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Interactions between upstream and core promoter sequences determine gene expression and nucleosome positioning in tobacco *PR-1a* promoter

Niraj Lodhi, Amol Ranjan, Mala Singh, Rakesh Srivastava, Sudhir Pratap Singh, Chandra Prakash Chaturvedi, Suraiya Anjum Ansari, Samir V. Sawant, Rakesh Tuli *

National Botanical Research Institute, Council of Scientific and Industrial Research, Rana Pratap Marg, Lucknow—226001, India

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ABSTRACT

The expression of *PR-1a* gene in tobacco is accompanied by changes in the chromatin architecture over its promoter region. The transcription initiates when the gene is induced in defense response, a condition that can be simulated experimentally by external application of salicylic acid. Mutagenesis of the core promoter sequence established that the TATA-box was critical to the expression of *PR-1a* gene. In order to study functional specificity between the core promoter and upstream activator region, the native core promoter was exchanged with that of a heterologous salicylic acid inducible promoter, *Pcec*. The core promoter and the activator region of *PR-1a* together determine its tightly regulated expression, slow kinetics of induction by SA and several fold induction of expression. In uninduced state, a single nucleosome was present over the core promoter of *PR-1a*. It masked both the TATA-box and the transcription initiation region. The transcriptional activation of the promoter by SA was accompanied by shift in the position of this nucleosome. The chimeric promoters failed to show inducibility or gave very low level of induction. They showed failure in shifting the nucleosome from the core promoter region. The promoter *Pcec* expressed constitutively at a high uninduced level in spite of a nucleosome over the TATA-box region. However, in this case, the nucleosome did not mask the transcript initiation region. The TATA-box nucleosome was shifted as the expression increased further, following induction by SA. A fully induced *Pcec* had the TATA-box fully exposed, though a weak nucleosome appeared on the +1 region. The results support a close relationship among promoter sequence architecture, nucleosome positioning and *PR-1a* expression.

1. Introduction

Although there are several reports suggesting the regulation of transcription by chromatin architecture, much remains unknown about the mechanism of regulation of promoters by chromatin structures formed over specific regions. The tobacco *PR-1a* is a well studied plant promoter. It remains completely repressed until it receives a signal elicited by pathogen infection. It can be experimentally induced by salicylic acid (SA). Regulation of its expression by the upstream activator sequences has been examined [1] in some details. However, functional relationships among the core promoter, the upstream activator region of *PR-1a* and the chromatin architecture in this region have not been studied.

Several general transcription factors associated with the development-specific regulation of distinct subsets of genes have been reported [2,3]. The diversity among the families of general transcription factors associated with such sets of genes suggests that besides gene specific upstream activator motifs, core promoter sequences may also be important in determining the recruitment of general transcription factors. Very little information is however, available on the role of core promoters *per se* in the regulation of gene expression. In *phaseolin* promoter, a rotationally and translationally positioned nucleosome over the three phased TATA-box was suggested to regulate gene expression [4]. In case of *FLOWERING LOCUS C* gene, chromatin acetylation and methylation [5–10] silence it after vernalization in *Arabidopsis*. The core promoter of mammalian *IFN-β* gene is masked by a nucleosome, preventing access to basal transcription factors [11].

The chromatin structures around *as-1*-like transcriptional motif have been implicated in regulation of *PR-1a* promoter [12]. In uninduced state, repression of *PR-1a* has been suggested to be due to the presence of a nucleosomal structure over the promoter [12]. We have analyzed in further details, the mechanism of regulation of *PR-1a* promoter. A conserved TATA-box, initiator and a DPE-like box are present in the core promoter of *PR-1* genes viz. *PR-1b* (GenBank accession number X17680), *PR-1c* (GenBank accession number X05454) and two pseudogenes *W38/1* and *W38/3* (GenBank accession numbers X52555 and X52556 respectively). Mutational analysis of the TATA and initiator regions in *PR-1a* was carried out to establish that *PR-1a* was a TATA-dependent promoter. The core promoter region of

* Corresponding author. Tel.: +91 522 2205848; fax: +91 522 2205839, 2205836.
E-mail address: rakeshtuli@hotmail.com (R. Tuli).

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PR-1a was exchanged with that of another SA inducible promoter, to obtain two chimeric promoters. These were analyzed for function and nucleosomal architecture. Our results show that the exchange of sequence domains influences the expression behaviour of these promoters. This is accompanied by changes in the state of chromatin organisation over the core promoter region.

