Journal of Plant Physiology 167 (2010) 1214–1218

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Short communication

Promoter activation by ACGT in response to salicylic and abscisic acids is differentially regulated by the spacing between two copies of the motif

Rajesh Mehrotra*, Sandhya Mehrotra

Birla Institute of Technology and Science, Pilani, Rajasthan 333031, India

ARTICLE INFO

Article history:
Received 26 December 2009
Received in revised form 4 April 2010
Accepted 6 April 2010

Keywords:
Abscisic acid regulation
ACGT motif
cis-Elements
Gene expression
Promoter regulation
Salicylic acid regulation

ABSTRACT

A variety of small sequence motifs, located upstream of minimal promoters influence gene expression. The function of the core sequence ACGT, present in a family of commonly occurring *cis* acting transcription regulatory motifs was investigated in the background of an artificially designed synthetic promoter sequence. The ACGT was placed in one or two copies and separated by different spacer lengths between the two copies, to study their affect on the expression of basal promoter in plant cells. The activation of transcription by the ACGT element was examined by transient and stable transformation in tobacco, using *gusA* as the reporter gene. The analysis shows that the expression of the reporter gene was influenced differently by spacing between two adjacent copies of the motif. Two copies of the ACGT element separated by 5 nucleotides gave highest activation. This configuration imparted salicylic acid inducibility to the basal promoter. However, two copies of ACGT separated by 25 nucleotides allowed the promoter to be induced by abscisic acid but not salicylic acid. Computational analysis of the *Arabidopsis thaliana* genome database showed the presence of these motifs in several genes associated with a variety of stress responses. The results on motif-related inducibility by salicylic acid and abscisic acid, as seen in a synthetic sequence background were validated by experiments on the expression of the native promoter of a protein phosphatase 2C-like gene of *Arabidopsis* in tobacco leaves. The study supports the importance of spacing between the ACGT sequence motifs on the elicitor specific modulation of gene expression and demonstrates the role of different ACGT motifs in the regulation of *PP2C* gene expression.

Introduction

The transcriptional control of gene expression depends on a variety of complex interactions mediated by sequence specific DNA binding proteins, their cognate promoter elements and the core promoter region (Johnson and Mcknight, 1989).

In plants, a rapidly increasing number of DNA binding proteins have been identified on the basis of their interactions with *cis* acting elements. A prominent group of *cis*-acting elements common to many plant promoters contains an ACGT core sequence (Armstrong et al., 1992). The ACGT family of elements (ACEs) is functionally important in a variety of promoters that respond to different stimuli like light (Donald and Cashmore, 1990), and hormones such as salicylic acid (Jupin and Chua, 1996), abscisic acid (Guiltinan et al., 1990). Deletions or mutations in the ACGT core dramatically reduce overall promoter activity and/or render the promoter unresponsive to a given stimulus. The core element occurs at different relative positions in one or more copies upstream of the minimal promoter region (Sawant et al., 2001). By transient transformation studies, we have earlier reported that the ACGT motif functions even when it is placed out of the native sequence context (Mehrotra et al., 2005). It contributes synergistically to gene expression by enhancing the stability of transcriptional complex formed on minimal promoter (Sawant et al., 2005). This report aims to analyze the activation behavior of the ACGT element in stable transgenic plants in an otherwise artificial promoter sequence background. The ACGT was placed upstream of a minimal promoter in one or two copies, separated by randomized nucleotide sequences of different spacer lengths to examine the contribution of the core motif in gene expression. Studies on multiple stable transgenic lines are important to establish the behavior of synthetic promoters in spite of the position effects associated with independent events of transgene integration.

Methods

Constructs

A minimal promoter *Pmec*, described earlier (Sawant et al., 2001) was used which is a 138 nucleotide long sequence as given below.

* Corresponding author at: Department of Biological sciences, Birla Institute of Technology and Science, Pilani, Rajasthan 333031, India. Tel.: +91 1596 245073x273; fax: +91 1596 244183.
E-mail address: rajmeh25@hotmail.com (R. Mehrotra).

