A Strong Promoter Fragment from the Large Noncoding Region of Soybean Chlorotic Mottle Virus DNA

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Soybean chlorotic mottle virus (SoyCMV) is a member of the caulimovirus group of plant viruses and infects only four species of legume plants¹³⁾. The double-stranded DNA genome of SoyCMV (8,175 bps) contains nine open reading frames (ORFs) and one large noncoding region¹²⁾. We previously reported that a promoter fragment (P IV) from the upstream of SoyCMV putative coat protein gene exhibited a comparable activity to the cauliflower mosaic virus (CaMV) 35S promoter¹²⁾. In the present paper, we show that another promoter fragment (P NCR) from the large noncoding region of SoyCMV genome caused approx. five-fold stronger expression activity than the CaMV 35S promoter. We also show that truncation of P IV fragment remarkably reduced the promoter activity. The relationships between structure and activity of P NCR and P IV are discussed, with comparison to those of CaMV 35S promoter^{3,17-19)} and figwort mosaic virus (FMV) 34S promoter²³⁾.

Three kinds of SoyCMV promoter fragments were prepared as follows. The P NCR promoter fragment of 486 bp from the large noncoding region was obtained by polymerase chain reaction (PCR) using two oligonucleotide primers: 5'-CCAAGCTTATGTAGGACATGCCAGCTGTAA-3' and 5'-CCGGATCCTTTCTTTCTCTTGTGCTTG-3', and pSSac as template DNA. These two primers corresponded to the start position (nucleotide no. 5979-6000) and near the end position (nucleotide no. 6443-6464) of the SoyCMV large noncoding region (nucleotide no. 5979-6474) and had additional *Hind*III and *Bam*HI sites for cloning. pSSac was a full-length SoyCMV DNA clone ligated with pUC18²⁴⁾. Amplification was done using a GeneAmp PCR Reagent kit (Perkin Elmer Cetus) in 50 μ l of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, each 200 μ M dNTPs, each 1 μ M primers, 1 ng template DNA and 5 units of AmpliTaq DNA polymerase. Thermal cyclings were carried out 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min by an ATTO Zymoreactor.

The P IV promoter fragment of 378 bp (nucleotide no. 689-1066) from the upstream of SoyCMV putative coat protein gene (ORF IV) was excised from ORF III (nucleotide no. 647-1223) as a *Hind*III-*Xba*I fragment as described previously¹²⁾.

The truncated promoter fragment of P IV (P Δ IV: nucleotide no. 689-981), lacking 85 bp of 3'-terminal region of P IV, was prepared as follows. A *Ssp*I site was introduced at nucleotide no. 978-983 (5'-AATATC-3') by converting the sequence into 5'-AATATT-3' using oligonucleotide-mediated site-directed mutagenesis²²⁾ and the P Δ IV fragment of 293 bp was excised from this mutated DNA clone as a *Hind*III-*Ssp*I fragment.

The promoter fragments, P NCR, P IV and P Δ IV, were inserted 5'-proximal to the β -glucuronidase (GUS) gene of pBI 221 (Clontech Laboratories)¹⁴⁾, from which the CaMV 35S promoter fragment (P 35S)

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had been removed previously by double digestion with *Hind*III and *Bam*HI (for P NCR), *Hind*III and *Xba*I (for P IV) or *Hind*III and *Sma* I (for P Δ IV). The resulting chimeric plasmids were named P NCR/GUS/pUC19, P IV/GUS/pUC19 and P Δ IV/GUS/pUC19, respectively. pBI 221 is called here P 35S/GUS/pUC19 for convenience, and was also used for comparison. The CaMV 35S promoter fragment in pBI 221 has approx. 800 bp length, of which the 3'-end is an original *Dde*I site of nucleotide no. 7575 of CaMV^{11,14}.

Electroporation of tobacco mesophyll protoplasts (*Nicotiana tabacum* L. cv. Xanthi NN) was carried out according to the procedures described previously¹², with the following modifications. The protoplasts were suspended at approx. 2.5×10^5 /ml in 0.5 M mannitol with 100 μ M MgCl₂ and mixed with the chimeric plasmids at 50 μ g/ml and carrier salmon sperm DNA at 40 μ g/ml at 4°C. The protoplast suspension was exposed at 4°C to five square DC pulses of 50 μ s duration at 800 V/cm in the electric field by a JASCO CET-200 continuous-flow electro-manipulator. The electroporated protoplasts were incubated for 40 hr at 25°C under approx. 1,000 lux continuous illumination. After incubation the protoplasts were collected by centrifugation at 600 rpm for 5 min, lysed with a Vortex mixer and prepared according to the modified procedures¹⁶ for the fluorometric GUS assay¹⁴. An aliquot of the samples was taken for the protein assay using a Bio-Rad protein assay kit. GUS activity was measured using a JASCO FP-777 spectrofluorometer at 365 nm for excitation (band width: 10 nm) and 455 nm for emission (band width: 5 nm). Protein content was measured using a Bio-Rad 450 microplate reader at 595 nm.

