A variety of synergistic and antagonistic interactions mediated by cis-acting DNA motifs regulate gene expression in plant cells and modulate stability of the transcription complex formed on a basal promoter

Samir V. Sawant, Kanti Kiran, Rajesh Mehrotra, Chandra Prakash Chaturvedi, Suraiya A. Ansari, Pratibha Singh, Niraj Lodhi and Rakesh Tuli*

National Botanical Research Institute, Rana Pratap Marg, Lucknow-226001, India

Received 14 February 2005; Accepted 16 May 2005

Abstract

Several synthetic promoters containing a variety of commonly found cis-acting DNA sequence motifs were constructed to study the motif–motif and motif–protein interactions involved in gene expression in plants. Transient expression of the reporter gene gusA in tobacco leaves was used to demonstrate that several sequence elements can be arranged upstream of a basal promoter to function synergistically in enhancing gene expression. A cis-acting DNA motif could function as an activator by itself as well as a synergizing activator in the presence of other homologous as well as heterologous motifs in the neighbourhood. The function of a complex promoter comprising several activation motifs was arrested nearly completely in vivo, following titration with any one of the motifs. The results suggested a hierarchical assembly of several motif-binding factors, leading to the stabilization of the transcriptional complex formed on the TATA-box.

Key words: Cis motif interactions, gene regulation, plant promoters, transcription activation in plants, transcription factors.

Introduction

A complex interplay of DNA and protein interactions activates transcription in plant promoters (Singh, 1998; Chen, 1999; Haralampidis et al., 2000; Benfey and Weigel, 2001). Computational analysis of the genome database reveals a large variety of conserved sequence elements present upstream of the TATA-box. These are present in variable copy numbers as variants of core motifs, positions and relative arrangements (Pilpel et al., 2001; Sawant et al., 2001a). A number of regulatory proteins that bind such elements have been identified (Riechmann and Ratcliffe, 2000). These modulate recruitment of the transcription machinery on the minimal promoter (Chen and Hampsey, 2002). Several other accessory proteins that do not directly bind specific DNA motifs facilitate contact between the sequence-specific activators and the basal transcription machinery (Näää et al., 2001).

Several groups are studying the role of individual sequence motifs in a combinatorial context of sequence architecture in promoter expression in plants. Transcriptional factors that bind known sequence motifs can be assembled on synthetic promoters to determine their function in vivo (Rushton et al., 2002; Bhullar et al., 2003). The design of an artificial expression cassette for the high level expression of transgenes in plants was reported earlier (Sawant et al., 2001a). It was developed by deploying core sequences of eight types of motifs and their variants. These included, the ACGT-box, OCS-element, W-box, GT1-element, GATA-box, CAAT-box, PU-element, and YY1-element that are commonly present in highly expressed plant genes. These motifs were employed in this study, singly and in combination, to construct artificial modules that were placed upstream of a minimal promoter. In vivo expression of the reporter gene gusA from such constructs was analysed following their delivery in tobacco leaves, in order to explore the mechanistic details of expression from a multifactorial expression cassette. In vivo titration of the motif-specific factors by co-delivery of specific oligonucleotides revealed that the activating factors were...
present intracellularly as protein complexes. The presence of cognate motifs in a given promoter and interactions among heterologous trans factors influenced gene expression. The study identified a variety of motif- and trans factor-related interactions that operate in the regulation of gene expression in plant cells.

Materials and methods

Construction of promoter modules

Conserved features in the TATA-consensus region, the transcript initiation site, and the translation initiation context (Sawant et al., 2001a) had been identified earlier (Sawant et al., 1999) in a dataset of highly expressed plant genes. A canonical sequence comprising these features (−38 to +100 in Fig. 1) was synthesized to construct a basal promoter, called Pmec—minimal expression cassette with the flanking BamHI and XbaI sites. The Pmec fragment along with the XbaI EcoRI gusA–nosT region of pBl101 was cloned into the pUC19 vector to obtain the construct Pmec–gusA. As reported earlier, Pmec–gusA expresses the reporter gene at a low level following transient transformation of plant cells (Sawant et al., 2001b). Multimers of the eight individual motifs, identified earlier as commonly present in highly expressed plant genes (Sawant et al., 2001b) were used for detailed studies. These are listed in Table 1. These were synthesized as complementary oligonucleotides which were annealed to obtain blunt ended 18-mer fragments and cloned at the BamHI site of the vector to obtain the construct Pmec–gusA. All constructs were sequenced on automated (ABI 377) sequencer (Applied Biosystem Inc., USA) to establish their authenticity.

