RESEARCH ARTICLE

DEVELOPMENT OF NOVEL SYNTHETIC PROMOTERS FOR GENE EXPRESSION IN TRANSGENIC SUGARCANE

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Abstract

Advances in synthetic promoter technology offer a framework for designing expression cassettes that could provide precise control of transgene expression. The use of synthetic promoters enables defined transgene regulation, reduces unwanted background expression and allows to overcome homology dependent gene silencing (HDGS) in transgenic plants. In the present study, a synthetic module was designed using characterized *cis* acting elements and two constructs, one with the synthetic module at the 5' end of the minimal promoter (SynS1) and the other with the module in both 5' and 3' ends (SynS2), were made and sub-cloned in pCAMBIA1305.1 by replacing CaMV 35S promoter so as to drive *GUS* (β-glucuronidase) expression. Transient studies in sugarcane revealed that both the promoters could confer *GUS* expression. Further, the constructs were stably transformed in sugarcane wherein both the promoters drove constitutive expression.

Keywords: Synthetic promoter, *GUS*,transgene expression, sugarcane

Introduction

Promoters are the key determinants in plant genetic engineering. Although several promoters have been characterized from different sources, there is a need to determine regulatory sequences to enable precise control of gene expression and this could also be achieved by the deployment of synthetically made promoters. Synthetic promoters can be designed using an array of *cis*-acting elements from different sources which would not only improve the expression characteristics but also reduce unwanted background expressions (Mehrotra et al. 2011). They can be developed by adopting many approaches such as: (i) combining a defined *cis* element with a strong constitutive promoter (Ito et al. 1998; Rushton et al. 2002) or using multiple enhancer regions with a strong promoter (Maiti et al. 1997); (ii) combining *cis* elements from diverse promoters (Sawant et al. 2001); (iii) fusing two strong constitutive promoters or using bidirectional promoters (Comai et al. 1990; Chaturvedi et al. 2006).

Synthetic promoters help to mitigate several limitations: they can increase promoter availability; help to control the expression of multiple transgenes; help to prevent homology-dependent gene silencing (HDGS) and ensure more refined control of transgene expression in a tissue and environment specific manner. Synthetic promoters have been successfully used in several studies to either reveal the role of *cis* regulatory elements or modulate targeted inducibility, independently and/or within a specific *cis*-motif arrangement. An array of *cis*-

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acting regulatory elements associated with heat shock, light, development, tissue specificity, mechanical wounding, pathogen attack, sugar sensing, reactive oxygen species and low temperature stress have been reported (Pietrzak et al. 1989; Comai et al. 1990; Gilmartin and Chua, 1990; Ni et al. 1995, 1996; Mitsuhara et al. 1996; Rushton et al. 2002; Geisler et al. 2006; Mazarei et al. 2008; Zhu et al. 2008). Furthermore, they may play a more prominent role in future biofuel strategies involving GM plants (Taylor et al. 2008).

Liu et al. (2011) constructed a series of synthetic promoters with inducible *cis* elements and analyzed their expression through transient studies. Shokouhifar et al. (2011) expressed *GUS* gene driven by a synthetic pathogen inducible promoter (SynP-FF) in transgenic canola and demonstrated that it could be used to impart resistance against *Sclerotinia sclerotiorum*. Ranjan and Dey (2012) have developed Caulimovirus based vascular tissue and stress inducible hybrid–synthetic promoters through Dof-1 (domain of function-1) motif rearrangement that performed better than CaMV35S, F20 and FS3 promoters.

The maize ubiquitin is the promoter of choice for sugarcane transformation and has been widely used for over two decades (Gallo-Meagher and Irvine 1996). Sugarcane ubiquitin promoters (Ubi4 and Ubi9) when expressed in sugarcane have led to post transcriptional gene silencing (PTGS) (Wei et al. 2003). The promoters used currently for the development of transgenic sugarcane are limited in number and only very few provide tissue specific expression. Hence, there is a need for identification of more promoters from unrelated sources for specific applications; synthetic promoters would be of great value for future genetic engineering studies in sugarcane.

