Analysis of promoter activity in transgenic plants by normalizing expression with a reference gene: anomalies due to the influence of the test promoter on the reference promoter

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Variations in transgene expression due to position effect and copy number are normalized when analysing and comparing the strengths of different promoters. In such experiments, the promoter to be tested is placed upstream to a reporter gene and a second expression cassette is introduced in a linked fashion in the same transfer DNA (T-DNA). Normalization in the activity of the test promoter is carried out by calculating the ratio of activities of the test and reference promoters. When an appropriate number of independent transgenic events are analysed, normalization facilitates assessment of the relative strengths of the test promoters being compared. In this study, using different modified versions of the Cauliflower Mosaic Virus (CaMV) 35S promoter expressing the reporter gene *β-glucuronidase* (*gus*) (test cassette) linked to a *chloramphenicol acetyl transferase* (*cat*) gene under the wild-type 35S promoter (reference cassette) in transgenic tobacco lines, we observed that *cat* gene expression varied depending upon the strength of the modified 35S promoter expressing the *gus* gene. The 35S promoter in the reference cassette was found to have been upregulated in cases where the modified 35S promoter was weaker than the wild-type 35S promoter. Many studies have been carried out in different organisms to study the phenomenon of transcriptional interference, which refers to the reduced expression of the downstream promoter by a closely linked upstream promoter. However, we observed a positive interaction wherein the weakened activity of a promoter led to upregulation of a contiguous promoter. These observations suggest that, in situations where the promoters of the test and reference gene share the same transcription factors, the activity of the test promoter can influence the activity of the reference promoter in a way that the test promoter's strength is underestimated when normalized by the reference promoter.

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1. Introduction

The introduction of a transgene via *Agrobacterium* sp.-mediated gene transfer is the most commonly used method for plant transformation. The introduced transfer DNA (T-DNA) cassette might integrate as a single copy or multiple copies in the genome. Further, in different transgenic plants transformed with the same construct, the site of integration at chromosomal locations is also different. This leads to extensive variation in the expression of the gene of interest in different independent transformation events. This has been referred to as 'the position effect' in earlier studies (Peach and Velten 1991; Iglesias *et al.* 1997). In experiments where the strengths of different promoters are compared by developing transgenics, variations due to position effect interfere with analysis of the *per se* strength of the promoters

Keywords. CaMV 35S promoter; normalizations; position effect; reference gene; synthetic promoters; transcriptional interference

Abbreviations used: 35S, Cauliflower Mosaic Virus 35S promoter; CAT, chloramphenicol acetyl transferase; GUS, glucuronidase; Mp, minimal promoter; MU, methylumbelliferone; NPT II, neomycin phosphotransferase; PCR, polymerase chain reaction; Pnos, nopaline synthase promoter; Pref, reference promoter; Ptest, test promoter; T-DNA, transfer DNA

(test promoter, Ptest). Normalizations of such variations have been carried out by the use of a reference gene (Sanger *et al.* 1990; Iglesias *et al.* 1997; Bhullar *et al.* 2003, 2007). In such experimental designs, the T-DNA constructs contain the Ptest placed upstream to a reporter gene (for example, *β-glucuronidase*, *gus*), and a reference promoter (Pref) drives a second reporter gene (as an example, *chloramphenicol acetyl transferase*, *cat*). While the Ptest-reporter cassette varies from one construct to another, the Pref-reporter cassette is kept constant for all the constructs (Ow *et al.* 1987; Fang *et al.* 1989; Lam *et al.* 1989; Bhullar *et al.* 2003, 2007). Normalization in the activity of the Ptest is carried out by calculating the ratio of Ptest and Pref activities. In such analyses, it is assumed that when an appropriately large number of independent transgenic events are analysed, the ratio of Ptest and Pref will give an assessment of the relative strength of the different test promoters. However, in our study, we report that the activity of the Pref could also be influenced by the activity of the Ptest, especially when both the Ptest and Pref promoters share common *cis*-elements. These observations have implications on the analysis of the strengths of promoters by normalizations and for the choice of the reference promoter. Further, several studies have reported the phenomenon of transcriptional interference, which implies negative *cis* effects between promoters (Adhya and Gottesman 1982; Ingelbrecht *et al.* 1991; Padidam and Cao 2001; Eszterhas *et al.* 2002; Callen *et al.* 2004; Martens *et al.* 2004). However, our observation is an example of a positive interaction, wherein the weakened activity of a promoter upregulates the activity of a contiguous promoter.

2. Materials and methods

2.1 *Creation of modified promoters*

Four synthetic promoters (Mod2A1T, Mod3A1T, Mod4A1T and ModB1A1T; figure 1) were created using recursive

Figure 1. **(A)** Sequence comparison of domain A (–90 to +1) of the 35S promoter with that of the Mod2A1T, Mod3A1T and Mod4A1T promoters. The repeat region of *as-1* element and TATA box are shown in bold italics. Domain A of Mod2A1T, Mod3A1T and Mod4A1T promoters show sequence similarity of 35%, 41% and 33%, respectively, with that of the 35S promoter in the regions flanking the identified *cis*-elements. The rest of the 35S promoter (domain B; –90 to –343) is the same in all the promoters. The region which is identical to the 35S promoter sequence is underlined. **(B)** Sequence of the subdomain B1 and domain A (–108 to +1) of the 35S promoter and ModB1A1T. *Cis*-elements of B1 and domain A are highlighted in bold italics. The nucleotide separating GATA/GATG in the *as-2* element was mutated and the direct repeat TGACG of the *as-1* element was placed in the synthetic DNA context with different flanking and spacer nucleotides. The rest of the 35S promoter (–109 to –343) is the same as its wild-type counterpart. CG and CNG that have been retained are underlined. The +1 site is marked in bold letters.

