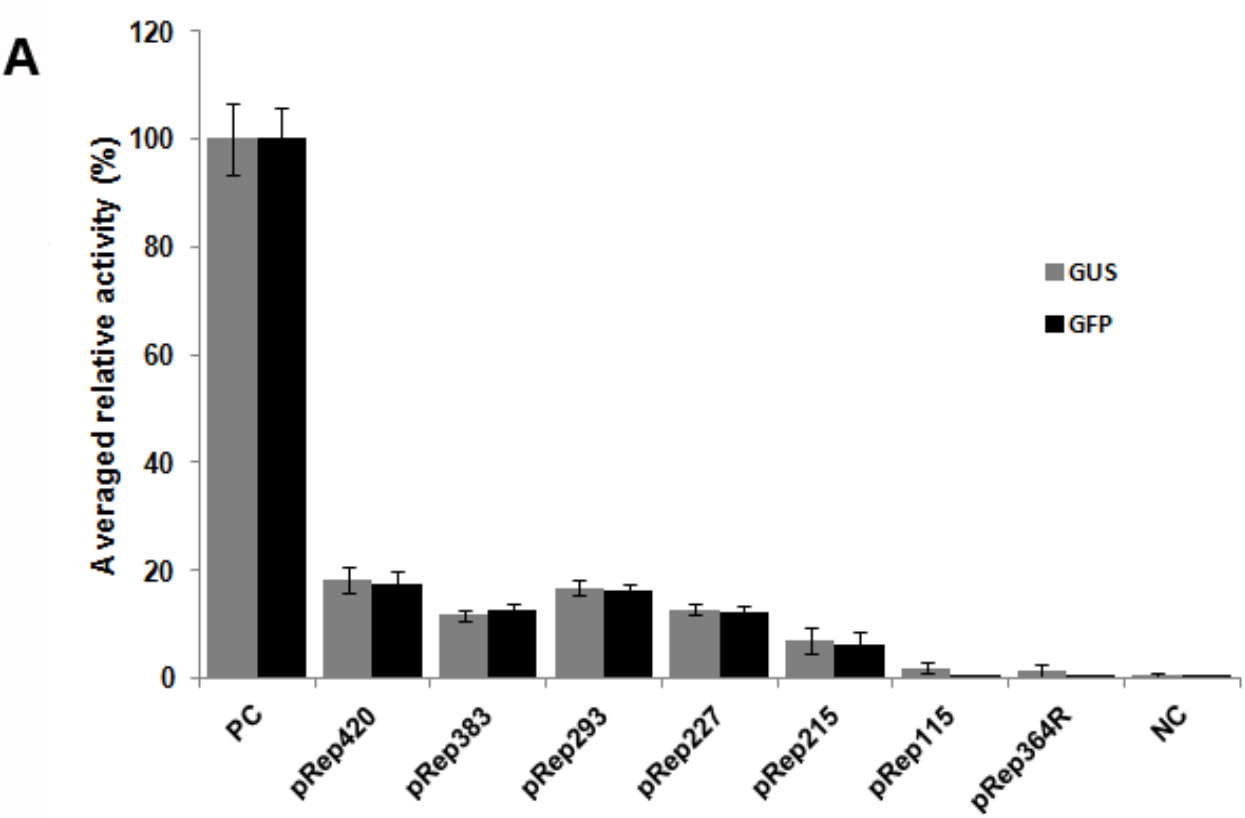

Figure 4. Functional characterization of the *Rep* promoter of TbCSA. (A) Averaged relative GUS or GFP activity of the promoter constructs. The CaMV 35S-GUS or GFP activity was considered as 100% and used to standardize the activities for other promoter constructs. PC, positive control, the *GUS* or *eGFP* gene driven by CaMV 35S promoter in binary vector pINT121 or pCHF3-eGFP, respectively; NC, negative control, promoter-less vector pBINGUS or pCHF3 vector without the *eGFP* gene. The error bars indicated the standard deviation of each construct. Nine replicates were used for each construct. (B) Fluorometric GFP activity analysis in *N. benthamiana* leaves after transient expression driven by promoter constructs. pCHF3-eGFP and pCHF3 were used as positive and negative control, respectively.

negative control (Figure 4B). The results obtained with GFP were consistent with the results obtained with the fluorometric GUS assay (Figure 4A).