2. Materials and methods

2.1. Chemicals

The synthetic oligonucleotides were obtained from Microsynth (Balgach, Switzerland). TaqMan master mix and fluorescence labeled probes for the *gusA* and *ubiquitin* genes were synthesized by Applied Biosystems (Foster City, CA, USA). Majority of the chemicals were purchased from Sigma Pharmaceuticals (St. Louis, MO, USA).

2.2. Generation of reporter gene constructs in tobacco with PR-1a and chimeric promoters

The *PR-1a* promoter was amplified from the genomic DNA of tobacco, using PRF (forward) and PRR (reverse) primers (Table 1). Its expression in tobacco leaves was compared with the SA inducible promoter *Pcec*. The *PR-1a* and *Pcec* were fused to the reporter gene *gusA* to give the plasmids p1329k and p527k respectively (Supplementary Fig. 1). The *PR-1a* core promoter was amplified along with *gusA* using MFS (forward) and NOST (reverse) primers. The core promoter of *Pcec* along with *gusA* was amplified by SMP (forward) and NOST (reverse) primers. The core promoters of *Pcec* and *PR-1a* with *gusA* were cloned in pBluescriptSK$^+$ (Stratagene, La Jolla, CA, USA) to give p800k and p1103k respectively. These core promoters were named as *Pmec* and *PR-1a*^{cp}. The *PR-1a* activator (−1533 to −38, *PR-1a*^{acti}) was amplified by PRF (forward) and PRB (reverse) primers and fused to *Pmec* (−33 to +77) along with *gusA* gene. Similarly the *Pcec* activator (from −350 to −34) was amplified by SAM (forward) and ENH (reverse) primers and fused to *PR-1a* core promoter (from −37 to +29, *PR-1a*^{cp}) along with *gusA* gene. The two chimeric promoters were cloned with *gusA* in pBluescript SK$^+$ (Stratagene, La Jolla, CA, USA) to give p1101k and p1104k. The chimeric promoters were named as *Pchim1* and *Pchim2* respectively (Supplementary Fig. 1).

A mutation in TATA region of *PR-1a* was introduced by PCR using PRHF (forward) and TGR (reverse) primers. The core promoter with *gusA* gene was amplified by TGF (forward) and NOST (reverse) primers. The mutation in the initiator region of *PR-1a* was inserted by PCR using PRHF (forward) and IPR (reverse) primers. The core promoter with *gusA* gene was amplified by IPF (forward) and NOST (reverse) primers. The complete TATA-mutated *PR-1a* promoter was obtained by fusing PCR product of PRHF and TGR to the PCR product of TGF and NOST primers. The initiator-mutated complete promoter was obtained by fusing the PCR product of PRHF and IPR to the PCR product of IPF and NOST primers. Both the mutated genes were cloned in pBluescriptSK$^+$ to give p1108k and p1106k. The promoters were named as $P_{PR1a\ TATA}^{M}$ and $P_{PR1a\ Inr}^{M}$ respectively. All the constructs were verified by DNA sequencing (Supplementary Fig. 1 A–H). The sequences of the primers used in cloning are given in Table 1.

2.3. Plant transformation and selection

The gene constructs were cloned into the binary vector pBI101.1 (Clontech, Palo Alto, CA, USA). *A. tumefaciens* mediated transformation was employed to examine the expression of various promoter constructs in stable transgenic lines of *Nicotiana tabacum* cv Petit Havana. Primary transformants were allowed to self-fertilize and the T_1 progeny plants were selected on medium containing 300 mg/l kanamycin. For each construct, 25–30 independent T_1 transgenic plants were developed. Transgenic lines with homozygosity and single locus of transgene insertions were identified on the basis of 3:1 segregation of kanamycin resistance in T_2 seeds. Twelve to twenty homozygous lines were grown in greenhouse under 16 h of 80 μmol m^{-2} s^{-1} light at 25±2 °C. Plants were grown for 8 weeks and then used for the experiments.