polymerase chain reaction (PCR) followed by subsequent cloning in the pPCRScript (SK+) vector (Stratagene, La Jolla, CA, USA). DNA sequences of the modified promoters were confirmed by sequencing. All clonings followed standard protocols as described in Sambrook *et al.* (1989). The modified promoters (Ptest; figure 2A) were cloned as *Hin*dIII-*Nco*I fragments upstream to the *gus* reporter gene with a 35S poly(A) signal in pPCR Script SK(+). The Ptest-*gus* expression cassette thus developed was cloned as a *Sac*I–*Sal*I fragment in the binary vector pPZP200 (Hajdukiewicz *et al.*1994) containing a *cat* gene under the control of the 35S promoter (P35S/Pref) at the *Hin*dIII site towards the right border to be used as a reference expression cassette. The binary vector additionally contained a nopaline synthase promoter (Pnos)-driven *neomycin phosphotransferase II* (*npt II*) gene, as a selection marker for plant transformation at an *Eco*RI site towards the left border of the T-DNA. The synthetic promoters Mod2A1T and Mod3A1T have been described in our previous report (Bhullar *et al.* 2003). Two binary vectors containing the promoter Mod2A1T and ModB1A1T were also developed without the reference expression cassette (35S-*cat* gene; figure 2B).

2.2 *Development of transgenic lines*

Binary vectors were mobilized into disarmed *Agrobacterium* sp. strain GV2260 by electroporation. The transgenic lines were developed in *Nicotiana tabacum* cv. Xanthi by *Agrobacterium* sp.-mediated transformation of leaf-disc explants following the protocol of Svab *et al.* (1995). For each of the constructs, 7–25 independent transgenic lines were developed. In order to reduce the influence of the physiological status of the plant on promoter activity, the transgenic lines transformed with the expression cassettes containing different promoters that were to be compared, were grown and maintained simultaneously under controlled conditions (16 h day and 8 h night, 28°C ± 2°C, relative humidity 70%) in soil in a growth chamber. Promoter activity was analysed at two growth stages using the leaves of 45- and 60-day-old (after transfer to soil) plants.

For the analysis of expression in different tissues, viz., stem and roots, the plants were grown in a tissue culture room (16 h day and 8 h night, 28°C ± 2°C) on Murashige–Skoog (MS) medium in glass bottles and the tissues were harvested 35–40 days after subculturing. Callus was raised from either stem or leaf tissue by placing 15–18 explants on MS agar supplemented with 2 mg/l α-naphthaleneacetic acid (NAA) and 0.5 mg/l 6-benzylaminopurine (BAP). Extracts were prepared 14–16 days after callusing from profusely growing calli.

Selfed (T_1) seeds were collected from the growth chamber-grown primary transformants (T_0) and germinated on germination paper. Approximately 150 7-day-old seedlings were harvested to make the total protein extract.

2.3 *Enzyme assays for estimation of promoter strength*

The total protein from different tissues of transgenic plants was extracted in GUS extraction buffer (Jefferson 1987).

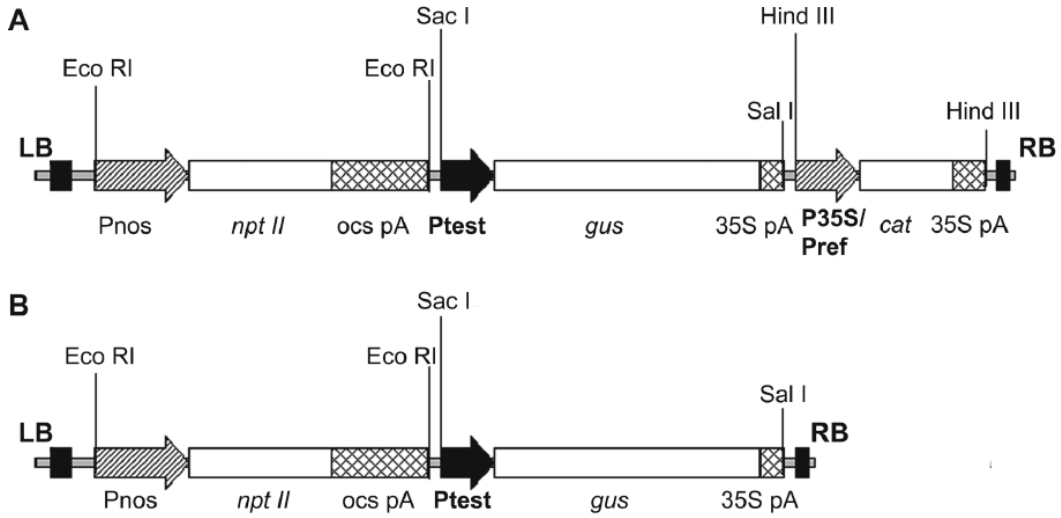

Figure 2. **(A)** Schematic representation of the T-DNA of the binary vector developed for transformation of tobacco. LB and RB are the left and the right borders, respectively, of the T-DNA. The selection marker gene *npt II* is driven by the *nos* promoter (Pnos) and contains the 3′ pA region of the *ocs* gene (ocspA). Ptest stands for the synthetic promoter driving the *gus* reporter gene or 35S promoter in the case of the wild-type 35S-driven *gus* gene and has the 3′ pA region of 35S. The *cat* gene is under the control of a second 35S promoter (P35S/Pref) and has a 3′ pA region from 35S promoter. **(B)** This binary vector is same as in A except that it lacks the reference 35S-*cat* reporter cassette.

