RESEARCH NOTE

Effect of copy number and spacing of the ACGT and GT *cis* elements on transient expression of minimal promoter in plants

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Introduction

A variety of *cis*-acting DNA sequences regulate gene expression from basal promoter. In this study, two types of regulatory motifs, called ACGT and GT elements were placed at different distances from TATA-box to examine their affect on reporter gene expression in transient transformation of tobacco leaf. The ACGT core motif is recognized by transcription factors of the bZIP family. The core ACGT element occurs at different relative positions in one or more copies upstream of the minimal promoter region. Protein-DNA interaction studies have shown that sequences flanking the ACGT core affect bZIP protein binding specificity. The bZIP transcription factors regulate a variety of processes like pathogen defence (Zhou *et al*. 2000), light (Weisshaar *et al*. 1991) and flower development (Chuang *et al*. 1999). The GT elements have a core sequence with T and A, preceeded by one to two G nucleotides on the 5′ side (Zhou 1999). Though high degeneracy makes it difficult to identify them by sequence search, a variety of such sequences are present in the genes encoding diverse functions like light regulation (Dehesh *et al*. 1990), tissue specificity (Villain *et al*. 1994) and pathogen defence (Buchel *et al*. 1996). These are recognized by a family of transcription regulatory proteins called GT-factors (Buchel *et al*. 1999). The differences in binding affinity and the nature of transcriptional complex formed on promoters have been related to differences in regulatory functions of the GT sequence motifs (Puente *et al*. 1996). The GT elements are usually present in tandem repeats within a relatively long promoter region. An increase in spacing between two elements by as little as 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. This study aims at establishing the activation behaviour of these two elements, when placed upstream of a minimal promoter in one or two copies, at different positions from the TATA-box.

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Materials and methods

A minimal promoter *Pmec*, described earlier (Sawant *et al*. 2001), and cloned in the plasmid pUC19 (New England Biolabs), was used in this study. The sequence, as shown below, contains a TATA-box and a transcription initiation site followed by the reporter gene *gusA*. TSGG ATCCTCACTATATATAGGAAGTTCATTTCATTTGG AATGGACACGTGTTGTCATTTCTCAACAATTACCA ACAACAACAAACAACAAACAACATTATACAATTA CTATTTACAATTACATCTAGATAAACAATGGCTTC CTCC-*gusA*.

A 50 nucleotide long random sequence, as underlined, (GGATCCGGCTATGGCGGAGCAAGATTCACTCTGC GAGGCCAAAGCTTACCCCGGAAGGATCC), or its dimer (100 nucleotide long) were cloned at the *BamH1* site (figure 1) of the *Pmec*. One or two copies of the activator motif ACGT or GTGGTTAG were placed 50 or 100 nucleotide upstream of the *Pmec*. In each of the two series, the space between two copies of the motif was also kept variable. The two copies were either placed in tandem with no nucleotide in between, or with five, ten or twenty-five nucleotide long random sequence separating them, to further examine the effect of such a spacer on the motif dependent activation of the minimal promoter. In each case, the promoter function was monitored on the basis of the expression of *gusA* reporter gene, following transient transformation of tobacco leaves.

Keywords. ACGT element; *cis* active motifs; GT element; minimal promoter; *Nicotiana tabacum*; transcription.