A 312 bp long, multifactorial combinatorial module comprising the eight core motifs and their variants was fused at −38 position of the Pmec to obtain the 450 bp long Pmec—complete expression cassette, as shown in Fig. 1 and reported earlier (Sawant et al., 2001b). Yet another construct, Pmec–2X was designed in which the 936 bp long region, placed upstream of Pmec comprised three copies of the −38 to −350 activation module, placed in head-to-tail fashion. All variations of the promoter cassette were cloned upstream of gusA in an otherwise uniform background of pUC19. All promoter modules described above and their abbreviated designations used in the text are listed in Table 1.

Plant material and transient transformation assays to study promoter expression

Leaves from the 3rd to 5th internode of 4-week-old Nicotiana tabacum var. Petit Havana plants were used to study the expression of the reporter gusA gene from different promoter modules. These were surface-sterilized with 0.1% HgCl₂, washed, and placed on MS agar medium. The leaves were incubated on the medium at 28°C in continuous light for 14–16 h before bombardment with DNA-coated gold microprojectiles. Coating of the gold particles and biolistic transformation were carried out by a method improved for high reproducibility as described (Sawant et al., 2000). The improved protocol involves ‘activation’ of the gold particles by heating and gives low variance, thus obviating the need for deploying an internal control. Ten μg plasmid DNA, purified on a Qiagen plasmid purification kit was coated on 3 mg ‘activated’ gold particles and bombarded on tobacco leaves at 1100 lbs cm⁻² pressure using a PDS1000He machine (Bio-Rad, USA). The 10 μg DNA-coated gold particles were bombarded on six independent leaves, taken as replicates for each treatment. After bombardment, the leaves were incubated in the light for 48 h before estimating the β-glucuronidase (GUS) enzyme activity, using the fluorogenic substrate, 4-methyl umbelliferyl β-D-glucuronide (Jefferson and Wilson, 1991). Total soluble protein in the leaf extract was quantified using the dye-binding assay (Bradford, 1976).

In vivo motif competition by co-bombardment

Double-stranded oligonucleotides comprising 18 copies of the individual motif (Table 1) were co-bombarded on leaf discs in several-fold molar excess, along with the desired promoter–gusA plasmid. This depleted the motif-specific intracellular trans active factors. The competing oligonucleotide was co-precipitated with the supercoiled promoter-reporter construct on gold particles in buffer containing 12% PEG 8000 (Sigma Chemical Co., St Louis). The PEG was added after adding the DNA, CaCl₂, and spermidine. The suspension was vortexed for 1 min and incubated on ice for 5 min. The particles were then washed with 500 μl 70% ethanol followed by 500 μl absolute ethanol and finally suspended in 60 μl absolute ethanol before bombardment, as described by Sawant et al. (2000). The bombarded leaves were incubated in light for 4 h before measurement of GUS activity.

Nuclear extract and electrophoretic mobility shift assay

The nuclear protein extract was prepared by the procedure modified from Green et al. (1987). After pelleting the nuclei from the homogenization buffer, the pellet was suspended in nuclei isolation medium (10 mM HEPES–KOH, pH 7.5, 20 mM potassium phosphate buffer, pH 7.5, 0.2 mM EGTA, 5 mM DTT, 4 mM Mg-acetate, 2 mM Na-ascorbate, 0.1 mM spermidine, 0.5 mM PMSF, 0.6 μM leupeptin, and 18% Ficoll 400). The suspension was repeatedly spun at 200 g for 10 min to remove cellular debris. Finally, pure nuclei were harvested at 3000 g for 20 min. The nuclei were then lysed and the protein was extracted in nuclear extraction buffer (NEB: 25 mM HEPES–KOH, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 5 μg ml⁻¹ antipain, and 0.6 μM leupeptin).