The protein concentration was estimated according to the method of Bradford (1976). Fluorometric GUS assays using 4-methyl umbelliferyl *β*-D-glucuronide (MUG) substrate were performed as described earlier (Jefferson 1987). The product released (methylumbelliferone [MU]) was estimated with a DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech, San Francisco). GUS activity was expressed as pmol MU min^{-1} mg^{-1} protein.

The amount of CAT and NPT II protein in the total protein extracts was measured by ELISA using kits from Roche Diagnostics (Cat. No.1363727, Mannheim, Germany) and Agdia Incorporated (Cat. No. PSP 73000, Indiana), respectively. CAT and NPT II levels were expressed as mg protein/mg of total protein. All reactions were carried out in duplicate.

2.4 *Statistical analyses of the data*

Statistical tests were performed using the JMP-SAS package (SAS Institute; Cary, NC). The Shapiro–Wilk test for normality was used to assess the distribution of promoter activities across the two promoters being compared. As there was a significant deviation from normality in some cases, the non-parametric Wilcoxon rank-sum test was applied to test whether the activities of the different promoters being compared were significantly different or not. *P* values <0.05 were accepted as significantly different.

3. Results and discussion

The Cauliflower Mosaic Virus 35S (35S) promoter consists of two domains – domain A that contains a minimal promoter (mp) and subdomain A1, and domain B that comprises five subdomains, B1–B5 (Benfey *et al.* 1990a, b). Apart from the TATA box in the mp, *cis*-elements of subdomains A1 and B1 have been functionally characterized. Transcription factors ASF-1 and ASF-2 have been shown to interact with the *as-1* (Lam *et al.* 1989) and *as-2* elements (Lam and Chua 1989) of subdomains A1 and B1, respectively (figure 1). In an earlier study to analyse ways of circumventing promoter homology-based gene silencing, we designed two synthetic 35S promoters (Mod2A1T and Mod3A1T) by modifying the domain A of the 35S promoter (Bhullar *et al.* 2003). These promoters, which were observed to be as active as the wild-type 35S, were generated by placing the core elements of the minimal promoter and subdomain A1 in the context of a divergent nucleotide sequence. Thus, the synthetic promoters shared the same transcription factors as those of 35S but differed in their sequence composition for domain A. As described in our early study (Bhullar *et al.* 2003), the synthetic context sequences were generated by keeping their length and GC content the same as that of 35S. However, the synthetic stretches lacked methylation-prone CG and CNG sites. In order to extend our earlier observations, we designed another promoter Mod4A1T on lines that were similar to Mod2A1T and Mod3A1T, but with a variant sequence context. While domain A of Mod2A1T and Mod3A1T had a sequence similarity of 35% and 41%, respectively, with that of the 35S promoter in the regions flanking the identified *cis*-elements (Bhullar *et al.* 2003), Mod4A1T was 33% similar to the 35S in this region (figure 1A).

The activities of the synthetic promoters Mod2A1T, Mod3A1T and their comparison with that of the wild-type 35S promoter in transgenic tobacco lines have been reported in our earlier study (Bhullar *et al.* 2003). Analysis of the activity of the reference promoter driving the *cat* gene and Pnos promoter expressing the *npt II* gene have been reported in the present study, in addition to the analysis of all the promoters in transgenic lines developed with Mod4A1T and ModB1A1T promoter constructs. The study of the different promoter activities in each of the transgenic lines was carried out by assaying for *β-glucuronidase* enzyme in the case of Ptest and by measuring CAT and NPT II levels by ELISA in the case of Pref 35S promoter, which is expected to function as reference, and the *nos* promoter Pnos for the *npt II* gene, respectively.

As reported in our earlier study (Bhullar *et al.* 2003), the activities of Mod2A1T and Mod3A1T were found to be equivalent to that of the 35S promoter based on the analysis of GUS activities (figure 3A, D). On the other hand, the activity of the promoter Mod4A1T was found to be significantly lower than that of the 35S promoter (figure 3G). That the fall in activity of Mod4A1T was not due to mutation in the promoter cassette following mobilization in *Agrobacterium* sp. was confirmed by amplifying and sequencing the Mod4A1T promoter from the transgenic lines. It thus appears that the drop in promoter activity could be due to an inadvertent incorporation of a strong negative regulatory element during the designing of the novel sequence, which could be the DNA structure *per se* or a binding site for some repressor protein. Although in our earlier study (Bhullar *et al.* 2003) we reported the possibility of creating a set of synthetic promoters with minimum sequence homology, but with expression levels comparable to the wild-type prototype by modifying sequences present between *cis*-elements, it seems that more information is required about promoter architecture, framework for transcription factor-binding sites and DNA bending *per se*, in order to universally use this strategy.