Figure 1 shows the results of expression activities for the four promoter fragments in electroporated tobacco mesophyll protoplasts after 40 hr of incubation as determined by transient GUS assay. SoyCMV P NCR fragment exhibited approx. five-fold stronger activity compared to CaMV 35S promoter fragment. SoyCMV P IV fragment showed a comparable activity to the CaMV 35S promoter fragment, as already reported¹². P Δ IV fragment exhibited only approx. one fourth activity of P IV.

The nucleotide sequences of CaMV 35S⁹, FMV 34S²¹, SoyCMV P NCR and SoyCMV P IV¹² promoter fragments are compared in Fig. 2. SoyCMV P NCR and P IV fragments have shorter upstream regions before the TATA box (approx. 170 bp and 230 bp, respectively) and the P NCR fragment has a

Fig. 1. GUS activities caused by four different promoter fragments in electroporated tobacco mesophyll protoplasts as determined by transient expression assay. Means and standard deviations from six independent experiments are shown. P 35S: P 35S/GUS/pUC19 chimeric plasmid, P NCR: P NCR/GUS/pUC19 chimeric plasmid, P IV: P IV/GUS/pUC19 chimeric plasmid, P Δ IV: P Δ IV/GUS/pUC19 chimeric plasmid.

	7280	
CaMV	5' CGAGGAGCAT <u>CGTGGAAAA</u> GAAGACGTTT CAACCACGTC TTCAAAGCAA <u>GTGGATTGAT</u>	
P 35S	6772	
FMV	5' GCAAGATATA AGCAGATTCC TCTAGTACAA <u>GTGGGGAACA</u> AAATAAC <u>GTG</u> <u>GAAAAGAGCT</u>	
P 34S	6023	
SoyCMV	5' <u>GTGGTAGTGG</u> ACGCTTTACT TTATTAATAA <u>TGGTTGGTCA</u> GTAATAATGT AAGAC <u>CCAC</u>	
P NCR	796	
SoyCMV	5' ACCTCATAAT CAACCTAACA CTAAGGTTTT AGACGATATA GAATGTATAG AGCAAGGAGG	
P IV		
CaMV	GTGATATCTC CACTGACGTA AGGGATGACG <u>CACAAT</u> CCCA CTATCCTTCG CAAGACCCCTT	
P 35S		
FMV	GTCCTGACAG <u>CCCACT</u> CACT AATGCGTATG <u>ACGAACGCG</u> <u>TGACGACAC</u> <u>AAAAGAATTC</u>	
P 34S		
SoyCMV	<u>TTCTTTTCTT</u> TTGCTTGACG GCGAAGGATG CCGCTCTACC CAGTTGTTAA GGCACCTATC	
P NCR		
SoyCMV	CCCATGGCCA CATGCATACC ATACATTTAA CC <u>CACAAT</u> TA AATAACATAT CTGACATGTT	
P IV		
CaMV	CCTC <u>TATATA</u> AGGAAGTTCA TTTCAATTTGG AGAGGACACG CTGAAATCAC CAGTCTCTCT	
P 35S		
FMV	CCTC <u>TATATA</u> AGAAGGCATT CATTCCCAT TGAAGGATCA TCAGATACTG AACCAATATT	35S RNA
P 34S		
SoyCMV	GCAT <u>TATAAA</u> TAAGAGACCA AGGACTCTAT TGTTCCTTGG AGTTGATTG AGTAAGGAAT	
P NCR		
SoyCMV	ATCA <u>TATATA</u> ATAAAAAAT GTTGTGCA <u>AAAACACAAG</u> <u>GAACATCAGG</u> AAACATCACC	
P IV		
CaMV	CTACAAATCT ATCTCTCTCT ATAATAATGT GTGAGTAGTT CCCAGATAAG GGAATTAGGG	
P 35S		
FMV	TCTCACTCTA AGAAATTAAG AGCTTTGTAT TCTTCAATGA GAGGCTAAGA CCCTAAGAG	
P 34S		
SoyCMV	ATAGCCAATA GTGCCGTGTA AGGCCAAGTG <u>CTTTATCCA</u> <u>TTTACACTCA</u> <u>CTCCAGTCG</u>	
P NCR		
SoyCMV	TGAATATCAA <u>TCTATACAAA</u> <u>ATAGACTGAC</u> <u>AAAGCTCTTA</u> <u>GAAGAACTAT</u> <u>CTAAGAAAGA</u>	
P IV		
CaMV	TTCTTATAGG GTTTCGCTCA TGTGTTGAGC ATATAAGAAA CCCTTAGTAT GTATTT 3'	7575
P 35S		
FMV	TTTCGAAAGA GAAATGTAGT ATAGTAAGAG TCCTCCCACT CCGGGAGATT GTAATAAGA	
P 34S		
SoyCMV	GTGGTTTAAA <u>AACCTGGACC</u> <u>GGCAAAGTCG</u> AGAGACTCTA AATTAGAAAA GGAGAAGTCC	
P NCR		1066
SoyCMV	<u>ACAATTTGAT</u> <u>AAAAAATCCA</u> <u>GTAAAATTTA</u> T 3'	
P IV		
FMV	GATCTTGTA TG 3'	7083
P 34S		
SoyCMV	<u>TTTATACTAT</u> <u>CAACAAGGA</u> GAGATCCTAA ATCTTAAACA <u>CAAAATCCTT</u> <u>TATGAATAAG</u>	
P NCR		
SoyCMV	<u>AAATTGTTCC</u> <u>AGCAACTACC</u> <u>AAGTCTTAAA</u> <u>AAGACCCAGG</u> <u>AAGCAAAAGC</u> <u>AAAGCAAGAA</u>	
P NCR		
SoyCMV	CAAGCACAG AGAAAGAAA GG 3'	6464
P NCR		