For electrophoretic mobility shift assays (EMSA), the DNA–protein binding was carried out at 25°C for 20 min in 20 μl NEB.
Modulation of plant promoters by cis-acting motifs

Table 1. Activation of reporter gene gusA expression by individual cis-motifs and their combinations, placed upstream the core promoter Pmec

<table>
<thead>
<tr>
<th>Abbreviated name used in the text</th>
<th>Activating sequence</th>
<th>GUS activity ((\times 10^3\text{ pmol MU min}^{-1}\text{ mg}^{-1}\text{ protein})\pm SD)</th>
<th>Fold activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pmec</td>
<td>Pmec ((-38\text{ to }+100))</td>
<td>2.67\pm0.18</td>
<td>1</td>
</tr>
<tr>
<td>TGACG</td>
<td>(TGACG)(_{18})Pmec</td>
<td>6.28\pm0.56</td>
<td>2.35</td>
</tr>
<tr>
<td>CAT</td>
<td>(CACATA)(_{14})Pmec</td>
<td>20.29\pm1.21</td>
<td>7.6</td>
</tr>
<tr>
<td>GT</td>
<td>(GTGGAAA)(_{18})Pmec</td>
<td>13.48\pm1.07</td>
<td>5.1</td>
</tr>
<tr>
<td>ACGT</td>
<td>(ACGT)(_{18})Pmec</td>
<td>9.76\pm0.61</td>
<td>3.7</td>
</tr>
<tr>
<td>GATA</td>
<td>(GATA)(_{18})Pmec</td>
<td>8.57\pm0.48</td>
<td>3.2</td>
</tr>
<tr>
<td>OCS</td>
<td>(ACGTAAAGCGCTTACGT)(_{18})Pmec</td>
<td>7.34\pm0.51</td>
<td>2.7</td>
</tr>
<tr>
<td>PU</td>
<td>(AGAAAAGG)(_{18})Pmec</td>
<td>12.36\pm1.09</td>
<td>4.6</td>
</tr>
<tr>
<td>YY1</td>
<td>(CGATCTGACCATCTCTAGATCG)(_{18})Pmec</td>
<td>6.60\pm0.46</td>
<td>2.5</td>
</tr>
<tr>
<td>Pcec</td>
<td>((-350\text{ to }-38\text{ in a single copy}))Pmec</td>
<td>293.6\pm28.62</td>
<td>110</td>
</tr>
<tr>
<td>Pcec+2X</td>
<td>((-350\text{ to }-38\text{ in three copies}))Pmec</td>
<td>4014.0\pm495.14</td>
<td>1503</td>
</tr>
</tbody>
</table>

supplemented with 1 mM MgCl\(_2\), 5 \(\mu\)g poly(dI)-poly(dC) (Amer- sham Pharmacia Biotech, Hong Kong) and 5.0 \(\mu\)g nuclear protein. Various oligonucleotides containing multimers of the individual motifs were employed to examine their binding to nuclear proteins. The length of such multimers is specified in the legend to Fig. 2. The binding of nuclear proteins to the multifactorial combinatorial module was examined by amplifying a part of the Pcec. For this purpose a 370 bp fragment \((-38\text{ to }+20\text{ in Fig. 1})\) was amplified by PCR. In studies involving TATA-box binding proteins, a 45 bp oligo \((-38\text{ to }+7\text{ in Fig. 1})\) was used. The oligonucleotides were end-labelled with \(\gamma\)-\(^{32}\)P-ATP (BRIT, Hyderabad, India) using T4 polynucleotide kinase (New England Biolabs, USA). For each reaction, 25 000–50 000 cpm of the labelled oligonucleotide were used. For competition experiments, the competitor oligonucleotide was added in increasing molar excess calculated on the basis of the motif under consideration. Binding of the motifs to the nuclear extract was allowed for 20 min at 25 °C. Subsequent to that, the 370 bp fragment was resolved on 1.5% agarose gel in 0.5× TBE. The individual motif element was resolved on 6% PAGE in 0.5× TBE. The gels were dried before exposing the X-ray film.