Of greater significance was the observation that the expression levels of CAT (the reference gene) varied, depending upon the comparative strength of the Ptest. In the cases of both Mod2A1T and Mod3A1T, wherein the activity of the synthetic promoter is equivalent to that of the 35S promoter (figure 3A, D), the activity of the 35S promoter

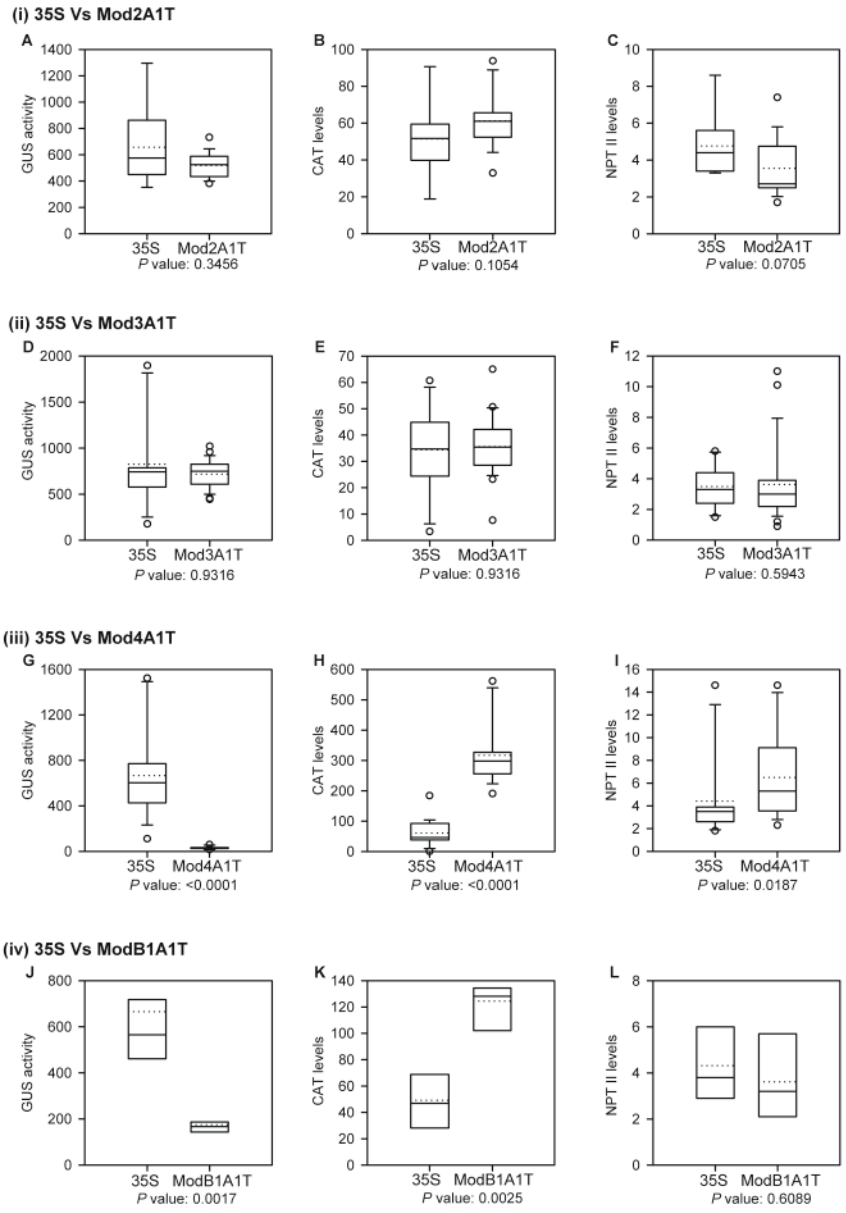

Figure 3. A comparison of the activities of different promoters in the leaves of primary tobacco transgenic plants (T_0). The transgenic lines transformed with the promoter cassettes to be compared were grown and maintained simultaneously under control conditions (16 h day and 8 h night, 28°C ± 2°C, relative humidity 70%) in the growth chamber. Promoter activity was analysed at two growth stages using the leaves of 45- and 60-day-old (after transfer to soil) plants. The box and whisker plot represents the comparison of the activities of promoters Ptest, Pref and Pnos in transgenic lines developed with a control 35S promoter construct and the modified promoters Mod2A1T, Mod3A1T, Mod4A1T and ModB1A1T. The horizontal lines in the 'box and whisker' plot signify 10, 25, 50, 75 and 90 percentiles. The dotted line represents the mean of the data. Extreme values are displayed as small circles at the top and bottom of the plot, known as outliers. **(A, D, G, J)** GUS activity from the 35S promoter and its modified promoter versions Mod2A1T, Mod3A1T, Mod4A1T and ModB1A1T, respectively; **(B, E, H, K)** CAT levels under the control of the Pref 35S promoter, and **(C, F, I, L)** NPT II levels as driven by Pnos. A total of 9 T_0 plants containing 35S–*gus* and 17 T_0 plants having Mod2A1T–*gus* were analysed as one set. In the second set, 11 T_0 lines with 35S–*gus* and 25 T_0 lines containing Mod3A1T–*gus* were analysed. The third set consisted of 19 T_0 plants harbouring 35S–*gus* and 16 T_0 plants having Mod4A1T–*gus*. In another set, 7 T_0 plants containing 35S-driven *gus* and 7 with ModB1A1T–*gus* were analysed. The *P* values are given below each plot. *P* values <0.05 reflect significant difference in the activity of the two promoters being compared. GUS activity was expressed as pmol MU min^{-1} mg^{-1} protein, while CAT and NPT II levels were expressed as mg protein/mg of total protein. Box and Whisker plot is available as an option in the software SigmaPlot.