0176-1617/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2010.04.005

Table 1
The upstream activator sequences used in this study. The core motif is shown in bold and the random spacer sequence is underlined.

Sequence	Code
TCTAGA**ACGT**TCTAGA	ACGT
TCTAGA**ACGTACGT**TCTAGA	$(ACGT)_2$
TCTAGA**ACGT**GGCTA**ACGT** TCTAGA	$(ACGT)_{N5}(ACGT)$
TCTAGA**ACGT**GGCTATGGCG**ACGT**TCTAGA	$(ACGT)_{N10}(ACGT)$
TCTAGA**ACGT**GGCTATGGCGGAGCAAGATTCACTC **ACGT**TCTAGA	$(ACGT)_{N25}(ACGT)$

GGATCCTCACTATATATAGGAAGTTCATTTCATTTGGAATGGACACGTGTTGT

CATTTCTCAACAATTACCAACAACAACAAACAACAAACAACATTATACAATT

ACTATTTACAATTACATCTAGATAAACA**ATGGCTTCCTCC-*gusA***

It was cloned in the plasmid pUC19 (New England Biolabs). A 50 nucleotide long random sequence (GGATCCGGCTATGGCG GAGCAAGATTCACTCTGCGAGG CCAAAGCTTACCCCGGAAGGATCC) was cloned at the *Bam*H1 site to the 5′ side of the *Pmec*. Single or two copies of the activator motif ACGT were placed at the 5′ end of this 50 nucleotide sequence. The space between two copies of the ACGT 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 of 50 nucleotide random sequence. The promoter-reporter cassettes were cloned in pBI101 between *Sma*I and *Eco*RI sites (Fig. 1), mobilized in *Agrobacterium tumefaciens* and used for plant transformation.

Cloning of PP2C-like promoter and introduction of mutations in its ACGT region

The genomic DNA was prepared from *Arabidopsis thaliana* using CTAB method (Murray and Thompson, 1980). The protein phosphatase 2C (*PP2C*) like promoter was amplified by PCR and cloned into SK(+/−) vector (Stratagene,USA) using primers P1 (CGGGATCCAAGTATTCACGCACCAAGGTTATATT) and P2 (GCTCTAGATGTGATCGTCAAAACTATTCAACAAAC). The gusA reporter gene was cloned downstream of the *PP2C* promoter. Deletion of the $(ACGT)_{N5}(ACGT)$ region was introduced by using primer P4 (ACGCGTCGACGGACACAATCAACGGTCAAGATTTC). Mutation (M1) in the 5′ ACGT box of $(ACGT)_{N5}(ACGT)$ was introduced by using primer P5 (ACGCGTCGACTACTGTTTACACGTGGACACAATCAACGGTCAAGATTTC) while the mutation (M2) in the 3′ ACGT box of $(ACGT)_{N5}(ACGT)$ was introduced by using primer P6 (5′ ACGCGTCGACTACGTTTTACACTGGGACACAATCAACGGTCAAGATTTCGTCGTCC 3′).

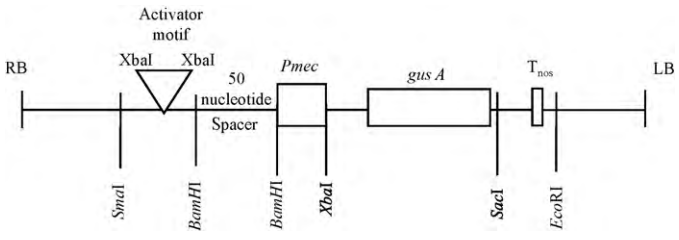

Fig. 1. A scheme of the reporter gene constructs used for plant transformation. It is not drawn in scale.