Results

Conserved cis-acting motifs function synergistically as activators of basal transcription

The effect of cis-acting motifs on transcription from the basal promoter (Pmec) was studied by placing these upstream of the TATA-box at the \(-38\) position (Fig. 1). Multimers of the eight different sequence elements were inserted, taking one at a time. Each of these caused activation of the basal transcription, though the extent of activation varied from 2–8-fold (Table 1). The results indicated that the putative trans activating factors that function through the motifs selected in this study were present in tobacco leaves. In agreement with this, the tobacco leaf nuclear extract retarded the electrophoretic mobility of each of the eight oligonucleotides (Fig. 2).

The artificially designed promoter cassette, Pcec comprises a 312 bp module placed at the \(-38\) position in Pmec (Fig. 1). The cassette consists of the above eight motifs and their sequence variants, arranged in an order representative of highly expressed plant genes (Sawant et al., 2001b). The results given in Table 1 show that the complete module enhances the basal transcription by 110-fold, suggesting that several individual elements when brought together, functioned synergistically in the activation of transcription. When copy number of the 312 bp activator module was increased to three, a further 14-fold enhancement of the reporter gene expression was obtained in the Pcec+2X, as compared to Pcec.

Heterologous transcription factors show complex interactions that influence gene expression in vivo

DNA–protein and protein–protein interactions involved in the activation of transcription were studied by co-bombardment of the promoter constructs along with an...
excess of the oligonucleotides representing homologous and heterologous motifs. The competitor motifs were included in 100-fold molar excess to allow titration of their cognate trans-acting factors in the cell. The results in Figs 3 and 4 show the motif-specific trans factors modulate transcription from the basal promoter through a variety of factor–factor and factor–motif interactions.

**Functionally competitive interactions among the motifs in trans:** A 100-fold molar excess of a given motif (horizontal axis) completely inhibited gusA expression from the promoter activated by the same element placed upstream of the Pmec (Fig. 3). Experiments involving multimers of three motifs, i.e. OCS, TGACG, and ACGT that contain a common sequence ACGT, suggested the ability of these motifs to compete with one another in binding their cognate trans activating factors (Fig. 3A). However, such competition was not always predictable on the basis of the primary sequence of the motifs. For example, the OCS motif, co-bombarded in 100-fold molar excess, suppressed gusA expression not only from the OCS–Pmec but also from the reporter constructs containing TGACG or ACGT as the upstream activating elements. Similarly, the expression of the reporter construct OCS–Pmec was suppressed by an excess of TGACG, ACGT, and OCS and that of TGACG-Pmec by TGACG, ACGT, and OCS elements. However, rather unexpectedly, the ACGT-Pmec was suppressed by ACGT and OCS but not TGACG.

**Functionally activating interactions among the motifs in trans:** A molar excess of the PU and YY1 oligonucleotides that shared no sequence homology, enhanced transcription from the YY1–Pmec and PU–Pmec, respectively. However, another heterologous oligonucleotide, like OCS showed no interaction with PU or YY1 (Fig. 3B). A similar reciprocal relationship was observed between the CAT and TGACG, ACGT and GT, and OCS and GT pairs of motifs (data not shown). The results suggest that certain heterologous trans-active factors may be present intracellularly as complexes with mutual suppression of activator function. The sequestering of one member of such a complex (by delivering molar excess of its cognate oligonucleotide) enhanced the functional competence of the other member to activate transcription from its cognate cis motif.

