RESEARCH COMMUNICATION

Effect of CWG methylation on expression of plant genes

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INTRODUCTION

The presence of two DNA methyltransferases in Pisum raises the possibility that they serve different functions. In vitro methylation of CWG sequences in the strong cauliflower mosaic virus 35S promoter had no effect on reporter gene expression. In contrast, in vitro methylation of CWG sequences in the relatively weak, CG-deficient Phaseolus vulgaris rbcS2 promoter inhibited transcription. Expression of both constructs was strongly inhibited by extensive CG methylation. A search of published plant promoter sequences revealed that the CG content of promoters is very variable, with some promoters having typical CG islands. In contrast, the distribution of CWG sequences is more even with little evidence for CWG islands.

Key words: CG island, in vitro methylation, methylcytosine, methyltransferase.

MATERIALS AND METHODS

Chimaeric promoter chloramphenicol acetyltransferase (CAT) constructs

pCAMVCN (Pharmacia) is a 35S–CAT construct containing 400 bp of the CaMV 35S gene promoter fused to the coding region of the CAT gene of Tn9 and the termination sequence of the nopaline synthase (NOS) gene.

The rbcS–CAT construct contained a promoter fragment (-1433 bp to +26 bp) of the bean rbcS2 gene fused to the CAT coding sequence and the NOS terminator of pCAMVCN [7].

In vitro methylation of reporter gene constructs

Prokaryotic DNA methyltransferases were used according to the manufacturer’s instructions. The pea CWG methyltransferase was purified as described previously [5], and was used to methylate plasmid DNA by incubation overnight at 30 °C in the presence of 0.5 mM S-adenosylmethionine. The DNA was re-purified, and the amounts were quantified using a microfluorimeter (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) before use in transfection.

Plant growth and protoplast isolation

This was performed essentially as described by Urwin and Jenkins [7]. Ph. vulgaris L cv. Tendergreen plants were grown for 10–15 days in continuous white light provided by warm white fluorescent tubes at a fluence rate of 10 μmol⋅m−2⋅s−1. Plants were transferred to darkness for 2 days (dark-adapted). All further steps prior to incubation of protoplasts were performed under a green safe light. The upper surfaces of primary leaves

Abbreviations used: CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase; NOS, nopaline synthase.

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were rubbed with alumina, which was subsequently removed by washing thoroughly in distilled water. Leaves were floated (upper-side down) on 0.8% (w/v) cellulase R10, 0.25% (w/v) macer-zyme R10 (both from Yakult Honsha, Nishinomiya, Japan) in culture buffer (0.4 M mannitol with CPW salts [8], and 50 μg ml⁻¹ ampicillin, pH 5.7) for 20 h at 22 °C in the dark. Protoplasts were released from leaves by brief, gentle agitation, and cells were filtered through one layer of muslin and a steel sieve (70-μm pore size). Protoplasts were allowed to pellet under gravity on ice for 1–1.5 h, after which they were washed twice in cold 0.4 M mannitol/CPW salts, pH 5.7.

**Transient expression assay**

Protoplasts were resuspended in electroporation buffer [0.4 M mannitol/4 mM Mes⁻ (pH 5.7)/20 mM KCl] at (1–3) × 10⁶ cells ml⁻¹. Aliquots of 0.5 ml were dispensed into cuvettes on ice, and 25 μg of plasmid DNA was added in < 30 μl. Cells were electrooporated with a single pulse at a capacitance of 100 μF and a field strength of 325 V cm⁻¹. Cells were then returned to ice for 5 min. Aliquots of 200 μl were added to 800 μl of culture buffer, and cells were then incubated in 24-well culture plates for 20 h in white light, as described above, at a fluence rate of 80 μmol m⁻² s⁻¹. Protoplasts were pelleted at 100 g for 5 min, and resuspended in 50 μl of 20 mM Tris/HCl, pH 8.0/2 mM MgCl₂. After freezing at −80 °C and heating at 65 °C for 15 min, debris was removed by centrifugation at 14 000 g for 5 min. CAT activity was assayed by the method of Seed and Sheen [9] using 40 μl of extract for all constructs, apart from 35S–CAT, where 40 μl of a 1:100 dilution of the extract was used.