driving the *cat* gene was also found to be equivalent in the transgenic lines containing the wild-type 35S and the synthetic 35S promoter driving the *gus* gene. The CAT levels varied between 18.8 and 90.7 mg/mg of total protein in 35S–*gus* containing transgenic lines, which were similar to that in Mod2A1T–*gus* containing transgenic lines (32.9 and 93.8 mg/mg of total protein; figure 3B). In the experiments with Mod3A1T–*gus* and 35S–*gus*, the CAT levels ranged between 3.4 and 60.7 mg/mg of total protein in 35S–*gus* containing transgenic lines, compared with 7.6–65.0 mg/mg of total protein in Mod3A1T–*gus* containing transgenic lines (figure 3E). However, in the case of Mod4A1T, where synthetic promoter activity showed a considerable drop in GUS activity compared with that of the 35S promoter (figure 3G), there was a significant enhancement in the activity of the reference 35S promoter as observed by the levels of CAT protein (figure 3H). While in transgenic lines containing the 35S–*gus* construct the CAT levels were observed to be between 0.3 and 183.9 mg/mg of the total protein, in lines with the Mod4A1T–*gus* construct, there was a significant increase in the range of CAT level expression, which varied between 190.6 and 561.5 mg/mg of total protein (figure 3H). In order to substantiate this observation, we analysed the expression levels of CAT in transgenic lines containing a fourth modified ModB1A1T promoter (figure 1B), which was observed to be a weak promoter in comparison with 35S. The drop in activity of ModB1A1T (figure 3J) was mainly due to changes introduced in the 7 bp spacer sequence between the direct TGACG repeats of the *as-1* element, which was observed to be critical for the activity of the 35S promoter (Bhullar *et al.* 2003). Consistent with our observations with the Mod4A1T promoter, the CAT levels were significantly higher in transgenic lines with the ModB1A1T–*gus* expression cassette in comparison with lines with the 35S–*gus* gene (figure 3 K). The NPT II levels in all cases except that of Mod4A1T remained similar (figure 3C, F, I, L). Although it is difficult to predict any linear correlation in the present study, the trend suggests that the weaker the Ptest-driving *gus* gene, the higher the level of enhancement of the Pref-driven *cat* gene.

In order to test whether similar trends in activity were observed in other tissues, the activity of Ptest, Pref and Pnos was monitored in stem, root and callus of transgenic lines developed with the Mod2A1T promoter (equivalent to 35S), ModB1A1T promoter (weaker than 35S) and 35S promoter constructs. As already reported (Bhullar *et. al.* 2003), the activity of Mod2A1T is equivalent to that of the 35S promoter in all the tissues tested (figure 4A, D, G). Further, the activity of the Pref as assayed by CAT levels (figure 4B, E, H) in transgenic lines with the Mod2A1T promoter was not significantly different from that observed in transgenic lines with the 35S promoter construct. As in the case of leaves, the activity of Pnos in other tissues also did not vary significantly between the transgenic lines with 35S and Mod2A1T promoter constructs (figure 4C, F, I). Our observations showed that in the Mod2A1T promoter-containing transgenic lines, all the three expression profiles, viz., GUS activity, CAT and NPT II levels were not significantly different as compared with transgenic lines with the 35S–*gus* construct (figure 4). However, in contrast, in ModB1A1T (figure 5), GUS activity (figure 5A, D, G) and CAT expression levels (figure 5B, E, H) were found to be significantly different than those of the 35S–*gus* construct containing transgenic lines, thus emphasizing that when Ptest is appreciably weaker than the 35S promoter, CAT levels show a marked increase in all the tissues tested, while no significant difference was observed in the activity of Pnos (figure 5C, F, I).

The 35S–*cat* gene cassette used as a reference gene in our study has also been used in many earlier studies (Ow *et al.* 1987; Fang *et al.* 1989; Lam *et al.* 1989), wherein characterization of the 35S promoter was carried out. In most of these studies, deletion analyses were carried out to delineate the various functional domains of 35S. However, variations in the CAT levels due to changes in the nearby Ptest could have led to erroneous conclusions following normalizations. For example, in the case of ModB1A1T, GUS expression in different independent transgenic lines showed a greater fall in activity when normalized with CAT levels (10.43–19.82 GUS/CAT units in case of the 35S promoter, and 1.11–1.97 GUS/CAT units in the ModB1A1T promoter; figure 6A) as compared with the drop in GUS expression when normalized by NPT II levels (85.5–219.1 GUS/NPT II units in case of the 35S promoter, and 26.7–89.0 GUS/NPT II units in the ModB1A1T promoter; figure 6B), which is not influenced by the Ptest. The increased drop in CAT-normalized GUS expression is due to higher CAT levels in ModB1A1T as compared with the 35S promoter. Therefore, the use of a Pref, which shares *cis*-elements and thus transcription factors with the Ptest, can lead to wrong normalizations.