In the present study, a synthetic module was designed to confer stem specific gene expression, fused with a minimal promoter at 5' and at both 5' as well as 3' ends and functionally validated in transgenic sugarcane plants. However, the promoters conferred constitutive *GUS* expression in transgenic sugarcane which necessitates the appropriate use of elements as well as the minimal promoter so as to obtain precise control of transgene expression.

Materials and methods

Designing and synthesis of stem specific module

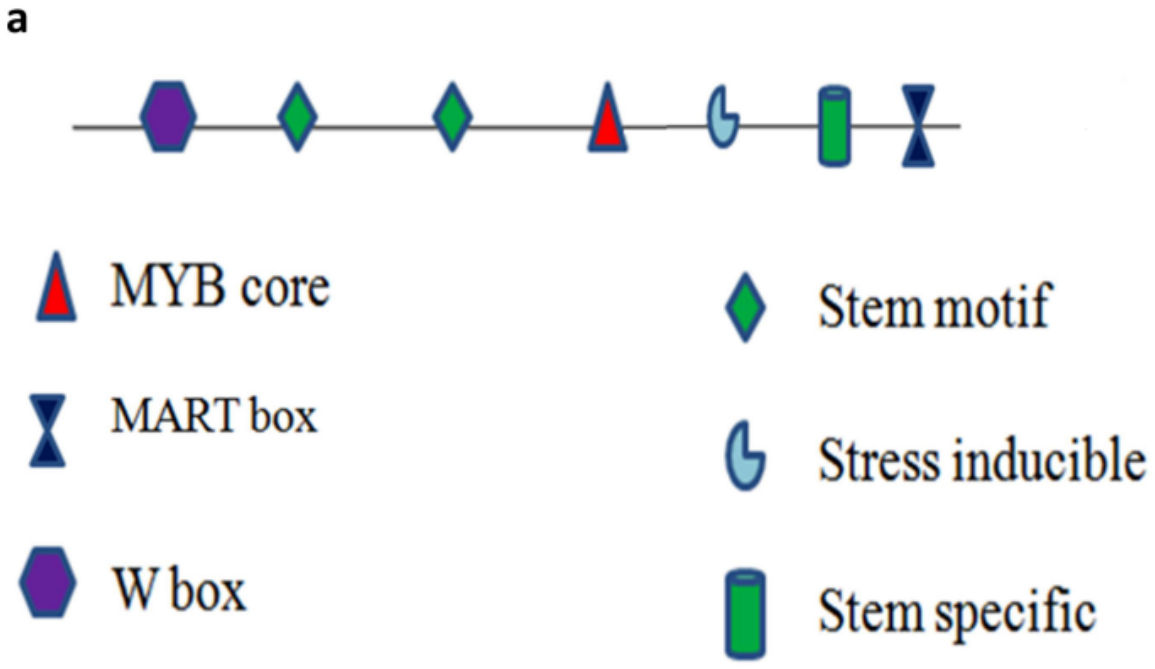
The *cis* acting regulatory elements responsive to stem specificity, expression enhancement and stress responsiveness were identified from literature. Table 1 lists the various *cis* elements incorporated in the synthetic module (SynS). Since spacing and copy number of the *cis* elements are the key players of tunable gene expression, two copies of the stem specific motif (AGCGGG) and a copy of another validated stem motif (ATAATGGGCCACACT GTGGGGCAT) were incorporated with a spacing of 10 bp between *cis* elements. One copy each of other *cis* acting elements, namely stress responsive, elicitor responsive, MART (matrix attachment region for enhanced expression) box were placed in the module. The spacer sequence was designed so that no *cis* acting elements were present and the design was checked using PLACE (Plant cis-acting regulatory DNA elements) database (Higo et al. 1999). The synthetic module was custom synthesized (Bioserve). The arrangement of *cis* elements in the synthetic module is depicted in Fig. 1a. The nucleotide sequence of the synthetic module is given in Fig. 1b.