The gel retardation experiments were conducted to examine if the interactions between motif-related trans factors were also noticed in *in vitro* studies. The representative results for the three motifs, i.e. the PU, YY1, and OCS elements are shown in Fig. 4. The PU motif makes multiple complexes, including one major and one minor complex (lane 2, Fig. 4A) with leaf nuclear protein. An excess of the unlabelled PU or YY1 element, but not the OCS element dissociated these complexes with increasing molar concentration of the competitor motif. The results for the YY1 element are shown in Fig. 4B. The YY1 complex (Fig. 4B) dissociated readily by molar excess of the YY1 element (lanes 3–5). The heterologous PU multimer also dissociated the complex (lanes 6–8), although at higher molar excess. The results on specificity of these interactions were substantiated by the controls in which the competitor oligonucleotide was selected as the motif that showed no effect on *in vivo* promoter expression by the activator motif being examined. For example, the OCS motif does not affect the PU and YY1 functions *in vivo* (Fig. 3B). It also failed to dissociate the PU– or YY1–protein complexes (Fig. 4A, B). However, while the *in vivo* results suggested activation by a heterologous sequence motif, the *in vitro* results suggested competition between such motifs for binding to trans activating factor(s). The contrasting results may be indicative of non-specific interactions in the *in vitro* mobility shift experiments.

**Transcription from a complex promoter becomes dependent on the availability of all cognitive factors**

To study *in vivo* interactions among cognitive factors and the several activating sequence elements in a complex promoter, the combinatorial promoter Pcec was examined in further details. The oligonucleotides representing the eight motifs were co-bombarded, one at a time, along with the Pcec to examine the extent to which the sequestering of
one trans-acting factor at a time affected the activation of transcription by a complex promoter. The results in Fig. 5 show that titration with any one of these motifs, with the exception of GATA, resulted in an incremental decline in transcription from the Pcec, as the competitor oligonucleotide was gradually increased to 100-fold molar excess. In spite of each motif being individually capable and sufficient in partial activation of the basal (Pmec) promoter (Table 1), depletion of any of the several trans factors causes nearly complete collapse of the promoter function. The results suggest that, the function of such motifs in a complex (Pcec) promoter may be substantially dependent on the hierarchical arrangement of each of the motif–specific trans factors. Such factors have to be arranged in an orderly manner to give a fully functional enhanceosom-like complex.

A combinatorial promoter requires complete occupancy of its cis elements for efficient function

The results presented in Fig. 6 show the kinetics of promoter expression, following delivery of a stoichiometrically

![Fig. 5](https://academic.oup.com/jxb/article-abstract/56/419/2345/531973)

**Fig. 5.** Fall in gusA expression in vivo from a multifactorial promoter, following co-delivery of Pcec with individual motifs. The GUS activity was estimated, 4 h following microprojectile mediated delivery of Pcec–gusA into tobacco leaves. The Pcec–gusA was co-bombarded with 100-fold molar excess of the non-specific oligonucleotide poly(dI)-(dC) (open squares), GATA (black squares), YY1 (open triangles), OCS (black triangles), CAT (open circles), PU (black circles), GT (open diamonds), ACGT (black diamonds), and TGACG (asterisks). The results show the decline in promoter activity by each of the eight motifs, except GATA. Readings for the ACGT and TGACG are overlapping in the above figure.

![Fig. 6](https://academic.oup.com/jxb/article-abstract/56/419/2345/531973)

**Fig. 6.** Fall in gusA expression in vivo with increasing intracellular concentration of the promoter–reporter constructs. Increasing amounts of DNA of the GT–Pmec–gusA (open squares) or Pcec–gusA (black squares) were bombarded on tobacco leaves. Fold change in GUS activity is plotted to show the molar-concentration-dependent expression from the monofactorial (GT–Pmec) and multifactorial (Pcec) promoters. The initial levels of GUS expression at 0.3 µg DNA bombarded per leaf in the case of GT–Pmec and Pcec were 3.36 and 368.2×10³ pmol of MU min⁻¹ mg⁻¹ protein, respectively.
increasing number of promoter molecules in leaf tissue. In the case of GT–P mec, taken as a representative promoter with a single upstream activator motif, the expression of gusA continued to increase, until it reached a plateau (when 3 μg of the plasmid representing 0.75 fmol of the promoter module had been delivered). A further increase in DNA showed a saturating level of activity or very little decline, if any. By contrast, the expression from the P mec reached saturation rapidly at 1 μg DNA that was equivalent to 0.46 fmol of the module. A sharp fall in promoter activity in the case of the multifactorial P mec is presumably caused by functional collapse of the activation complex. One or more transcriptional factor(s) may become limiting as the molar concentration of the plasmid DNA received by a cell is increased. This may result in the formation of partial transcriptional complexes on P mec. Such complexes may be functionally inactive, suggesting co-operative and hierarchical assembly of the activation complex in the case of multifactorial promoters.