Further, many studies have been carried out to analyse the phenomenon of transcriptional interference, which refers to the reduced expression of the downstream promoter by a closely linked upstream promoter in different organisms (Adhya and Gottesman 1982 in prokaryotic systems; Ingelbrecht *et al.* 1991 in transgenic tobacco calli; Padidam and Cao 2001 in protoplasts prepared from tobacco cell suspension line BY2; Eszterhas *et al.* 2002 in mouse cell lines; Callen *et al.* 2004 in *Escherichia coli*, Martens *et al.* 2004 in *Saccharomyces cerevisiae*, to name a few and latest reviewed by Shearwin *et al.* 2005). Many models such as promoter competition (Hirschman *et al.* 1988, Conte *et al.* 2002), sitting duck interference (Callen *et al.* 2004), occlusion (Adhya and Gottesman 1982), collision (Prescott and Proudfoot 2002) and roadblock (Epshtein *et al.* 2003)

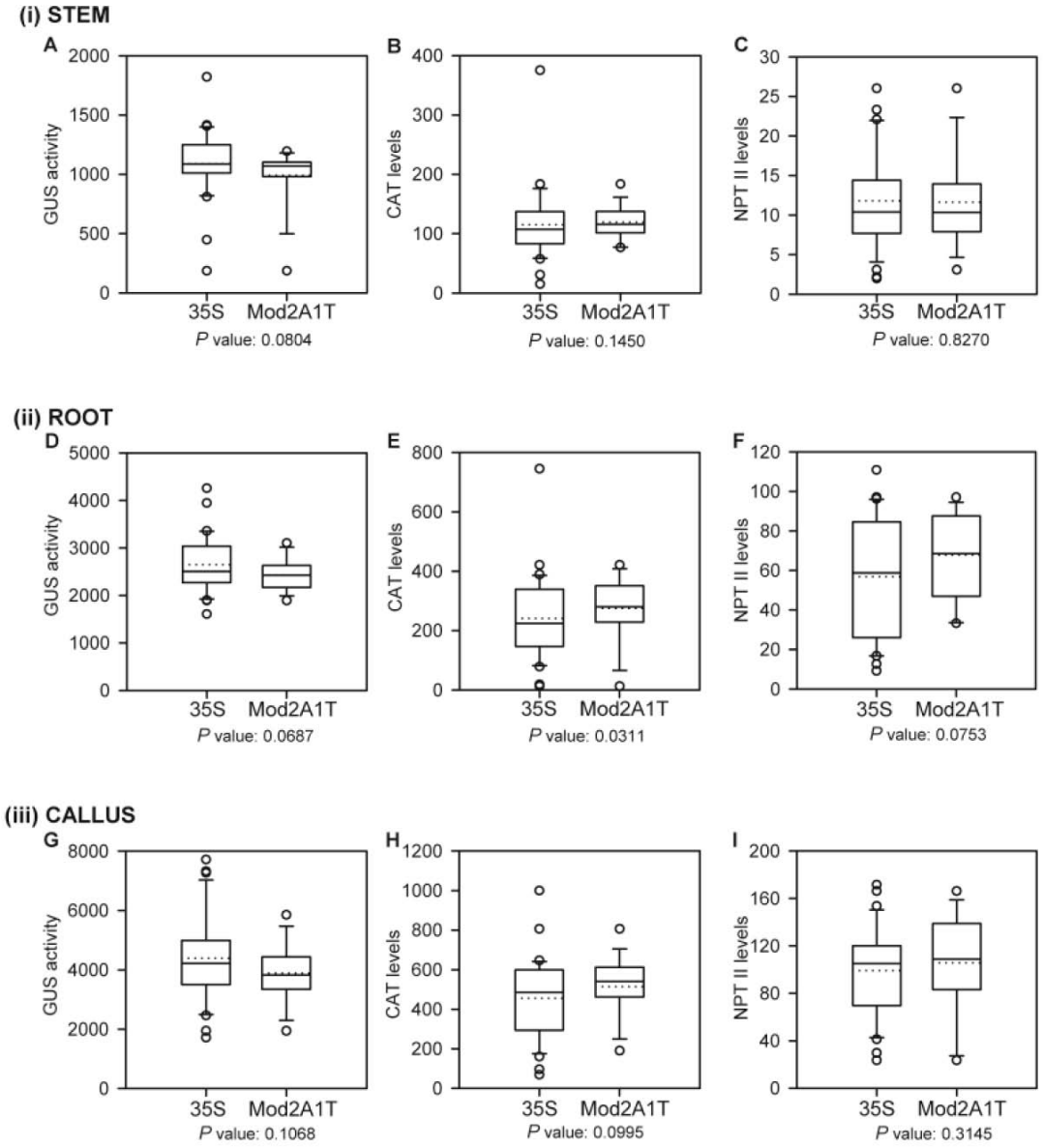

Figure 4. A comparison of the activities of different promoters (Ptest, Pref and Pnos) in different tissues of T_0 plants with 35S and Mod2A1T promoter constructs. A minimum of 13 and maximum of 21 independent T_0 plants, grown and maintained simultaneously in tissue culture were used for expression analysis in stem **(A, B, C)**, root **(D, E, F)** and callus **(G, H, I)**.

have been proposed to explain the suppressive influence of one transcriptional process on a second one. In all these reports, although the operative mechanism has been found to be different, the outcome has been the repression of one promoter by a contiguous second promoter. Only one report (Opel *et al.* 2001) in *E. coli* showed transcriptional coupling in which a promoter is activated by the activity of the divergent promoter. This activation was due to the release of negative helical twists caused by DNA supercoiling. In the present study, we observed a positive interaction, wherein the weakened activity of a promoter led to the upregulation of a contiguous promoter, as opposed to the suppression that is the underlying mechanism in transcriptional interference.