Recombinant plasmid constructs

Two synthetic promoters were made containing the synthetic modules fused either at the 5' (SynS1) or

Table 1. List of various *cis* elements incorporated in the synthetic module (SynS)

Cis element	Sequence motif	Function	Reference
SE1PVGRP18	ATAATGGGCCA CACTGTGGGGCAT	Stem specific	Keller and Heierli1994
MYBCOREATCYCB1	AACGG	Stress responsive	Planchais et al. 2002
ELRECOREPCRP1	TTGACC	Elicitor responsive	Rushton et al. 1996
ARFAT	TGTCTC	Auxin responsive	Ulmasov et al. 1999
LEAFYATAG	CCAATGT	Root meristem specific	Kamiya et al. 2003
MARTBOX	TTTATTTTTTT	Enhances expression	Gasser et al. 1989



b

5′
AGCAGAATACATCGAGAACC**TTGACC**TCATAACGCT**AGCGGG**TCATAACGCT**A**
GCGGGTCATAACGCT**AACGG**TCATAACGCT**CACATG**TCATAACGCT**ATAATGG**
GCCACACTGTGGGGCATTCATAACGCT**TTATTTTTTT**AGAAGCCAGGCAGTC
CACAA 3′

Fig. 1. Synthetic stem module:(a) arrangement of *cis* elements in the synthetic module; (b) nucleotide sequence of the SynS module; the *cis* acting elements are in bold letters

both at the 5' and 3' end (SynS2) of the minimal promoter (Philip et al. 2013). The primers used for amplifying the minimal promoter and the synthetic module are given in Table 2. The minimal promoter was amplified with PMF and PMR primers whereas the synthetic module was amplified with S1F and S1R primers. The amplified products were restricted with *Sac*I enzyme for 1 h at 37ºC and separated on 1% agarose gel through electrophoresis. The restricted minimal promoter and synthetic module were eluted and ligated overnight at 4ºC. A 1:10 dilution of the ligated mixture was used as a template for PCR to amplify the SynS1 promoter with *Eco*RI anchored forward primer (S1F) and *Nco*I anchored reverse primer (PMR).

In order to prepare the SynS2 promoter, the stem specific synthetic module was fused to the 3' end of SynS1 promoter. The synthetic module was PCR amplified with S2F and S2R primers (Table 2). Also the SynS1 promoter was amplified using S1F and PMR primers. The promoters and the synthetic module were restricted with *Nco*I enzyme at 37ºC for 1 h and the restricted products were separated on 1% gel through electrophoresis. The restricted products were eluted and ligated overnight at 4ºC. The ligated mixture was diluted at 1:10 ratio and used as a template for PCR to amplify the SynS2 promoter with *Eco*RI anchored forward primer (S1F) and *Bgl*II anchored reverse primer (S2R).

Table 2. Primer sequences used in the study

Primer	Sequence 5'- 3'
PMF	GCCGAAGCTTCCAATAAAT
PMR	GATCCCATGGGTACATGTCT
S1F	GATCGAATTCAGCAGAATAC
S1R	GATCAAGCTTCCTGGCTTCT
S2F	GATCCCATGGATCGAGAACC
S2R	GATCAGATCTTTGTGGACTG
*GUS*R	GATCAATGTCGTGAAAGCCCGCA

The pCAMBIA1305.1 vector and the amplified products were restricted for 1 h at 37ºC and separated on 1% agarose gel through electrophoresis. The restricted vector and promoters were eluted, ligated overnight at 4ºC and transformed in *E. coli* DH5α strain. The recombinant plasmids pSynS1 and pSynS2 were isolated and individually mobilized into *Agrobacterium* LBA4404 for stable transformation.

Transient expression in sugarcane

Transient expression was performed as described in Chakravarthi et al. (2015). Shoot tips of 6–8 months old sugarcane variety Co86032 were used for the transient expression studies and biolistic bombardment was carried out in triplicates with pSynS1 and pSynS2 along with pCAMBIA1305.1 and pPortUbi882 as controls. In each replicate, 10–15 leaf bits of 0.5 cm^2 were used. Gold particles without plasmids were used for bombardment as negative control. The bombarded tissues were incubated in dark at 25ºC for 24 h prior to histochemical staining for *GUS* activity (Jefferson et al. 1987). The leaf bits were viewed under stereo light microscope for recording the number of blue foci. Transient *GUS* expression was expressed as mean number of *GUS* foci/0.5cm^2 explant.