DISCUSSION

In this study, using fluorometric assays in transient and stable transgenic systems in tobacco plants, we characterized the putative promoter region of the TbCSA *Rep* gene. Although many promoters from begomoviruses and nanoviruses have been characterized (Dinant et al., 2004; Hermann et al., 2001; Schünmann et al., 2003a; Shirasawa-Seo et al., 2005a, b; Sunter and Bisaro, 1997; Usharani et al., 2006; Xie et al., 2003), and three promoters from beta-satellites were isolated and studied (Guan and Zhou, 2005; Ding et al., 2009; Eini et al., 2009), to our knowledge, this was the first description of a promoter sequence of an alpha-satellite. Alpha-satellites associated with begomoviruses are believed to evolve from nanoviruses. Previous reports have shown that most of characterized promoters from nanoviruses directed predominantly tissue-specific expression (Hermann et al., 2001; Schünmann et al., 2003a; Shirasawa-Seo et al., 2005a, b). However, histochemical GUS analysis showed that GUS expression driven by pRep420 was observed in almost all tissues of transgenic tobacco leaves. We presumed that *Rep* promoter of TbCSA displayed a constitutive expression pattern and required no begomovirus and/or beta-satellite factors to work. Obviously, our results identified that alpha-satellite promoter shared different expression pattern when compared with nanoviruses.

Transient expression assays indicated that 5'-terminus deletion construct pRep115 almost lost the GUS activity, and 5'-terminus deletion in pRep215 increased the GUS expression to 7% level of the CaMV 35S promoter. As shown in Figure 2, a TATA-box, a DOF motif and a binding site (TGTCA), which are homologous to the *cis*-acting elements identified in plants, are found in the region from 116 to 215 nt upstream of the *Rep* gene. Interestingly,DOF motif had been identified as a binding

