**RESEARCH NOTE**

# Dimerization of GT element interferes negatively with gene activation

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## Introduction

Specific sequence elements in DNA regulate the transcription by recruiting transcription factors. A family of sequences called GT elements is present in the promoter region of genes encoding diverse functions like light regulation (Dehesh *et al*. 1990), pathogen defense (Buchel *et al*. 1996) and tissue specificity (Eyal *et al*. 1995; Villain *et al*. 1996). In addition, a GT element called site 1 (GT-1), found in the ribosomal protein gene *rps1* promoter, has been shown to repress transcription in non-photosynthetic tissues or cells (Zhou *et al*. 1992; Villain *et al*. 1994). The GT elements have a core sequence rich in A and T, preceded by one to two G nucleotides on the 5′ side (Zhou 1999). The high degeneracy makes it difficult to identify them by sequence search. GT elements were first identified in the pea ribulose 1, 5-biphosphate carboxylase/oxygenase (Rubisco) small subunit gene (*rbcs-3A*) promoter, as box II (5′ GTGTGGTTAATATG) (Green *et al*. 1987). Box II sequence is involved in light activation or dark repression of synthetic promoters in transgenic plants (Lam and Chua 1990). Recent studies have shown that several copies of box II are responsive to calcium/calmodulin stimulation in the phytochrome A-regulated light signalling pathway (Wu *et al*. 1996). Functional analyses have shown that box II related sequences may have either a positive or negative role in the transcription of different genes in different organs (Villain *et al*. 1994, 1996; Eyal *et al*. 1995), and GT-1 may be involved in the regulation of transcription of many different genes. The degenerated GT elements are either bound by different GT factors or have a different binding affinity for an individual GT factor, resulting in differential regulatory functions. The differences in binding affinity and the nature of transcriptional complex formed on promoters have been related to the differences in regulatory functions of the GT sequence motifs (Chattopadhyay *et al*. 1998). The GT elements are present in tandem repeats within a promoter region. The spacing between two GT elements seems to be critical for their activity. An increase in spacing between two GT elements even by 2 bp can dramatically reduce the transcript level (Gilmartin and Chua 1990), as in the case of *rbcS-3A*, suggesting critical sequence requirements for the activation of gene expression by the GT elements. However, deletions of 5–7 bp between them do not affect the *rbcS-3A* transcript level. None of the changes in spacing affects the binding of GT-1 *in vitro*. This might imply that the appropriate binding conformation of GT factors is needed for activity.

By transient transformation studies, we have earlier reported that the GT element functions even when it is placed out of native sequence context (Mehrotra *et al*. 2005). It contributes synergistically by enhancing the stability of transcription complex formed on minimal promoter (Sawant *et al*. 2005). Hamilton *et al*. (2000) showed, using *Zm13* pollen-specific promoter, that there is around an eight-fold higher expression in pollen of transgenic plants than transient transformation by microprojectile bombardment. They further explained that this may be due to the titration of limited number of transcription factors by the large number of potential binding sites in the promoter fragment along with the large number of promoter copies introduced during transient transformation. Using transient assays, Basu *et al*. (2003) showed that the reporter gene activity is significantly higher in mature leaves compared to young leaves in three of the four promoter constructs which they studied. Diana *et al*. (1993) showed that the DNAse level in different tissues is different and significantly higher in roots. The introduced DNA as a result of transient transformation can be degraded at a higher rate in such tissues. The transient transformation data may not be reflecting the real biological situation and hence the need to study the promoter reporter constructs using stable transgenic lines. This report aims at establishing the activation and repression behaviour of the GT element in stable transgenic plants, when placed upstream of a minimal promoter in one or two copies, separated by different spacer lengths.

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## Materials and methods

### *Constructs*

A minimal promoter *Pmec*, described earlier by Sawant *et al*. (2001) was used. It contains a 13 nucleotide long TATA-box (underlined) region and a transcription initiation site (arrow) leading to the *gusA* reporter gene (bold letters). It is a 138 nucleotide long sequence given below.

GGATCCTCACTATATATAGGAAGTTCATTTCATTTGGA
↓
ATGGACACGTGTTGTCATTTCTCAACAATTACCAACA
ACAACAAACAACAAACAACATTATACAATTACTATTTA
CAATTACATCTAGATAAACA**ATGGCTTCCTCC-*gusA***.

It was cloned in the plasmid pUC19 (New England Biolabs, Massachusetts, USA). A 50 nucleotide long random sequence (GGAT CCGGCTATGGCGGAGCAAGATT CACTCTGCGAGGCCAAAGCTTACCCCGGAAGGATCC) was cloned at the *Bam*H1 site, to the 5′ side of the *Pmec*. The space between two copies of the GT motif was also kept variable. In different constructs, the two copies were placed in tandem with no nucleotide in between or with 5, 10 or 25 nucleotide long random sequence (table 1) to examine the effect of such a spacer on the motif dependent activation of the minimal promoter. An *Xba*I fragment carrying the activator sequences listed in table 1 was inserted upstream. The promoter reporter cassettes were cloned in pBI101 between *Sma*I and *Eco*RI sites (figure 1), mobilized in *Agrobacterium tumefaciens* and used for plant transformation.