Agrobacterium-mediated transformation

Agrobacterium mediated transformation in sugarcane was performed as described by Arvinth et al. (2010). Maize ubi1-*GUS* construct was also transformed for use as a positive control.

PCR confirmation of transgenics

Genomic DNA from putative transgenics was isolated using DNeasy plant mini kit (Qiagen, Germany). PCR was carried out using a thermo cycler (Eppendorf, Germany) to confirm integration of the SynS1 and SynS2 synthetic promoter and

GUS using promoter specific forward primer (S1F) and *GUS*-specific reverse primers (*GUS*R) respectively. The reaction mixture contained 25 ng of template DNA with 1.5 mM dNTP (Merck Biosciences, Darmstadt, Germany), 0.25 µM each of the primers, and one unit of the *Taq* polymerase enzyme (Merck Biosciences, Darmstadt, Germany) along with the *Taq* buffer. PCR products were separated in 1% agarose gel by electrophoresis and the molecular weight of the products was determined by comparing with 1 kb DNA marker (Thermo Scientific, USA).

In situ histochemical localization of *GUS* expression

GUS staining was performed following Jefferson et al. (1987). The tissues were excised from the plant using a sterile blade. The explants were washed in 50 mM sodium phosphate buffer and incubated in phosphate buffer with 1% Triton X-100 at 37ºC for 1 h. The explants were then transferred to 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) staining solution and vacuum infiltrated for 5 min. The tissues were incubated for 16 h at 37ºC. After de-staining in 70% ethanol, the tissues were observed under a stereo light microscope (Zeiss, Germany).

***GUS* fluorometric assay**

Fluorometric *GUS* assay was carried out following Jefferson et al. (1987) in different transgenic plant parts (leaf, stem and root) using untransformed plants as control. Two hundred and fifty milligrams of tissue was ground in *GUS* extraction buffer [50 mM $NaPO_4$ (pH 7.0), 10 mM dithiothreitol, 1 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, and 0.1% triton X-100] and 50 µl of the extract was added to 0.5 ml aliquots of pre-warmed (37ºC) assay buffer (1 mM 4-methyl umbelliferyl b-D-glucuronide in extraction buffer). From this, 100 µl was transferred to a microfuge tube containing 0.9 ml stop buffer (0.2 M Na_2CO_3) at room temperature. The liberation of 4-methyl umbelliferone (4-MU) was assayed by measuring the fluorescence with excitation at 365 nm and emission at 455 nm in a fluorometer (Promega, USA). The assays were performed in triplicates for 10 independent transgenic events (per construct) and *GUS* activity was calculated as nanomoles of 4-MU hydrolyzed/minute/milligram of total protein.

Statistical analysis

The data from transient assays and fluorometric analysis were subjected to statistical analysis using one-way ANOVA. A probability (*P*) value of ≤ 0.05 was considered to be statistically significant.

Results

Designing of synthetic promoters

Two vector constructs were designed specifically for expression in stem. SynS1 promoter was prepared by fusing the SynS module to the 5' end of minimal promoter whereas in SynS2 promoter, the SynR module was fused to both 5' and 3' ends of the minimal promoter. The schematic representation of the cloning strategy is depicted in Fig. 2.

Construction of synthetic promoters-*GUS* fusion vectors

Both the minimal promoter and the synthetic module were amplified separately using restriction sites anchored forward and reverse primers. The amplified promoter and synthetic module were restricted with *Sac*I enzyme, ligated and the ligated mixture was diluted and used as a template to amplify the synthetic module-promoter fusion and sub-cloned into pCAMBIA1305.1 by replacing the CaMV 35S promoter so as to drive *GUS* reporter gene. The newly constructed recombinant plasmid was named