**Upstream activation complex increases stability of the PIC formed on minimal promoter**

The mechanism of transcriptional activation by the upstream module was studied by *in vivo* as well as *in vitro* experiments aimed at examining the stability of the transcription initiation complex formed at the TATA-box. This was monitored *in vivo* as the inhibition of GUS expression, following competition with molar excess of a 45 bp oligonucleotide (−38 to +7 in Fig. 1) that contained the TATA-box motif. The results given in Fig. 7 show that promoter expression declined sharply when the P mec−gusA was co-bombarded with increasing molar excess of the homologous TATA-box oligonucleotide. At 2-fold molar excess, the TATA-oligo completely arrested expression from the P mec. However, in case of P cece−gusA (that contains a single copy of the 312 bp combinatorial activation module), the transcriptional complex was substantially stabilized. The gusA expression from the P cece was not arrested completely until the competing TATA-box oligonucleotide was delivered in 10-fold molar excess (Fig. 7).

The nature of the interaction between the upstream activation complex and the transcription initiation complex became more apparent when two additional copies of the 312 bp combinatorial module were included upstream of the P mec. As shown in Table 1, P cece+2X gave about 1500-fold higher transcription compared with P mec. In agreement with this result, a substantially higher stability of the transcriptional initiation complex at the TATA-box was seen in P cece+2X. It continued to express *in vivo* until the competing TATA-box oligonucleotide reached 50-fold molar excess (Fig. 7).

Stabilization of the transcription complex formed on the TATA-box by the upstream activation module was examined further by experiments based on EMSA (Fig. 8). The large nucleoprotein complex formed following the incubation of P mec or P cece with nuclear extract failed to enter the agarose gel and was therefore not noticeable. The formation

![Fig. 7. Stabilization of the transcription complex in vivo by the upstream sequences. Fall in GUS activity is plotted against increasing molar excess of the TATA-box (−38 to +7 in Fig. 1) oligonucleotide, co-bombarded with the promoter−gusA reporter constructs. The expression from P mec−gusA (open triangles), P cece−gusA (black squares) and P cece+2X−gusA (open squares) is plotted. The results show improved stability of the transcription complex with increase in copy number of the upstream activating sequence.](image-url)
of such complexes could indirectly be inferred by examining the failure in the complex formation in vitro at increasing molar excess of the individual motifs or the TATA-box oligonucleotide (supplementary information can be found at JXB online). The ability of the TATA-box oligonucleotide to destabilize the complex formation on Pmec or Pcec and, instead, form a complex with itself, was examined by including a $^{32}$P-labelled TATA-box oligonucleotide in the incubation mixture containing the nuclear extract and the unlabelled promoter cassette. The TATA-box–protein complex, being of lower molecular weight than the promoter–protein complex, becomes visible on the gel. As seen in Fig. 8, the TATA-box could form a complex in vitro in competition with the Pmec (lanes 4–6), but not with the Pcec (lanes 7–10). The results substantiated the formation of a tighter complex on Pcec as compared to Pmec.