Fig. 2. Comparative nucleotide sequences in the promoter fragment of CaMV 35S, FMV 34S, SoyCMV P NCR and SoyCMV P IV. box: CAAT and TATA boxes, double underline: GT motifs, single underline: TGACG motifs, dotted underline: bowl sequence, wavy underline: translational enhancer core-like sequences triangle: P Δ IV lacks a 3'-terminal region here after.

longer downstream region after the TATA box (approx. 310 bp) than CaMV 35S and FMV 34S promoter fragments. The 34S promoter fragment of FMV, one of caulimoviruses, has been reported to have an approx. 63% homologous sequence to CaMV 35S promoter core (around the TATA box) and a comparable activity to that of CaMV 35S promoter fragment in protoplast transient expression assay²³. Both CaMV 35S and FMV 34S promoter fragments have more than two GT motifs (SV 40 transcriptional enhancer core-like sequence)^{5,17,19}, two TGACG motifs (histone gene transcriptional regulation motif)^{2,5,23}, CAAT-like boxes and TATA boxes^{17,21,23}.

The SoyCMV P NCR fragment also contained two GT motifs, CAAT-like and TATA boxes but no

TGACG motif. SoyCMV P IV fragment had also CAAT and TATA boxes but no GT or TGACG motifs, as described previously¹²⁾.

Although the sequence homology between SoyCMV P NCR and CaMV 35S promoter is lower than that between FMV 34S and CaMV 35S promoter and the transcripts of SoyCMV have not yet been analysed, SoyCMV P NCR is assumed to be a promoter corresponding to the CaMV 35S promoter. This is based on its location in the viral genome and its fundamental structural similarity to CaMV 35S promoter, including the existence of a bowl sequence as discussed previously¹²⁾. Bowl sequences were reported to be present also in the downstream of large noncoding regions of three other caulimoviruses and not to affect transient gene expression in electroporated protoplasts⁷⁾.

SoyCMV P IV is assumed to be a promoter for the coat protein gene¹²⁾.

In addition to the above-mentioned sequences, SoyCMV P NCR fragment contained four characteristic sequences (at approx. 80, 110, 170 and 220 nucleotide downstream of the TATA box) which, at the RNA level, had more than 60% of homology with the 5'-terminal region of tobacco mosaic virus (TMV) Ω sequence (Figs. 2 and 3). Three similar sequences were located at approx. 15, 60 and 85 nucleotide downstream of the TATA box in the SoyCMV promoter IV fragment¹²⁾, but were absent in CaMV 35S and FMV 34S promoter fragments. The SoyCMV P Δ IV fragment lacked 85 bp of 3'-terminal region of P IV containing the latter two sequences of the three. The transcription starting sites in these SoyCMV promoter fragments have not yet been shown. But, considering from the corresponding position of CaMV 35S promoter, the all Ω -like sequences of SoyCMV promoter fragments are assumed to be