Transient transformation

The *PP2C-gusA* cassettes were coated on gold microparticles and bombarded on to tobacco leaves at 1100 psi, using biolistic gun (Bio Rad PDS-1000/He). The leaves were taken from 3rd or 4th node from the top of 9–10-week-old plants of *Nicotiana tabacum* cv. Petit Havana. A highly reproducible protocol, published earlier for microprojectile bombardment was used (Sawant et al., 2000). 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. To examine induction of the promoters, leaf discs (3 cm diameter) were placed on Hoagland medium (Draper et al., 1988) containing salicylic acid (100 μM) or abscisic acid (100 μM) and incubated at 26 °C under 16 h light and 8 h dark conditions. The glucuronidase activity was estimated, after 48 h of the treatment.

Stable transformation

Tobacco (*N. tabacum* cv. Petit Havana) plants were transformed with the promoter reporter constructs using *Agrobacterium* mediated transformation. The shoots were regenerated on medium containing 200 μg/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 (MS) medium (Murashige and Skoog, 1962). The seedlings were maintained at 26 °C under 16 h light. Eight to eleven single copy, homozygous independently transformed T_2 transgenic plants were analyzed in each case. Lateral roots were excised from 7–8-week-old potted plants growing in MS medium supplemented with 3% sucrose, 0.7% agar and 200 μg/ml kanamycin. Mature seeds were soaked in water for 2 h before performing the glucuronidase enzyme assay.

Estimation of reporter protein activity

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 min at 4 °C. The glucuronidase activity was assayed in cell free extract using 4-methyl umbelliferyl glucuronide (Jefferson et al., 1987) as the substrate. The product, 4-methyl umbelliferone (MU) was quantified using fluorimeter (Perkin Elmer LS55). The GFP activity was measured as described previously (Reichel et al., 1996). Protein concentration was determined using BioRad dye. The GUS activities were assayed on 9–10-week-old transgenic plants in leaves, roots and mature seed extracts. The data on gene expression were statistically analyzed by two-way analysis of variance.

Computational analysis

The *Arabidopsis* genome database (NCBI Version, 19 February 2004) was analysed to compute the status of $(ACGT)_{N5}(ACGT)$ and $(ACGT)_{N25}(ACGT)$ motifs in the intergenic regions. The sequences 1000 bp upstream of the ATG initiation codon were extracted and

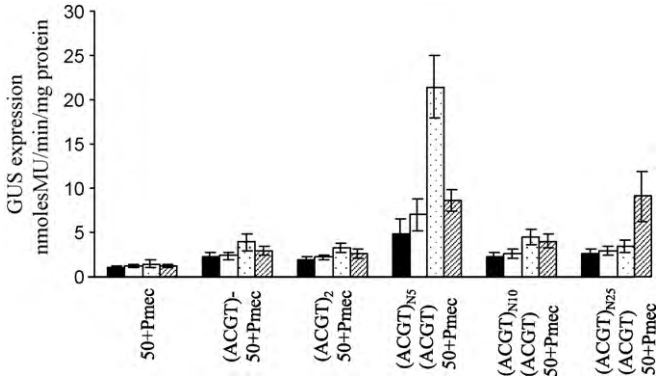

Fig. 2. Effect of placement of the ACGT elements on expression of the minimal promoter, *Pmec* in different constructs under uninduced and induced conditions in leaves of stable transgenic plants. Uninduced leaf disc immediately frozen in liquid nitrogen (■), uninduced and kept in Hoagland medium for 48 h, without (□) or with salicylic acid (⬚) or abscisic acid (▨). Each reading is an average of measurements on 8–11 independent transgenic lines. The bars indicate S.D. for the population. Gus activity was measured in nmol of MU/min/mg protein.