The promoter-reporter cassettes containing the 50 (i.e. 50 + *Pmec*) or 100 (i.e. 100 + *Pmec*) nucleotide long random sequence upstream of *Pmec* were cloned in the pBluescript SK (+/–) Phagemid (Stratagene, USA). A fragment carrying combinations of the activator sequences listed in Table 1 was inserted upstream of the *Xba*I site (figure 1). The activator–*Pmec-gusA* cassettes were coated on gold microparticles and bombarded onto tobacco leaves at 1100 psi, using biolistic gun (Bio Rad PDS-1000/He) as in Sawant *et al*. (2000). The leaves were taken from the third or fourth node from the top of 9–10 week old plants of *Nicotiana tabacum* cv Petit Havana. An internal control comprising *gfp* expressed from 35S CaMV promoter was included in some experiments to establish reproducible delivery of gold particles in the leaves. Following bombardment, the frozen 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 minutes at 4°C. The glucuronidase activity was assayed in cell free extracts using 4-methyl umbelliferyl glucuronide (Jefferson *et al*. 1987). The product, 4-methyl umbelliferone (MU) was quantified using a fluorimeter (Perkin Elmer LS55). Protein concentration was determined using BioRad dye. The data on gene expression were statistically analysed by two way analysis of variance, followed by Newman Keuls test (Zar 1974).

Results and discussion

Tobacco leaves bombarded with 50 + *Pmec* and 100 + *Pmec* cassettes without any activator sequence upstream, gave similar low levels of *gusA* gene expression (figure 2). This was considered as the non activated, minimal level of expression driven by *Pmec*. A single ACGT element, placed upstream of the 50 + *Pmec* or 100 + *Pmec* cassettes enhanced promoter expression by 1.81 and 2.39 fold, respectively. The trend in the change in level of activation in the two series was comparable, even when two ACGT elements were included in tandem, without or with a spacer of 5, 10 or 25 nucleotides (figure 2). The highest levels of expression in the two series were obtained in the presence of (ACGT)N5(ACGT) *cis* element. In the 50 + *Pmec* series, the expression was enhanced 6.31 fold, while in case of 100 + *Pmec* series, the enhancement was 5.21 fold.

A single copy of the GTGGTTAG element enhanced *gusA* expression by about 2 fold in case of both 50 + *Pmec* and 100 + *Pmec* cassettes. However, unlike in the case of the ACGT element, when two GT elements were placed in tandem or with a spacer of 5, 10 or 25 nucleotides, a fall in the level of expression was noticed. The fall was most notable in case of (GT)N5(GT) configuration (figure 2).

Pairwise comparisons following the two way analysis of variance (ANOVA tables not shown) in the 50 + *Pmec*

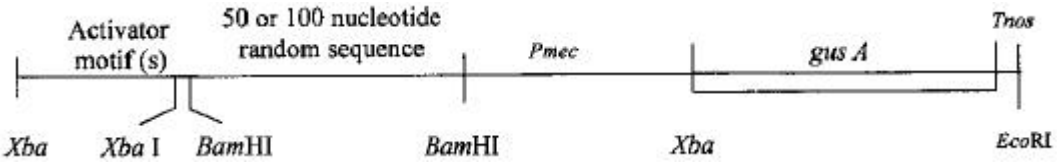

Figure 1. A scheme of reporter cassettes constructed to examine the effect of promoter architecture on *gusA* expression.

Table 1. Oligos containing one or two copies of the activator motifs with different lengths of the spacer sequence used in this study.

Activator sequence	Code
1. Activator sequences with ACGT core motifs	
TCTAGAACGTTCTAGA	ACGT
TCTAGAACGTACGTTCTAGA	$(ACGT)_2$
TCTAGAACGTGGCTAACGT TCTAGA	$(ACGT)_{N5}$ (ACGT)
TCTAGAACGTGGCTATGGCGACGTTCTAGA	$(ACGT)_{N10}$ (ACGT)
TCTAGAACGTGGCTATGGCGGAGCAAGATTCACTCACGTTCTAGA	$(ACGT)_{N25}$ (ACGT)
2. Activator sequences with GT core motifs	
TCTAGAGTGGTTAGTCTAGA	GT
TCTAGAGTGGTTAGGTGGTTAGTCTAGA	$(GT)_2$
TCTAGAGTGGTTAGGGCTAGTGGTTAGTCTAGA	$(GT)_{N5}$ (GT)
TCTAGAGTGGTTAGGGCTATGGCGGTGGTTAGTCTAGA	$(GT)_{N10}$ (GT)
TCTAGAGTGGTTAGGGCTATGGCGGAGCAAGATTCACTCGTGGTTAGTCTAGA	$(GT)_{N25}$ (GT)

series suggested no significant difference in promoter activation between single ACGT and GTGGTTAG elements. (q value = 2.59, $P > 0.01$). In the 100 + *Pmec* series, the difference between activation by the single ACGT and GTGGTTAG *cis*-elements was significant (q value = 12.37, $P < 0.01$). The ACGT element was a more efficient activator than the GT element.