### *Stable transformation*

The promoter reporter constructs were used for transforming tobacco (*Nicotiana tabacum* cv. Petit Havana). The shoots were regenerated on medium containing 200 mg/mL kanamycin (Rogers *et al*. 1986). After rooting, the transgenic plantlets were transferred to soil. The primary transformants were allowed to self-fertilize. The seeds were collected, surface sterilized, and germinated on Murashige and Skoog (1962) (MS) medium. The seedlings were maintained at 26°C under 16 : 8 h LD cycle. Seven to eight single copy, homozygous independently transformed $T_2$ transgenic plants were analysed in each case. The seeds of $T_0$ transgenic lines were grown in Hoagland solution containing kanamycin (300 mg/L). The ratios of kanamycin resistant to sensitive plants were recorded. The lines with segregation ratio of 3:1 (kanamycin resistant : kanamycin sensitive), indicating single copy insertion of the transgene, were used for further studies. $T_2$ seeds were first germinated in Hoagland medium containing kanamycin (300 mg/L) on petridish for three weeks to identify (nonsegregating) homozygous line.

### *Estimation of reporter protein*

Leaf tissue was ground in liquid nitrogen, extracted with buffer (50 mM $Na_2HPO_4$ pH 7.0, 1 mM EDTA, 0.1% v/v Triton X-100, 1.0 mM DTT and 0.1% SLS), and centrifuged for 20 min at 4°C. The glucuronidase activity was assayed in cell free extract using 4-methyl umbelliferyl glucuronide as the substrate. The product, 4-methyl umbelliferone (MU) was quantified using fluorimeter (Perkin Elmer LS55, Perkin Elmer, USA). Protein concentration was determined using BioRad dye. The GUS activities were assayed in leaves and mature seed extracts of 9–10 week-old-transgenic plants. Mature seeds were soaked in water for 2 h before performing the glucuronidase enzyme assay.

### *Statistical analysis*

Standard errors of means were calculated and Student's *t*-test was carried out (Zar 1974).

**Table 1.** Activator sequences with GT core motifs.

| Sequence | Abbreviation used |
|---|---|
| TCTAGAGTGGTTAGTCTAGA | GT |
| TCTAGAGTGGTTAGGTGGTTAGTCTAGA | $(GT)_2$ |
| TCTAGAGTGGTTAGGGCTAGTGGTTAGTCTAGA | $(GT)_{N5}$ (GT) |
| TCTAGAGTGGTTAGGGCTATGGCGGTGGTTAGTCTAGA | $(GT)_{N10}$ (GT) |
| TCTAGAGTGGTTAGGGCTATGGCGGAGCAAGATTCACTCGTGGTTAGTCTAGA | $(GT)_{N25}$ (GT) |

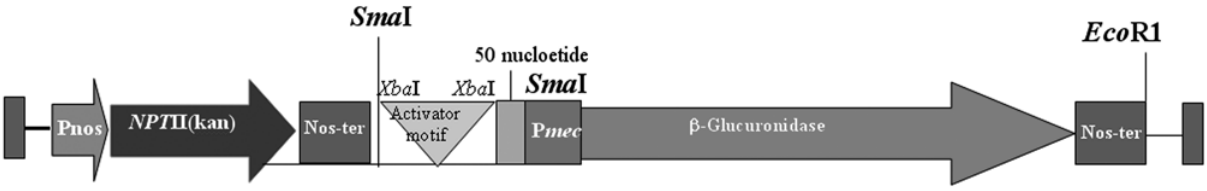


**Figure 1.** A scheme of reporter construct used for stable transformation of tobacco.

## Results and discussion

The aim of this work was to investigate the activation and/or repression behaviour of GTGGTTAG sequence in integrated state on chromosome. The octanucleotide sequence was placed in one or two copies separated by different spacer lengths. Two different kinds of spacer are used in this study. The first one is 50 bp upstream of the minimal promoter. The logic of using this spacer was to expose the *cis* regulatory elements that were used in this study. The TATA binding protein and the associated factors will cover around 30–35 nucleotides upstream of TATA box to form initiation complex. In the minimal promoter used in this study, only 10-flanking nucleotides upstream of the TATA box were present and, hence, this spacer was very essential to expose the *cis* regulatory element. Without the spacer there was a great possibility that the *cis* regulatory elements would have been concealed. The second spacer used in this study was in between the binding sites for GT-1 factor to see the effect of binding of transcription factor in or out of phase.