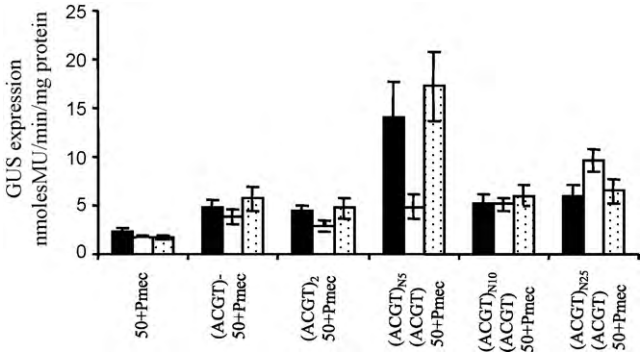

Fig. 3. Effect of the placement of the ACGT elements on the expression of *Pmec* in leaf (■), seed (□) and root (⬚) in stable transgenic plants. Each reading is an average of measurements on 8–11 independent transgenic lines. The bars indicate S.D. for the population. Gus activity was measured in nmol of MU/min/mg protein.

analysed for the occurrence of these motifs, using software provided by Genecraft, India.

Results

The aim of this work was to investigate the activation behaviour of ACGT, which is the core sequence in a variety of transcription activating *cis* elements. The tetranucleotide sequence was placed in an artificially constructed nucleotide sequence to be able to resolve the effect of different configurations of the ACGT motif in the absence of several other motifs that occur commonly in native promoters. One or two copies of ACGT were separated by different spacer lengths.

The trends noticed in the transient expression study were validated by repeating the experiments on stable transgenic lines (Fig. 2). The stable transgenic lines with $(ACGT)_{N5}$(ACGT) showed a 1.5-fold increase in activity with immediately frozen leaves when the freshly cut leaves were incubated for 48 h in Hoagland solution. A further 3-fold increase in the reporter gene expression was noticed when salicylic acid was included in the incubation medium. The increase in the activity without SA in case of $(ACGT)_{N5}$(ACGT), and a further increase following the SA treatment suggested that the increase in the former case may be the natural response of leaf to wounding during the process of preparing leaf discs. The two copies placed 25 nucleotides apart, i.e. $(ACGT)_{N25}$(ACGT) gave 3-fold induction of the minimal promoter by abscisic acid. As seen in the data presented in Fig. 2, the $(ACGT)_{N5}$(ACGT) gave marginal induction by ABA and $(ACGT)_{N25}$(ACGT) by SA. All other configurations, with single or two copies of ACGT gave insignificant induction by SA or ABA. The results suggest that the SA and ABA response of $(ACGT)_{N5}$(ACGT) and $(ACGT)_{N25}$(ACGT) respectively were specific to the configuration of the motifs used in these promoters.

Comparison of the reporter gene expression in different plant parts (Fig. 3) revealed that the ACGT led to a higher level of gene expression in roots and leaves than in seeds. The highest expression in roots (17.22 nmol/min/mg protein) was observed in case of $(ACGT)_{N5}$(ACGT) transgenic plants. The highest expression in leaves (14.06 nmol/min/mg protein) was also observed for the cassette $(ACGT)_{N5}$(ACGT). The ACGT motif failed to activate the promoter expression substantially in seeds. The highest expression in the seeds was (9.63 nmol/min/mg protein) which was observed for the cassette $(ACGT)_{N25}$(ACGT).

Since $(ACGT)_{N5}$(ACGT) and $(ACGT)_{N25}$(ACGT) gave high elicitor specific induction of the basal promoter, we searched the *Arabidopsis* genome sequence database for the presence of such motifs in the intergenic regions. Seventy three promoters having $(ACGT)_{N5}$(ACGT) element were identified. The literature survey showed that most of these genes were stress responsive. The examples included one or more members of the gene families comprising beta-1,3-glucanases, WRKY proteins, heat shock proteins, disease resistance protein, MAP kinases, monoxygenase-2, late embryogenesis proteins, 4-coumarate-co-A ligases, jasmonate inducible proteins, Ca^{2+}/H^{+} exchangers, sugar transporters, chalcone synthases and many others. Similar analysis for $(ACGT)_{N25}$(ACGT) yielded 151 genes that resemble protein phosphatases, leucine rich repeat proteins, cold stress acclimation proteins, glutathione-S-transferases, protein kinases, mannitol dehydrogenases, abscisic acid responsive GTPases, early dehydration stress proteins and many others (data not shown). One of these genes, At5g59220 was identified to code for protein phosphatase 2C-like protein. It has three ACGT motifs located in 1 kbp upstream region. Two of these make an $(ACGT)_{N5}$(ACGT) motif, located 325 bp upstream of translation start site (ATG). The third ACGT motif makes an $(ACGT)N_{30}$(ACGT) with the 5′ ACGT of the former pair. This 1 kbp region containing *PP2C* promoter was cloned and sequenced. It contains a putative TATA-box, 121 nucleotide upstream translation start site. The putative At5g59220 promoter was fused to the reporter gene *gusA*. Expression of the reporter gene from the promoter was examined in tobacco leaves, following transient transformation. The promoter activity measured as the *gusA* expression is given in Table 2. The results show that the At5g59220 promoter was induced nearly 6-fold by SA and 13.5-fold by abscisic