TMV	1	GUUUUUUACAACAAUACCAACAACAACA	31
AlMV-4 5'-NTR	15	* * * * * UUUUCUUUCAAAUACUCCAUC	36
PVX 5'-NTR	1	* * * * * G-AAAACUAAACCAUACCAACAACA-CAA	29
TEV 5'-NTR	4	* * * * * AUAACAAUUCUACA-CAACA	24
	25	* * * * * UAUACAAACAAACGAAUCUCAA	47
	68	* * * * * UAUUGCAGCAAUUUAAAUCA	88
	110	* * * * * UUUUCACCAUUUACGAACGAUAGCA	144
SoyCMV P NCR	6234	* * * * * UUUUA-UCCAUUUACACUCACUCCCA	6258
	6267	* * * * * UUUAAAAACCGGACCGGCAA	6287
	6323	* * * * * UUUAUACUAUCA-AACAAGGA	6342
	6382	* * * * * AUUGUCCAGCAACUACCAAGUCUUAAAA	6410
SoyCMV P IV	937	* * * * * UUGUUGCAAAAAACAAGGAACAUCA	963
	985	* * * * * AUCUAUACAAAAGACUGACAA	1007
	1011	* * * * * UCUAGAAGACUAUCUA-AGAAAGAA	1036

Fig. 3. Homologous regions in the 5'-nontranslated leader sequences of TMV, AlMV-4, PVX and TEV and in SoyCMV promoter fragments. *: identical to TMV Ω sequence.

transcribed into 5'-nontranslated regions of mRNA (Fig. 2).

The Ω sequence is a 68-nucleotide fragment of the nontranslated 5'-leader sequence of TMV-RNA, which has been reported to enhance the translation of contiguous foreign gene transcripts in electroporated tobacco protoplasts and has been named a translational enhancer⁸⁻¹⁰. The sequence of the first half of this fragment is conserved among several TMV strains.

Similar translational enhancements have been reported also for a 36-nucleotide fragment of 5'-leader sequence of alfalfa mosaic virus (AIMV) RNA⁴¹⁵, a 83-nucleotide leader sequence of potato virus X (PVX) RNA²⁰ and a 144-nucleotide fragment of 5'-nontranslated region (5'-NTR) of tobacco etch virus (TEV) RNA⁴. For TEV, it was reported that, when cDNA of the 5'-NTR was ligated between 3'-terminal of CaMV 35S promoter fragment and 5'-terminal of GUS gene, 5'-NTR resulted in approx. Eight-fold stronger translational enhancement than a chimeric gene without 5'-NTR in transient expression assay in electroporated tobacco mesophyll protoplasts⁴. Also these AIMV¹⁵, PVX²⁰ and TEV⁴ fragments have highly homologous sequences to the first half of TMV Ω sequence⁸ (Fig. 3). Therefore it is possible that these highly homologous regions are translational enhancer core signals, even though they have no consensus sequence. However, some other factors, like secondary structures of the 5'-nontranslated regions may also relate to the translational enhancement. The highly homologous sequences to TMV Ω sequence are called here, for convenience, translational enhancer core-like sequences (TLEs).

The four promoter fragments used in this study contain the following numbers of GT motifs and TLEs:

	GT motifs	TLEs
CaMV 35S	3	0
SoyCMV P NCR	2	4
SoyCMV P IV	0	3
SoyCMV P Δ IV	0	1

Comparing this table with the promoter activities shown in Fig. 1, it is suggested that TLEs are present also in the promoter core-downstream regions of SoyCMV double-stranded DNA genome and that the TLEs might participate in translational enhancement after they are transcribed into 5'-nontranslated regions of mRNA, while the promoter core-upstream GT motifs might be involved in transcriptional enhancement¹⁹. Detailed analyses on the actual activities of the possible transcriptional and translational enhancer core-like sequences in the SoyCMV P NCR fragment are now in progress.

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和 文 摘 要

L.R. Conci・西沢洋子・斎藤美保・伊達朋子・長谷川明・三木敬三郎・日比忠明：ダイズ退緑斑紋ウイルスゲノムの非翻訳領域に存在する強力なプロモーター

ダイズ退緑斑紋ウイルス (SoyCMV) の2本鎖DNAゲノムから、非翻訳領域に存在し、TATA配列の上流に2個のGTモチーフ、下流に4個のTMV- Ω 様配列を有するP NCR、外被蛋白質遺伝子上流に存在し、3個の Ω 様配列を有するP IV、P IVの3'端側から2個の Ω 様配列を削除したP Δ IV、の3種のプロモーター断片を調製し、GUS遺伝子に接続した後、タバコ葉肉プロトプラストにおける各プロモーターの発現活性を測定した。その結果、CaMV 35Sプロモーターに比べ、それぞれ、P NCRが5.4倍、P IVが1.1倍、P Δ IVが0.3倍の活性を示し、プロモーターコア下流に存在する Ω 様配列が、mRNAの先導配列に転写されることによって翻訳活性を促進している可能性が示唆された。

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