Comparison of the reporter gene activity in leaves and seeds of different promoter constructs revealed that the placement of *cis* elements in single copy or two copies has a significant effect over 50+*Pmec* (figure 2). Single GT element cloned upstream of 50+*Pmec* enhanced the reporter gene activity from 2260 to 5265.8 pmole $min^{-1}mg^{-1}$ protein (2.33-fold increase) in leaves. A reduction in *gus* activity was observed in promoter constructs having two GT elements. When compared with 50+*Pmec*, a reduction of 0.82, 0.47, 0.67 and 0.76 fold was observed in case of $(GT)_2$, $(GT)_{N5}(GT)$, $(GT)_{N10}(GT)$ and $(GT)_{N25}(GT)$, respectively. Two-tailed Student's *t*-tests revealed the placement of *cis* element over 50+*Pmec* is significant at $P<0.05$. The table value of *t* at 12, 13 and 14 d.f. were 2.179, 2.160 and 2.145, respectively at $P < 0.05$. When compared with 50+*Pmec*, *t*-values obtained were 6.52, 3.78, 4.04, 3.28 and 3.30 for constructs GT, $(GT)_2$, $(GT)_{N5}(GT)$, $(GT)_{N10}(GT)$ and $(GT)_{N25}(GT)$, respectively. However non-significant differences were observed in pair-wise comparisons among $(GT)_2$, $(GT)_{N10}(GT)$ and $(GT)_{N25}(GT)$.

The results indicate that the introduction of second GT element in near proximity leads to a reduction in activation of the minimal promoter. This result agrees with the behaviour of GT elements in *rbcS* promoter, where it has been reported that closer spacing of the two native GT-1 binding sites reduced transcription (Gilmartin and Chua 1990). Gilmartin and Chua (1990) also reported that the distance of 33 bp between two GT elements in the native pea *rbcS-3A* promoter is optimal. By changing the 10 bp sequence between box II and III, a very little effect on transcription was observed suggesting that the sequence as such between the two elements may not be critical to their function. The GT element sequence used in this study (GTGGTTAG) resembles the factor GT-1 binding box II motif (GTGTGGTTAATATG) present in several promoters in both monocots like rice (Kay *et al*. 1989), and dicots like pea *rbcS-3A* (Green *et al*. 1987). The box II motif used in the earlier studies binds GT-1. Its sequence has several variations in native promoters which may bind different members of the GT-1 family.

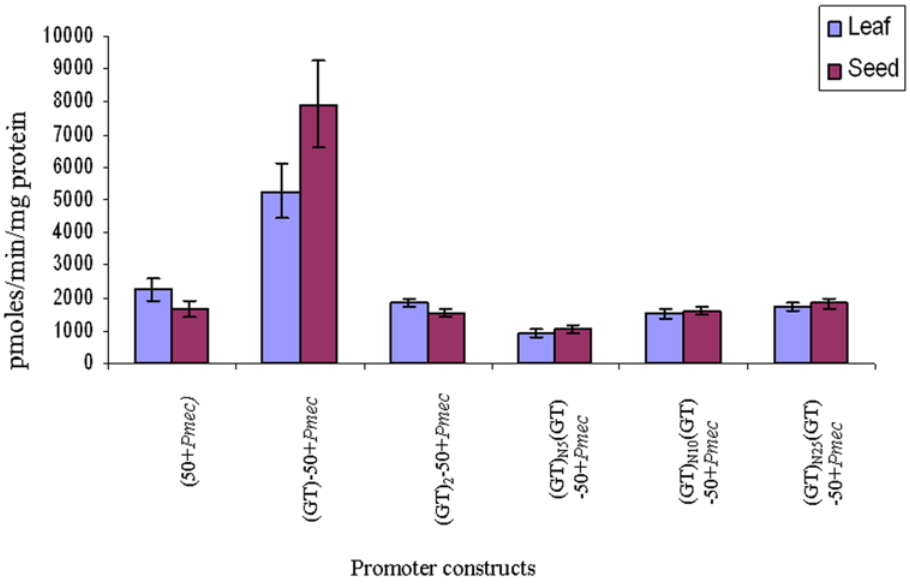


**Figure 2.** Effects of the placement of *cis* element on the expression of minimal promoter, *Pmec* in leaves and seeds in different constructs. Vertical bars are standard errors of the means. Each experiment was done thrice with three replicates. Each reading is an average of seven or eight independent transgenic lines.

The single GT element also governed high level of reporter activity in seeds. It enhanced the *gus* gene expression by 4.95-fold when compared with 50+*Pmec*. Smalle *et al*. (1998) reported that the AtGTLImRNA is more abundant in siliques; although the expression of all other cloned GT factors appears to be ubiquitous. Our results demonstrate that the presence of a second GT element in close proximity leads to a reduction in activation of the minimal promoter.

The maximum repression was noticed for $(GT)_{N5}(GT)$ in stable transgenic plants. A distance of 33 bp between two GT elements in the native pea *rbcs-3A* promoter was considered as optimal (Gilmartin and Chua 1990). On the other hand in the 5′ upstream region of phytochrome genes in rice (Kay *et al*. 1989), two GT-1 binding sites occur tandemly with the first GG in the binding centres being 14 nucleotides apart. The results suggest the role of spacer length and other features besides copy number of the GT element in promoter activation. Our study substantiates that the two closely located GT elements leads to repression and a single GT element confers high level of expression in seeds. This report shows that a single *cis* element cloned upstream of a minimal promoter can govern a high level of *gus* expression. The data obtained in this study can be used to design constructs with a single GT *cis* regulatory element for a high level of transgene expression in seeds.

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