Table 2
The reporter gene (*gusA*) expression from *PP2C* promoter and its ACGT mutations under uninduced and induced conditions, following transient transformation of tobacco leaves.

Cassette	Uninduced (nmol/min/mg protein ± S.D.)	Fold activity as compared to PP2C	Salicylic acid (nmol/min/mg protein ± S.D.)	Fold induction	Abscisic acid (nmol/min/mg protein ± S.D.)	Fold induction
PP2C (At5g59220)	19.76 ± 2.0	1	117.37 ± 10.02	5.94	266.76 ± 12.0	13.50
PP2C (M1)	18.92 ± 1.73	0.95	57.26 ± 4.29	3.02	140.29 ± 11.73	7.41
PP2C (M2)	18.22 ± 1.87	0.92	55.92 ± 5.03	3.06	239.79 ± 15.21	13.16
PP2C (D)	16.72 ± 2.08	0.84	18.27 ± 1.69	1.09	20.29 ± 1.76	1.21

acid, thus validating the role of ACGT motif suggested by the earlier described experiments, based on artificially constructed promoters. Mutation in either of the ACGT box of $(ACGT)_{N5}(ACGT)$ of PP2C promoter leads to 2-fold reduction in inducibility by SA. The mutation in the 5′ ACGT box of $(ACGT)_{N5}(ACGT)$ leads to a 2-fold loss of inducibility by ABA. The mutation in the 3′ ACGT box has no effect on inducibility by ABA. Deletion of the $(ACGT)_{N5}(ACGT)$ region results in complete loss of inducibility both by ABA or SA.

Discussion

We have earlier reported (Mehrotra et al., 2005) that the ACGT element placed upstream of the 50+*Pmec* in single copy, two tandem copies or two copies separated by a spacer of 5, 10 or 25 nucleotides enhanced *gusA* gene expression in transient transformation of tobacco leaves. This study validates the earlier results by examining the promoter function in chromatin integrated state by establishing the function of the ACGT motif in stable transgenic plants. The results obtained with the ACGT motifs tested in an artificial promoter were also true for the native PP2C promoter of *Arabidopsis*, tested in transient transformation of tobacco leaves.

The $(ACGT)_{N25}(ACGT)$ cassette was abscisic acid inducible both in transient and stable transgenic plants developed in this study. The ACGT containing ABA-response elements (ABREs) have been functionally identified in the promoters of various genes. Most promoters of ABA-inducible genes contains ACGTGGC motif within 300 bp upstream of the transcription start site (Shinozaki et al., 1989; Mundy et al., 1990). A second *cis* acting element (TGCCACCGG), called coupling element1 (CE1) was identified by (Shen and Ho, 1995), who conducted a linker-scan analysis of a 44 bp ABA responsive fragment from the barley HVA22 promoter. In this fragment, CE1 functions with a single copy of an ACGT containing ABRE, together constituting a minimal ABA response complex. Similar analysis with another barley promoter (HVA1) identified a second coupling element CE3 (ACGCGTGTC) (Shen et al., 1996). The CE3 is approximately 30 bp downstream of the ACGT containing motif. The second ACGT, located 25 bp apart in our study may provide a function similar to the coupling elements as indicated by the studies of Shen et al. (2004). In other instances, multiple ACGT containing ABREs and other elements have been identified (Busk and Pages, 1998). Multiple but not single copy of ACGT containing ABREs have been reported to confer ABA-responsiveness to a heterologous minimal promoter (Vasil et al., 1995).