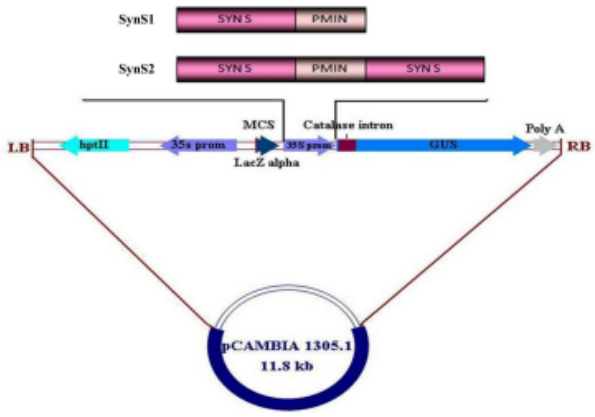


Fig. 2. Schematic representation of binary vectors generated with synthetic promoter-*GUS* fusions

pSynS1. In order to fuse the synthetic modules to the 3' end of the minimal promoter, pSynS1 and the synthetic modules were amplified separately and restricted with *Nco*I enzyme, ligated and the ligated mixture was diluted and used as a template to amplify the module-promoter fusion. Sub-cloning was done in pCAMBIA1305.1 by replacing the CaMV 35S promoter so as to drive *GUS* reporter gene. The newly constructed recombinant plasmid was named pSynS2.

Functional validation of the synthetic promoters

Transient assay

Meristematic leaf tissues of sugarcane were bombarded with pCaMV 35S-*GUS*, pPortUbi882-*GUS*, pSynS1 and pSynR2 constructs and subjected to histochemical staining for *GUS* expression after a day of incubation at 37°C. Blue foci were observed in tissues bombarded with all the constructs which indicated that the synthetic promoters could drive *GUS* expression (Fig. 3). pSynS1 had significantly highest number of blue foci whereas pSynS2 had an expression equivalent to that of the CaMV 35S promoter (Fig. 4).

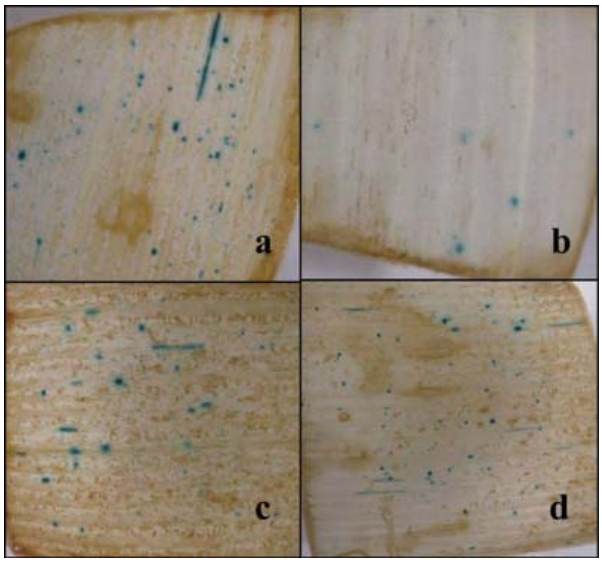


Fig. 3. Transient *GUS* assay in leaf tissue of sugarcane transformed with different constructs: (a) pCaMV 35S-*GUS*; (b) pPortUbi882-*GUS*; (c) pSynS1; (d) pSynS2

Stable transformation in sugarcane

The chimeric plasmids containing synthetic promoters-*GUS* gene fusions were introduced into competent *Agrobacterium* strain LBA4404 by freeze thaw method and the colonies obtained were

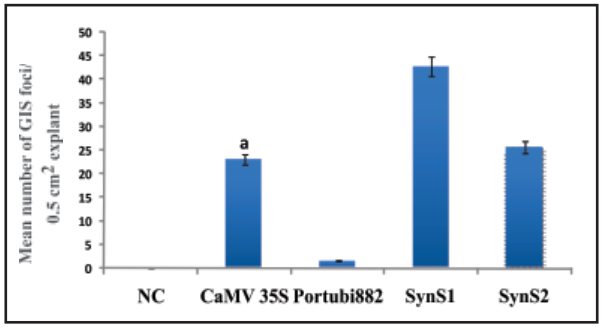


Fig. 4. Quantification of transient *GUS* expression driven by CaMV 35S, PortUbi882, SynS1 and SynS2 promoters:NC represents untransformed control; each value represents the mean of three replicates; error bars indicate SD; means marked with different letters are significantly different at $p \leq 0.05$