In the presence of single or two tandem copies of ACGT, and two ACGT elements separated by 25 nucleotides, the activation of *Pmec* was higher when the activator motif was 100 nucleotides away. However, as the distance between the two copies was changed to 5 and 10 nucleotides, the *Pmec* placed 50 nucleotide away functioned better than the *Pmec* placed 100 nucleotides away. Statistical analysis based on q values (figure 2) suggested that the activation by the ACGT but not by the GT element was influenced by its distance from the promoter.

The results show that the minimal core sequences of the commonly occuring *cis* elements can enhance promoter expression, even when used out of their native contexts. Krawczyk *et al*. (2002) examined the effect of spacing between the two palindromes in the native sequence context and concluded that promoter activation was maximum when the two palindromic centres were 12 bp apart, as in the case of *as-1* and octopine synthase (OCS) elements in pathogen-induced plant promoters. We noticed maximum expression when the palindromic centers were 9 bp apart. Feldbrugge *et al*. (1994) reported that the two ACGT elements separated by 12 bp functioned as a negative element, depending on their position and context. Deletion of the two ACGT *cis* elements from the common plant regulatory factor 1 (CPRF 1) 5′ region, resulted in a significant increase in expression of the gene downstream. Hence, the ACGT elements can function as negative regulatory elements also. In our experiments, negative regulation by the ACGT element was not noticed, suggesting a possible role of other features in the primary sequence or the chromatin in modulating the function of *cis* elements.

The single GT element enhanced *gusA* expression by about 2 fold in case of both 50 + *Pmec* and 100 + *Pmec* cassettes. This was in agreement with the role of GT element as a positive activator of transcription. In the 5′ upstream region of phytochrome genes in rice (Kay *et al*. 1989) and oat (Hershey *et al*. 1987), two GT-1 binding sites occur tandemly with the first GG in the binding centres being fourteen nucleotides apart. In our study, the GG in the binding centres were six, eleven, sixteen and thirty-one nucleotides apart. Our results show that the presence of a second GT element in close proximity leads