Expression of the *PP2C*-like gene (At5g59220) of *A. thaliana* has not been studied earlier. Our results show that Protein phosphatase 2C-like gene has an ABA and salicylic acid inducible promoter. This agrees with the prediction made on the basis of our results on promoter activation by ACGT motif placed in an artificially constructed promoter sequence. The PP2C promoter has both $(ACGT)_{N5}(ACGT)$ and $(ACGT)N_{30}(ACGT)$ elements. The sequence of $(ACGT)_{N5}(ACGT)$ is **ACGT**TTTAC**ACGT** while that of $(ACGT)_{N30}(ACGT)$ is **ACGT**AAGTGTTTCGTATCGCGATTTAGGAGAAGT**ACGT**. The former motif is 325 bp upstream and the latter is 359 bp upstream of the translation start site. In agreement with our studies on the artificially designed promoters, the *PP2C* promoter was salicylic acid as well as abscisic acid inducible. Though our study did not exceed spacing beyond $(ACGT)_{N25}(ACGT)$, the results on Arabidopsis *PP2C* suggest, a longer distance may also be favourable to ABA mediated induction. In case of *Fagus sylvatica*, protein phosphatase 2C gene is ABA inducible (Lorenzo et al., 2002) though the promoter sequence is not published. Mutation in the 5′ ACGT box of $(ACGT)_{N5}(ACGT)$ resulted in 7.4-fold induction by ABA and 3-fold by SA, while mutation in the 3′ ACGT box of $(ACGT)_{N5}(ACGT)$ resulted in 3-fold reduction in the inducibiltiy by SA of the PP2C promoter and no effect on the inducibiltiy by ABA. The deletions of any of the two members of $(ACGT)_{N5}(ACGT)$ resulted in the loss of inducibility either by SA or ABA.

Our study substantiates that two closely located ACGT elements are often associated with stress responsive gene promoters and that, difference in the spacing between the two ACGT elements leads to differential elicitor response. Two ACGT elements separated by five base pair is a feature associated with salicylic acid induction but when the distance between these motifs is increased to 25 nucleotides, the induction by SA is prevented while that by ABA is not. The differential induction is expected to involve the recruitment of different bZIP transcription factors. For example, the TGA factors have been shown to activate transcription in response to SA (Lam and Lam, 1995). The TRAB1 factor of bZIP family specifically binds to *cis*-ABREs (abscisic acid response elements) containing the ACGT core sequence (Hobo et al., 1999). Our study suggests that the change in spacing between two copies of a given motif can alter the signal pathway to which a promoter responds and shows the participation of different members of the ACGT family of motifs in the regulation of *PP2C* promoter in *Arabidopsis*.

Acknowledgements

The authors are thankful to the Director, National Botanical Research Institute, Lucknow, India for providing facilities and encouragement. RM is thankful to Department of Science and Technology (under DST fast track scheme for young scientist) and CSIR, Government of India for financial support. Special thanks to Coral GeneCraft, Noida (arunsharma@genecraft.in) for the software used in computational analysis.