Table 1
List of primers used in this study

Name	Sequence
For cloning and chimeric constructs	
1. PRF[a]	5′ TCA ACT **CTG CAG** GTC GAC GGA CTA AGA TTA CGA GGA T 3′
2. PRR[b]	5′ TAC TAG **TCT AGA** ATC GAT GAC TAT AGG AGA 3′
3. SAM[c]	5′ ATG C**GTC GAC** CAT CAT TTG AAA GGG 3′
4. MFS[c]	5′ ATG C**GT CGA C**CT CCT ATA AAT ACC CTT GGT AGT 3′
5. PRB[d]	5′ GC**G GAT CC**A AAT CTT GAA GAT TTC ACG GTG A 3′
6. SMP[d]	5′ GC**G GAT CC**T CAC TAT ATA TAG GAA GT 3′
7. ENH[c]	5′ ATG C**GT CGA C**AG TAG CGT CAA GTG GCG TGG 3′
8. NOST[e]	5′ GT**G AAT TC**C CGA TCT AGT AAC ATA GAT GAC ACC GCG CGC GAT 3′
For TATA and Inr mutated constructs	
9. PRHF[f]	5′ CCC **AAG CTT** GGA CTA AGA TAT ACG AGG ATG TC 3′
10. TGF	5′ TCC GAG AAA GAC CCT TGG TAG 3′
11. TGR	5′GAA ATC TTG AAG ATT TCA CGG TG 3′
12. IPF	5′TGA AAG GAA AGA TAC AAC ATT TCT CCT ATA GT 3′
13. IPR	5′AAA CTA GAT TTA CTA CCA AGG GTA 3′
For detection of nucleosome	
14. PF3	5′ TGA TAT TAC CAT GTC AAA AAA TTT AGT 3′
15. NR1	5′ CAA TTG TGA AAA GAG AAC AAA T 3′
16. PRAF	5′ ATA ATA ATC ACC GTG AAA TCT TC 3′
17. PMCR	5′ AAT TGT TGA GAA ATG ACA ACA CG 3′
18. PCAF	5′ AAT TGA CGC ACA ATG ACG CC 3′
19. PRMP	5′ GAG AAA ATG TTG TAT CTT GAA TGG 3′
20. PMEF1	5′ GTC GAC CAT CAT TTG AAA GGG CCT CGG TAA TAC CAT 3′
21. PMER1	5′ ACG CAA TCC ACA ACT TTT CCA CAC CTT TTC TGG ATG ATG AAT CT 3′
22. PMEF2	5′ TGG ATT GCG TGG AAA AAG TTC GAT CTG ACC ATC TCT 3′
23. PMER2	5′ GCT TCT TTT TCC ACC GAT GCC TCC GTC TTC TTT TTC CAC AAT CCA CAT ATG 3′
24. PMEF3	5′ TCG GTG GAA AAA GAA GCT TGT ACG CTG TAC GCT GAC GAT AGA TAG 3′
25. PMER3	5′ CTT CCT ATA TAT AGT GAA GTA GCG TCA AGT GGC GTC ATT GTG C 3′
26. PMEF4	5′ TTC ATT TCA TTT GGA TTG GAC ACG TGT 3′
27. PMER4	5′ ATC TAG ATG TAA TTG TAA ATA GTA ATT GTA TAA TGT T 3′
28. NTP303F	5′ TCC TCA GAG GGA TTT CCC TT 3′
29. NTP303R	5′ CAA GAA GAA GAG GAG ACG CTT 3′
30. NPAF1	5′ TAT AAA TAT GGA AGT AAA AAT TAA TC 3
31. NPAR1	5′ TAA TTT TAA ATT GTC AAT GCA TGA 3′
32. NPAF5	5′ CTG ATA GAT CAA AAA AGT GTT TAA CT 3′
33. NPAR5	5′ ATG TAA TAT ATC CTG TTA TAG ATA A 3′
34. NPCF1	5′ TTC ATT TCT TCT TGT CTC TAC AC 3′
35. NPCR1	5′ GCT ACC TGG TCG TCC CAG 3′
36. NPCF6	5′ CAG ATG TAG GTG TAG AAC CTT 3′
37. NPCR6	5′ GCC GTC ATG AAA TCG CCA CT 3′
For quantitative RT-PCR	
38. GUSF	5′ CCG GGT GAA GGT TAT CTC TAT GA 3′
39. GUSR	5′ CGG GAA GCG GGT AGA TAT CA 3′
40. GUS probe FAM[g]	5′ TGT GCG TCA CAG CCA AAA GCC AG 3′ MGB[b]
41. UBIF	5′ GAA GCA GCT CGA GGA TGG AA 3′
42. UBIR	5′ CCA CGG AGA CGG AGG ACA A 3′
43. UBI probe VIC[h]	5′ ACC TTG GCT GAC TAC AA 3′ MGB[b]

5′ of GUS and UBI probes labeled by FAM and VIC respectively.
3′ of both the probes labeled by MGB.
Bold nucleotides indicate restriction sites.
[a] PstI.
[b] XbaI.
[c] SalI.
[d] BamHI.
[e] EcoRI.
[f] HindIII.
[g] FAM and VIC: Fluorescent dyes.
[h] MGB: Quencher.