screened by PCR and the positive clones were used for plant transformation. Transgenic sugarcane plants were developed and all the putative transgenics were subjected to PCR with SynS1 and SynS2 promoter specific forward and *GUS* reverse fusion primers. PCR analysis showed that all the putative transgenic plants that survived through stringent hygromycin selection were positive for the transgene integration. The transgenic plants were further subjected to histochemical staining using X-gluc substrate. (Fig.5) shows the *GUS* expression driven by maize ubi1, SynS1 and SynS2 promoters in stems and rinds of transgenic sugarcane. In transgenic sugarcane, 10 independent transgenic events analyzed for *GUS* expression through fluorometric assay showed that both SynS1 and SynS2 promoters drove significantly higher *GUS* expression in stem and roots compared to maize ubiquitin promoter; in leaves, however, the expression was lower than that driven by maize ubi1 promoter (Fig.6).

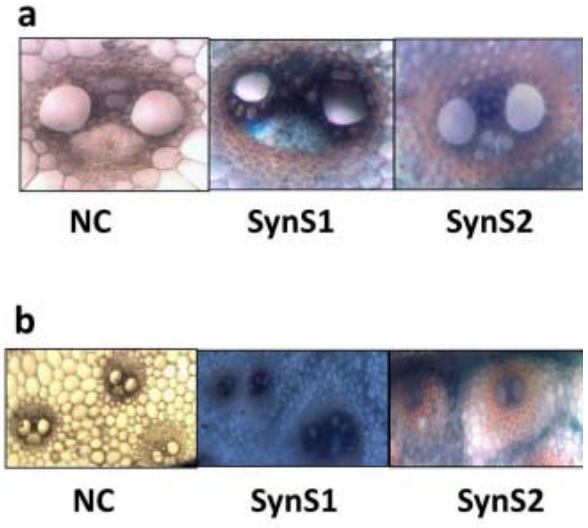


Fig. 5. Histochemical localization of *GUS* activity driven by different promoters in transgenic and non-transgenic sugarcane: (a) stem section of NC (untransformed control), SynS1 and SynS2 promoters; (b) rind section of NC, SynS1 and SynS2 promoters

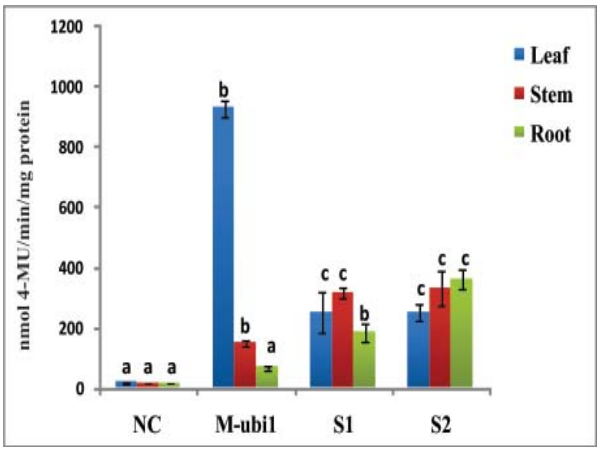


Fig.6. Fluorometric *GUS* analysis in transgenic sugarcane plants: NC - untransformed control; M-ubi1–transgenic sugarcane expressing *GUS* driven by m-ubi1 promoter; S1 - transgenic sugarcane expressing GUS driven by SynS1 promoter; S2 - transgenic sugarcane expressing *GUS* driven by SynS2 promoter;each value represents the meanof 10 independent transgenic events; error bars indicate SD; means marked with different letters are significantly different at $p \leq 0.05$ for the same plant part

Discussion

Synthetic promoters provide an efficient and flexible strategy to regulate transgene expression in a desired spatial and temporal manner and reduce the complexity of the expression pattern of natural promoters (Venter 2007; Gurr and Rushton 2005; Rushton et al. 2002). Recent reports show that individual pathogen responsive *cis*-acting elements when fused with a minimal promoter can locally direct reporter gene expression in response to pathogenesis (Cazzonelli and Velten 2008; Mazarei et al. 2008; Rushton et al. 2002). In the present study, synthetic promoters to confer stem specific expression were designed using an array of *cis*-acting elements from various sources as building blocks along with a minimal promoter and validated in sugarcane.