**Discussion**

This study reveals a variety of interactions that may influence gene expression in plant cells. The *cis*-acting elements selected for this study are the DNA sequences that occur most commonly in highly expressed plant genes (Sawant et al., 2001a). [Results of the analysis of transcriptional motifs in the first 100 genes of *Arabidopsis* chromosome 1 (taken as a representative sample) can be seen on www.geocities.com/rakeshtuli. The computational analysis was done by software developed by Coral GeneCraft (arunsharma@geneCraft.in). The analysis illustrates that a large number of sequence motifs are present in plant genes, often in multiple copies in predicted promoter regions.] This analysis of the *Arabidopsis* genome database confirmed that these elements occur preferentially in promoter regions. Some of them have been reported to determine the genomic response to a variety of physiological and developmental cues. The ACGT element, commonly called a G-box, regulates gene expression under diverse physiological and environmental conditions (Foster et al., 1994). The TGACG element, commonly known as a W-box has been assigned functions in transcriptional activation by auxin, salicylic acid, and light (Terzaghi and Cashmore, 1995). The GT1 group of elements has been implicated in the transcriptional response to light (Kuhlemeier et al., 1988), tissue specificity (Eyal et al., 1995), defence (Lawton et al., 1991), and circadian rhythm (Anderson et al., 1994). These motifs not only occur at high frequency but also tend to co-occur in the promoter regions of several plant genes. For example, an alternating combination of CACAAT and TGACG forms an E-box which activates transcription synergistically with a G-box in the phaseolin gene (Kawagoe et al., 1994). Combinatorial interactions among such elements have been reported to specify expression in a number of plant genes (Benfey and Chua, 1990; Singh, 1998; Chen, 1999; Haralampidis et al., 2000). Molecular details of how these motifs interact to bring out combinatorial regulation are largely unknown.

Transient expression, following the biolistics-mediated delivery of DNA in tobacco leaves demonstrated that several *cis*-motifs function co-operatively to facilitate the formation of a multifactorial complex whose member proteins synergistically activate expression from the downstream promoter in plants. Formation of large protein complexes that activate gene expression has been reported in animal cells and these have been named as enhanceosomes (Bazette-Jones et al., 1994). All the eight motifs selected in this study contributed to the formation of a DNA template that nucleated the formation of such an activation complex. These core motifs functioned in principle, both by themselves and in combinations even when deployed out of their native contexts. The depletion of any one of the several motif-specific activation factors led to a substantial decline in the combinatorial function of a multifactorial promoter. This was not true for a monofactorial promoter. The presence of only a single type of *cis*-motif gave activation that continued to increase until the *trans* factor presumably became limiting. Unlike the multifactorial promoter Pcec, partial occupancy of the motifs in a monofactorial promoter did not significantly influence its competence in transcriptional activation.

Molecular details of the co-operative function of multiple regulatory motifs have been best studied in human IFN-β gene expression (Thanos and Maniatis, 1995). Multiple motifs result in in vitro assembly of an enhancer complex comprising NF-κB, HMG1(Y), ATF-2, and C-Jun proteins. Depletion of any of the several transcription factors in the titration experiments severely compromise the enhanceosome formation (Yie et al., 1999a). HMG1(Y) mutants that could not recruit one of these factors, fail to facilitate enhanceosome-dependent transcription. In agreement with these results, the availability of all the members was also noticed as critical to the formation of a fully functional enhanceosome in this study. Presumably due to hierarchical assembly, the depletion of any one member led to inactivation of its function.

Depletion of transcriptional factors can be achieved intracellularly in several ways to ensure a well-orchestrated regulation of gene expression. In activated transcription, activators are often synthesized *de novo* only in response to external cues, like abscisic acid (Nakagawa et al., 1996). Alternatively, a pre-existing activator may be modified in response to a signal. For example, the YY1-binding proteins in mammalian embryos interact with the histone acetyl transferase–P300 complex (Yao et al., 2001). This complex acetylates YY1 protein at various places, resulting in a fall in the YY1-DNA binding activity and destabilization of the enhanceosome (Munshi et al., 2001). The bZIP transcription factor CRP2 is modulated by the photoreceptors phyA/phyB and is then, localized to the nucleus (Kircher et al., 1999). Modifications leading to the degradation of
transcription factors or their exit from the nucleus, or failure to bind to their motifs or interact with other proteins may result in depletion of certain transcription factors required for the formation of transcription activating complex, thus resulting in the desired fall in gene expression in response to a variety of cellular cues.