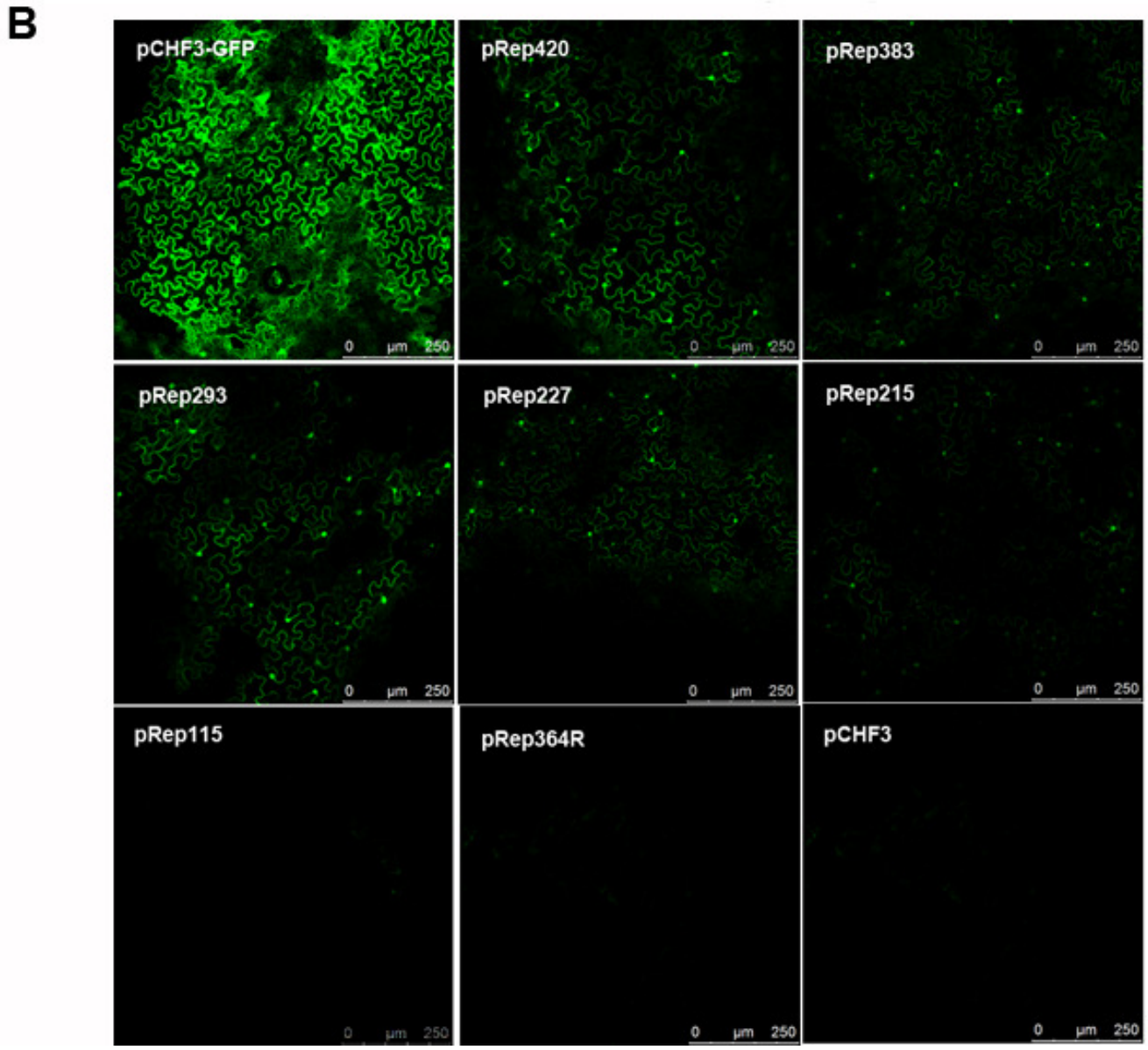

Figure 4. Contd.

domain of DOF proteins reported to be a family of transcription factors (Yanagisawa and Schmidt, 1999), and they might determine the difference in expression profiles between non-Rep and Rep promoters derived from *Milk vetch dwarf virus* (MDV; Family: *Nanoviridae*) (Shirasawa-Seo et al., 2005b). The TGTCA motif was also confirmed to be bound by the recombinant homeo-domain type transcriptional factor OsBIHD1 protein (Luo et al., 2005). Therefore, based on all these previous reports, we suggested that the two putative regulatory motifs might act as positive regulatory elements for *Rep* transcription and that alpha-satellites might share a similar transcription regulation mechanism with other organisms.

A-rich regions, which shared by alpha-satellites and beta-satellites (Briddon et al., 2003, 2004; Xie et al., 2010), possibly are the only feature to make a distinction between alpha-satellites and nanoviruses. Also, the deletion of A-rich region positioned at 215 to 383 nt resulted in significant difference in promoter activity between pRep383 and pRep215 ($P<0.01$), indicating that A-rich region might regulate *Rep* promoter activity. This is consistent with those results described by Guan and Zhou (2006). To further identify the key elements within the A-rich region involved in regulating the activity of *Rep* promoter, a series of deleted mutations were constructed. Fluorometric analysis showed that no statistically significant differences in promoter activity were found among the constructs of pRep420, pRep293 and pRep227 (Figure 4). These results suggested that *Rep*

promoter elements might not be included in the entire non-coding region, and a 227 nt fragment upstream of the *Rep* gene of TbCSA should be regarded as the minimal promoter. Interestingly, Eini et al. (2009) reported that the promoter construct β207 from Cotton leaf curl Multan beta-satellite (CLCuMB) rather than the entire non-coding region β989, showed the highest promoter activity. All these suggest that promoter sequence might not absolutely contain the entire non-coding region of satellites associated with begomoviruses.

Transient expression assays showed that the promoter activity of pRep227 was much higher than that of pRep215 ($P<0.01$). Sequence analysis identified that a TGA-element and a CGACG-element existed in the region from 215 to 227 nt upstream of the *Rep* gene, suggesting the elements might involve in the transcriptional regulation of *Rep*. This is consistent with the result that CGACG-element was found to be as part of an enhancer region in the promoter of *Arabidopsis* pathogen responsive gene, *PDF1.2* (Brown et al., 2003). Similarly, CGACG-element identified in the promoter of the *Amy3D* and *Amy3E* amylase genes was suspected to have the function as a coupling element for the G-box element (Hwang et al., 1998). Therefore, this TGA-element and CGACG-element may act as positive regulatory elements in *Rep* transcription. However, more studies are required to elucidate the function of them.

In conclusion, TbCSA *Rep* promoter directs a constitutive expression pattern in tobacco plants and not the entire non-coding region, but a segment of 227 nt located upstream of the *Rep* gene is considered as the minimal promoter. Considering particular features of alpha-satellite and the low promoter activity of pRep227 (13% that of CaMV 35S promoter), this construct might be applied in special plant genetic engineering studies for low-level gene expression.

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