References

Armstrong GA, Weisshaar B, Hahlbrock K. Homodimeric and heterodimeric leucine zipper proteins and parsley nuclear factors recognize diverse promoter elements with ACGT cores. Plant Cell 1992;4:525–37.
Busk PK, Pages M. Regulation of abscisic acid-induced transcription. Plant Mol Biol 1998;37:425–35.
Donald RGK, Cashmore AR. Mutation of either G box or I box sequences profoundly affects expression from the Arabidopsis rbcS-1A promoter. EMBO J 1990;9:1717–26.
Draper J, Scott R, Armitage P, Walden R. Plant-genetic transformation and gene expression: a laboratory manual. Oxford: Blackwell Scientific Publications; 1988.
Guiltinan MJ, Marcotte WR, Quatrano RS. A plant leucine zipper protein that recognizes an abscisic acid response element. Science 1990;250:267–71.
Hobo T, Kowyama, Hattori T. A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. Proc Natl Acad Sci USA 1999;96:15348–53.
Jefferson RA, Kavanagh TA, Bevan MW. Gus fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 1987;6:3901–7.
Johnson PF, Mcknight SL. Eukaryotic transcriptional regulatory proteins. Annu Rev Biochem 1989;58:799–839.
Jupin I, Chua N-H. Activation of the CaMV as-1 *cis* element by salicylic acid: differential DNA-binding of a factor related to TGA1a. EMBO J 1996;15:5679–89.
Lam E, Lam YKP. Binding site requirements and differential representation of TGA factors in nuclear ASF-1 activity. Nucl Acids Res 1995;23:3778–85.
Lorenzo O, Nicolas C, Nicolas G, Rodriguez D. Molecular cloning of a functional protein phosphatase 2C (Fs PP2C2) with unusual features and synergistically up-regulated by ABA and calcium in dormant seeds of *Fagus sylvatica*. Physiol Plant 2002;114:482–90.
Mehrotra R, Kiran K, Chaturvedi CP, Ansari SA, Lodhi N, Sawant SV, et al. Effect of copy number and spacing of the ACGT and GT *cis* elements on transient expression of minimal promoter in plants. J Genet 2005;84:183–7.
Mundy J, Yamaguchi-Shinozaki K, Chua N-H. Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *rab* gene. Proc Natl Acad Sci USA 1990;87:1406–10.
Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:473–97.
Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucl Acids Res 1980;8:4321–5.
Reichel C, Mathur J, Eckes P, Langenkemper K, Koncz C, Schell J, et al. Enhanced green fluorescence by the expression of an Aequorea Victoria green fluorescent protein mutant in mono- and dicotyledonous plant cells. Proc Natl Acad Sci USA 1996;93:5888–93.
Rogers SG, Horsch RB, Fraley RT. Gene transfer in plants: production of transformed plants using Ti plasmid vectors. Meth Enzymol 1986;118:627–40.

Sawant SV, Kiran K, Mehrotra R, Chaturvedi CP, Ansari SA, Singh P, et al. 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 basal promoter. J Exp Bot 2005;56:2345–53.

Sawant SV, Singh PK, Madanala R, Tuli R. Designing of an artificial expression cassette for high level expression of transgenes in plants. Theor Appl Genet 2001;102:635–44.

Sawant SV, Singh PK, Tuli R. Pretreatment of micropojectiles to improve the delivery of DNA in plant transformation. Biotechniques 2000;29:246–8.

Shen QJ, Casaretto JA, Zhang P, Ho T-HD. Functional definition of ABA-response complexes: the promoter units necessary and sufficient for ABA induction of gene expression barley (*Hordeum vulgare* L.). Plant Mol Biol 2004;56:111–24.

Shen Q, Ho T-HD. Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and novel *cis*-acting element. Plant Cell 1995;7:295–307.

Shen Q, Zhang P, Ho T-HD. Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. Plant Cell 1996;8:1107–19.

Shinozaki KY, Mundy J, Chua N-H. Four tightly linked *rab* genes are differentially expressed in rice. Plant Mol Biol 1989;14:29–39.

Vasil V, Marcotte WR, Rosenkrans Jr L, Cocciolone SM, Vasil IK, Quatrano RS, et al. Overlap of Viviparous1 (VP1) and abscisic acid response elements in the Em promoter: G-box elements are sufficient but not necessary for VP1 transactivation. Plant Cell 1995;7:1511–8.