The CaMV 35S promoter has been a workhorse model for *cis* engineering in plant promoters. Earlier studies conducted by Bhullar et al. (2003, 2007, 2010) have reported that rearrangement of *cis*-elements in CaMV 35S promoter region creates synthetic CaMV 35S promoters with minimum sequence homology whose transgene activity is equivalent to that of the wild type CaMV 35S promoter. Such promoters can be used to prevent homology based gene silencing which is a critical problem in transgenic technology. Rushton et al. (2002) found that defense signaling could be well conserved across species at the promoter element level. Several *cis*-acting elements (boxes W1, W2, GCC, JERE, S, Gst1 and D) recognized by specific transcription factors can mediate local gene expression in plants upon pathogen attack. Hence, defined synthetic promoters containing tetramers of only a single *cis* element were constructed and the expression was monitored during interactions with a number of pathogens, including compatible, incompatible and non-host interactions. Since the effect of spacing between individual *cis*-acting elements on transgene expression is quite difficult to predict (Wray 1998), one needs to study it experimentally. The spacing of *cis* acting elements leads to differences in the inducibility of various promoters for the pathogen tested, the speed of induction and the basal expression levels. Improved second-generation pathogen inducible promoters were made by varying several parameters like the number of copies of an individual *cis* element in a promoter and variations in the strength and inducibility of the promoter obtained (Rushton et al. 2002).

Sawant et al. (2005) illustrated the synergistic effect of *cis* acting elements by placing eight *cis*-acting motifs upstream of the TATA-box (at the -38 position as in plant genes) of the basal promoter (Pmec). Multimers of the *cis* elements were inserted, taking one at a time such that each of these caused 2 to 8 fold activation of the basal transcription. The complete module brought enhancement of 110-fold in transcription levels. This study proved that the use of many *cis*-elements together may provide additional TF (transcription factor) binding sites and contribute to the stability of Pre Initiation Complex (PIC) at TATA-box. A strategy for tunable gene expression was developed by Jensen and Hammer (1998), Mijakovic et al. (2005) and Hammer et al. (2006). They attempted to control gene expression through construction of synthetic promoter libraries by introducing changes in the sequences flanking the -35 and -10 consensus sequences of bacterial promoters. Alper et al. (2005) constructed a library of synthetic promoters of varying strength through mutagenesis of a constitutive promoter. Based on GFP (green fluorescent protein) fluorescence, a functional library of 22 mutants was obtained with a broad host range.

In the present study, the transgenic sugarcane plants transformed with the synthetic promoters showed higher *GUS* expression than those with maize ubiquitin promoter in stem and roots but lower expression in leaves. However, constitutive *GUS* expression was observed in all the transgenics driven by the synthetic promoter which was evident through histochemical and fluorometric analysis. The reason for this may be the minimal promoter which contained other *cis* elements pertaining to constitutive expression (an ATATT motif). Further, the minimal promoter needs modification for stem specific expression. Similarly, the stem specific module can be arranged in different combinations by taking into account the spacing, orientation and copy number in order to obtain a perfect synthetic stem specific promoter.

Also, generation of large synthetic promoter libraries and screening them for tissue specificity will aid in

constructing tissue specific synthetic promoters. However, there are still several combinatorial mechanisms of regulatory context and signaling that are largely unknown, which prevent the optimal design of synthetic tissue specific promoters (Venter 2007). Advances in bioinformatics, and in depth studies of plant transcription factor (TF) networks and *cis*- and/or trans-synergistic interactions could greatly accelerate design strategies for the construction of effective synthetic promoters. A high throughput promoter designing strategy adhering to all the critical factors mentioned above, combined with in silico methods would be a solution to generate synthetic promoters with tunable transgene expression. Such synthetic promoters, with their ability to minimize gene silencing, would replace the existing routine promoters and be of great use in genetic engineering approaches for crop improvement.

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