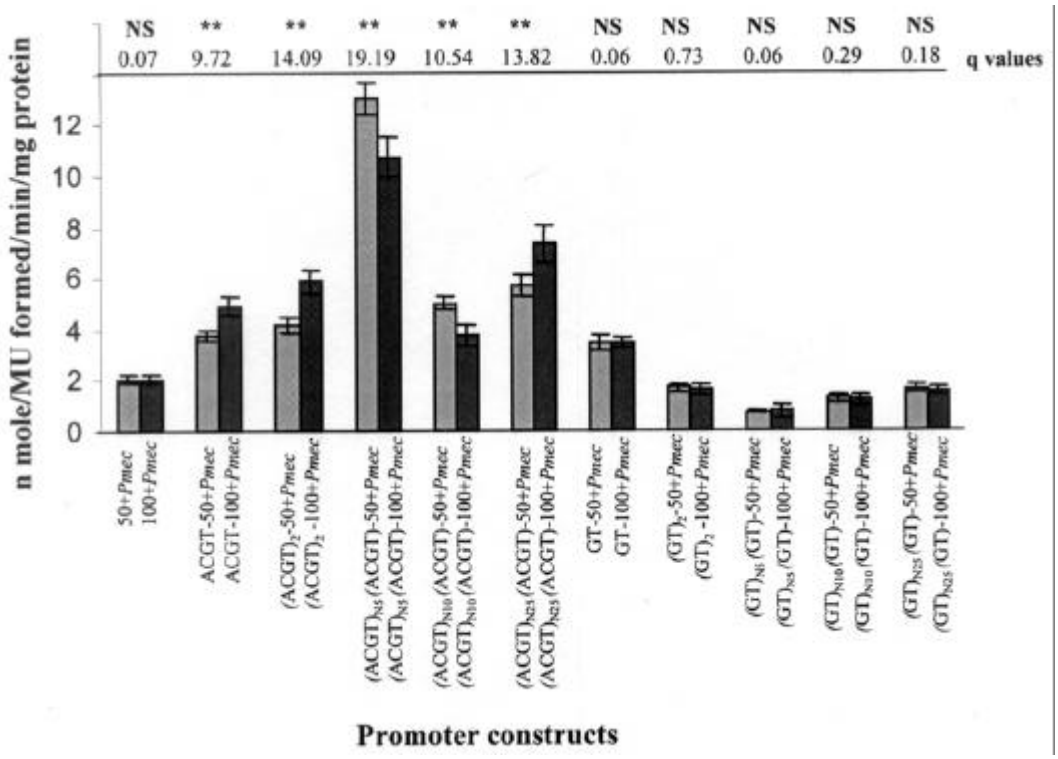

Figure 2. Effect of the placement of *cis* elements on the expression of minimal promoter, *Pmec*. The table '*q*' value at 0.01 level of significance is 3.64 at 176 degree of freedom. The calculated '*q*' values between the related pairs and the significance (**) or non significance (NS) of the data at $P < 0.01$ are indicated.

to a reduction in activation of the minimal promoter. The results agree with the behaviour of GT elements in *rbcS* promoter where, close spacing of two native GT-1 binding sites reduced transcription (Gilmartin and Chua 1990). Davis *et al*. (1989) reported that despite the presence of four tandem GT-1 binding box II sites, the configuration could not activate transcription when fused to the normally inactive – 50 deleted *rbcS-3A* promoter. *In vitro* binding of GT-1 factor is stronger to three to four tandem copies of box II than to the monomer and dimer (Green *et al*. 1988). The results suggest a role for spacer length and other features besides copy number of the GT element in promoter activation. In one case, where *P. hybrida rbcS-911* promoter has two GT-1 binding sequences with a spacing of 36 bp, their contribution to the *rbcS* transcript pool was only 1% (Dean *et al*. 1985; Dean *et al*. 1987). The low level of activity may be due to excessive distance between box II and box III-like elements in this promoter. A distance of 33 bp between two GT elements in the native pea *rbcS-3A* promoter was considered as optimal (Gilmartin and Chua 1990). Changes in the 10 base pair sequence between box II and III caused very little effect on transcription suggesting that the sequence *per se* between the two elements may not be critical to their function. *In vitro* study by Gourrierec *et al*. (1999) suggested that transcriptional activation by *Arabidopsis* GT-1 may be through TFIIA-TBP-TATA complex. The GT element sequence selected in this study (GTGGTTAG) resembles the factor GT-1 binding box II motif (GTGTGGTTAATATG) present in several promoters including dicots, like the pea *rbcS-3A* (Green *et al*. 1987) and the monocots like the rice (Kay *et al*. 1989). 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. No change in activation behaviour was noticed in transient assay in our study, by changing the distance of GT element from 50 to 100 bp from the TATA-box.

Our study substantiates that different *cis* elements function differently when their copy number, distance between multiple copies and the distance from the TATA box are altered. The ACGT *cis* element in single or two copies functioned as a positive activator of transcription. However, in case of the GT *cis* element, a second copy interfered negatively with the positive effect of the first copy. Further, investigations are planned to study how faithfully the *in vivo* results reported here by transient expression studies reflect the *cis* element function in chromatin integrated state in stably transformed plants.

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