Enhanceosomes activate gene expression by allowing multiple rounds of transcription (Yie et al., 1999b) and by increasing the rate at which the PIC is formed (Yie et al., 1999a). The time required for the PIC assembly was reported to be considerably lower, even in the presence of Sarkosyl, when the template contained cis elements that could form enhanceosomes. The enhanceosomes enhanced in vitro recruitment of TFIIB and the CBP-RNA-PolII complex to the promoter (Kim and Maniatis, 1998). The multifactorial complex formed upstream of the core promoter in the case of Pcec functions by anchoring the TBP on the TATA-box and hence improves stability of the transcriptional complex. Such a function is supported by the in vivo experiments that demonstrate (Table 1) a 1500-fold increase in reporter gene expression between Pmec and Pcec+2X and a 75-fold (Fig. 7) increase in in vivo stability of the PIC complex formation on the TATA-box.

Enhanced stabilization of the PIC complex by the multifactorial DNA sequence in Pcec is also suggested by the in vitro motility shift experiments (Fig. 8) where even 100-fold excess of the TATA-box oligo oligo could not competitively dislodge TBP from the Pcec. However, the in vitro motility shift data does not necessarily reflect the in vivo functions faithfully. For example, Pcec is inhibited nearly completely in vivo at 5-fold excess of the TATA oligo (Fig. 7), the in vitro complex formation does not show dissociation at even 100-fold excess (Fig. 8). The inhibition of function in vivo is presumably more sensitive to subtle conformational interactions that remain unnoticeable in the gross changes detected in the mobility shift experiments.

The in vivo experiments show that several commonly occurring heterologous factors may exist as protein complexes inside the cells. This can contribute to the modulation of gene expression. For example, a transcription factor like PU can suppress (co-suppressor-like function) or negatively regulate the function of a heterologous factor like YY1, if its cognate cis-motif is not present, as in the case of a monofactorial promoter. Such heterologous factors may interact in vivo among themselves to destabilize the binding of one another to their corresponding cis motifs on a promoter. Alternatively, two factors may share a common co-activator. Such negative interactions have been noticed earlier and referred to as ‘transcriptional squelching’ (Ptashne, 1988). The heterologous motif activation seen in vivo (Fig. 3) is not in agreement with the competition suggested by the mobility shift experiments (Fig. 4). The differences may be due to several reasons, like the loss of specificity, hierarchical binding kinetics, or the absence of other cellular factors in the in vitro experiments.

This study suggests a variety of interactions mediated by an enhancer-like multifactorial upstream region that may govern gene expression in plant cells. The results reflect various ways in which motif-factor-based architecture in the promoter region and heterologous interactions among cognate proteins can modulate gene expression. The stereospecific assembly of enhanceosomes has been reported as essential for efficient recruitment of TFIIB and RNA polymerase in in vitro transcription (Kim and Maniatis, 1998) in the case of the IFN-β promoter. In the chromatin integrated state, the enhanceosome binding region in the IFN-β gene remains nucleosome-free. The assembly of enhanceosome catalyses a series of interactions required for nucleosome repositioning on chromatin, which in turn leads to the formation of PIC and the start of transcription (Agalioti et al., 2000). Some of the proteins that bind to the motifs selected by us and activate promoter function synergistically, may also interact with the components of chromatin remodelling machinery in an integrated state. Future studies on the effect of individual motifs on gene expression vis-a-vis nucleosome positioning in stable transgenic lines will reveal additional details of their function and the validity of transient expression studies reported here.

Supplementary material

Supplementary material can be found at JXB online.

Acknowledgements

We thank Arun Sharma, Coral Gene Craft, Noida, India for providing software for computational analysis. The work was supported with research grant from The Council of Scientific and Industrial Research, Government of India under the NMITLI programme.

References


Modulation of plant promoters by cis-acting motifs