4. A hypothesis

The observed variation in the activities of the reference 35S promoter vis-à-vis that of the synthetic promoter could possibly be due to the wild-type 35S promoter competing away transcription factors from the adjacent modified promoter, which is impaired for its transcriptional activity.

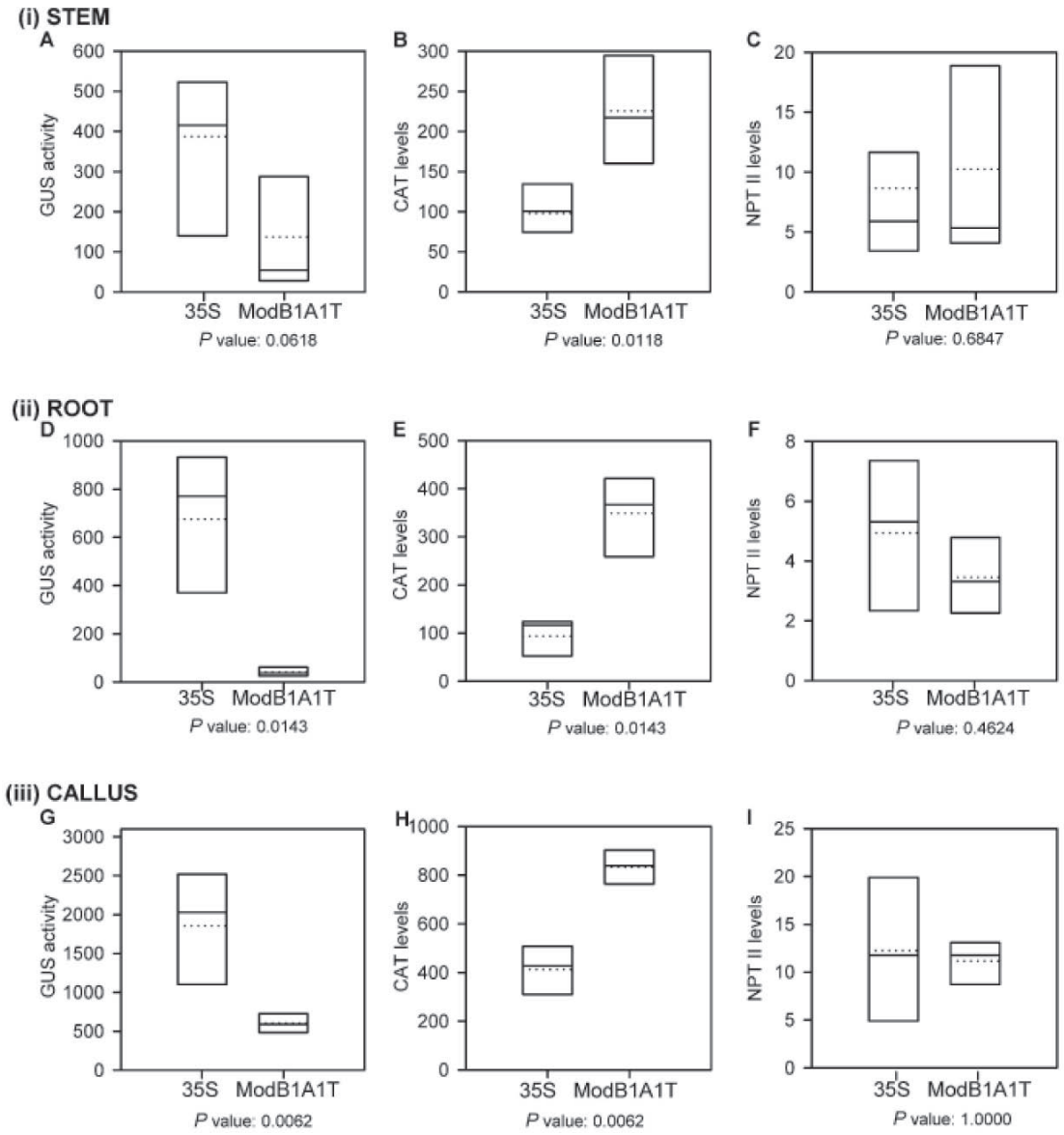

Figure 5. A comparison of the activities of different promoters (Ptest, Pref and Pnos) in different tissues of T_0 plants with 35S and ModB1A1T promoter constructs. A minimum of 4 and maximum of 7 independent T_0 plants, grown and maintained simultaneously in tissue culture were used for expression analysis in stem **(A, B, C)**, root **(D, E, F)** and callus **(G, H, I).**

Such a situation is likely to arise when there is a limitation of transcription factors locally. Thus, transcription factors, while interacting with the first promoter, are precluded from interacting with the second promoter as demonstrated in studies by Hirschman *et al.* (1988) in *S. cerevisiae* and Conte *et al.* (2002) in retroelements from *D. melanogaster*. In such a scenario, the modified promoter is expected to show higher levels of activity when the competing 35S promoter is missing. A comparison of normalized GUS activity (GUS activity/NPT II) in 7-day-old T_1 seedlings of transgenic lines containing Mod2A1T with (figure 2A) and without the neighbouring 35S–*cat* reference gene cassette (figure 2B) was carried out and showed no enhancement in Mod2A1T activity in the absence of a competing 35S promoter (figure 7A). The GUS/NPT II range varied from 54.2 to 113.9 units in Mod2A1T with *cat*, similar to the 56.9–105.7 units in Mod2A1T without the *cat* gene. We further analysed the expression of GUS under the ModB1A1T promoter (ModB1A1T–*gus*) in the presence and absence of the neighbouring 35S–*cat* cassette in transgenic lines, and the results again showed no significant difference (figure 7B). Although one can argue that since the activity of the ModB1A1T promoter is hampered, no increment in its expression is observed, our observations on Mod2A1T

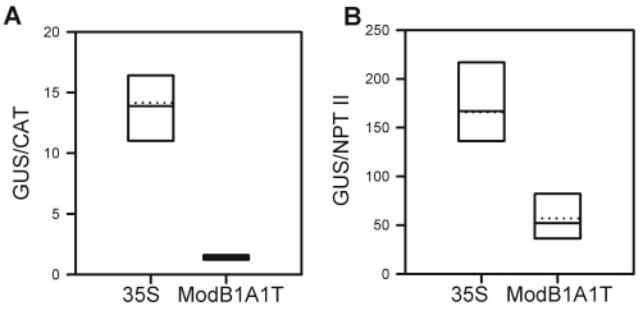

Figure 6. Comparison of **(A)** CAT-normalized GUS activities (GUS/CAT); **(B)** NPTII-normalized GUS activities (GUS/NPT II) between leaves of transgenic plants with 35S and ModB1A1T promoter constructs. The experimental design was the same as in figure 3.

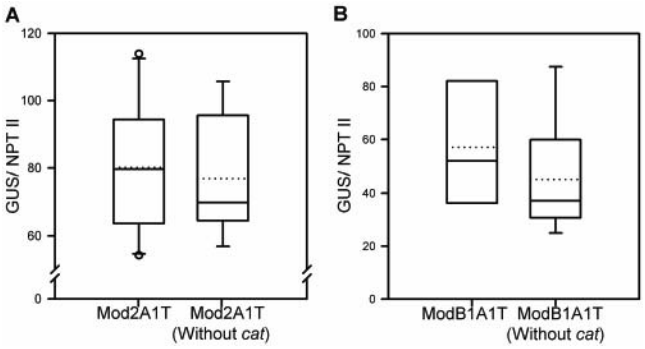

Figure 7. Comparison of GUS-normalized activities (GUS activity/NPT II levels) between transgenic plants with **(A)** Mod2A1T and Mod2A1T (without 35S-*cat*) promoter constructs in 7-day-old seedlings (T_1) of 10 representative transgenic lines containing Mod2A1T–*gus* along with the 35S–*cat* cassette, and 9 transgenic lines with Mod2A1T–*gus*, but lacking the 35S–*cat* cassette. Seedlings were grown on germination paper and harvested 7 days post germination. **(B)** ModB1A1T and ModB1A1T (without 35S–*cat*) promoter constructs in the T-DNA cassette of the binary vector. A minimum of 8 T_0 plants containing ModB1A1T–*gus* and 9 containing ModB1A1T–*gus* but lacking the 35S-*cat* cassette, were analysed as a set. The experimental design was the same as in figure 3.

reflect that there is probably no competition between wild-type 35S and the modified promoter.

A second plausible explanation for our observation is that the impaired modified promoter functions as an enhancer for the neighbouring wild-type 35S promoter by increasing the local concentration of transcription factors. In such a case, the impaired modified 35S promoter driving the *gus* gene can recruit transcription factors at domain B, which has not been modified in synthetic promoters, but is not able to utilize them efficiently, and the neighbouring 35S promoter is able to use these sequestered factors to drive better expression of its own gene (*cat* gene in this case). The weak promoter thus acts as a sink to attract factors that can be utilized by the stronger promoter present adjacent to it.

This could also be the way in which the enhancer element of 35S works. It has been shown in earlier studies that the –90 to –343 region of the 35S promoter functions as an enhancer (Kay *et al.* 1987; Odell *et al.* 1988), wherein multiple copies (1–5) of the –90 to –343 region progressively increase the activity of the 35S promoter. However, the 35S enhancer is sensitive to orientation, and shows decreased enhancing activity in a reverse orientation even when placed ~580 bp upstream to a promoter (Odell *et al.* 1988). Several general models have been proposed to explain enhancer activity (although not for 35S promoter), which utilize protein–protein contacts, covalent modifications of proteins, chromatin structure and nucleosome remodelling, to name a few (Blackwood and Kadonaga 1998; Bulger and Groudine 1999; Calhoun *et al.* 2002; Hatzis and Talianidis 2002). We envisage a simple model wherein the enhancing effect of the 35S promoter may simply be due to the presence of an increased number of *cis*-elements in the 35S double/multiple enhancer promoters, which ensures higher local concentration of transcription factors, which are then used by a functional minimal promoter in its immediate vicinity.

In conclusion, in situations where the promoters of the test and the reference gene share the same transcription factors, the activity of the Ptest can influence the activity of the Pref in such a way that the promoter strength recorded for the Ptest by normalization can underestimate its strength. These findings on the enhancement of synergistic activity for downstream promoters have a significant impact not only on the analysis of promoter activity but also on the development of transgenic plants where multiple transgenes are expressed under similar promoters.

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