**RESULTS**

**Effect of methylation on expression from different promoters**

We have investigated the effect of *in vitro* methylation on the transient expression of plasmids containing promoters with differing CG and CWG contents. We have used the CAT reporter gene under the control of either the CaMV 35S promoter (a typical CG-island promoter) or the bean rbcS2 promoter (a promoter lacking a CG island, but containing the expected number of CWG sequences). Transient expression was assayed in bean-leaf protoplasts. In order to prevent possible confusion with any pre-existing methylation, the plasmids were grown in an *Escherichia coli* strain (GM2163; New England Biolabs, Beverly, MA, U.S.A.) that was dam⁻ and dcm⁻ (i.e. lacking methylation of GATC and CCWGG sequences). Complete plasmids were methylated *in vitro* by using either the purified pea CWG methyltransferase [5] or one of a variety of prokaryotic methyltransferases (M. *Hpa*II, M. *Msp*I or M. *Sss*I) that would lead to the introduction of methyl groups into the following sequences: CmCGG (M. *Hpa*II); =CGG (M. *Msp*I); or =CG (M. *Sss*I). The prokaryotic CG methyltransferase, i.e. M. *Sss*I, was used in preference to the pea CG enzyme because (i) it is a much more active methyltransferase *de novo* and (ii) it is more readily available. Compared with the rbcS2 promoter, the 35S promoter confers a 1000-fold greater level of CAT expression with the control, unmethylated plasmids (i.e. the rbcS2 is a weak promoter relative to the CaMV 35S promoter). A diagram of the proximal 425 bp of the promoter regions is shown in Figure 1 (upper panel).

Methylation of CCGG sequences has little effect on gene expression from either promoter (Figure 1, lower panel). This is not surprising, since neither promoter contains CCGG target sites, and what sites there are in the CAT gene and the vector are well dispersed. In contrast, methylation of all CGs with M. *Sss*I leads to a very strong inhibition of expression from both promoters. Whether this is a direct effect of promoter methylation or an indirect effect mediated partly by vector methylation [10] is discussed below.

Methylation of CWG sequences leads to a dramatic inhibition of expression from the rbcS2 promoter, but has only a limited effect on expression from the 35S promoter (Figure 1, lower panel). Since the plasmids used are very similar, differing substantially only in their promoter regions, this difference must be attributable to promoter methylation.
and a higher-than-average CWG content. A low CG content is not, however, a characteristic of all rbcS promoters, since the tomato rbcS3A promoter has 11.5% of its cytosines in CG dinucleotides (accession number S44160). In contrast, the 12 promoters with 15% or more of their cytosines in CG dinucleotides show no deficiency in CG (average CG:GC ratio of 1.1), and a range of values for the proportion of cytosines in CWG trinucleotides that have an average value (9.6%) that is very close to that found for all the promoters (9.3%).

**DISCUSSION**

Ribulose bisphosphate carboxylase/oxygenase (or Rubisco) is an essential plant enzyme and the expression of the small subunit (RbcS) is controlled by light. The bean rbcS2 promoter contains a CG dinucleotide in the sequence CACGTG (known as a G-box) that binds the transcription factor GBF, which is implicated in regulation by a variety of environmental signals [2,12] and is required for high levels of expression [7,13]. Southern blot analysis, using a probe stretching from —1433 to —4 bp relative to the transcription start site of the bean rbcS2 gene, indicates an absence of CG methylation of the G-box sequence in genomic DNA from dark-grown, dark-adapted or light-grown leaves, roots or seeds. This analysis was made possible since we have shown that Eco72I (Promega) is sensitive to methylation of the internal cytosine in the sequence CACGTG (results not shown). The pea rbcS3A promoter contains only a single CG dinucleotide, and this is not in the G-box, which therefore lacks the CG dinucleotide discussed above [14]. It is therefore very unlikely that inhibition of expression is a result of interference in the binding of transcription factors (e.g. GBF), but it is likely that the very strong inhibitory effect of CG methylation in transient expression assays is mediated via the formation of an inactive chromatin structure [10]. This could also be true for the 35S promoter, and Dieguez et al. [15] have shown that none of the promoter CG sites are essential for expression, even though their methylation status correlates inversely with expression.

In contrast, the selective effect of CGW methylation on the weak rbcS2 promoter implies a direct effect on the promoter, and this might well involve inhibition of transcription factor binding. Although the bean rbcS2 promoter contains very few CG dinucleotides (CG:GC ratio is 0.2), it has very similar levels of CGW sequence relative to the 35S promoter. As the expression of the 35S·CAT plasmid is resistant to methylation of over 80% of its CGW trinucleotides, it is unlikely that CGW methylation of either the vector or the CAT gene can bring about the formation of inactive chromatin [10], perhaps indicating an alternative role for CGW methylation.

Southern blot analysis indicates that there is no more than a very low level of in vivo methylation of the EcoRII site (CCGG) at —850 bp in the rbcS2 promoter in DNA from all bean plant tissues tested. A similar complete lack of methylation was observed at the BalI site (TGGCCA) at —138 bp that overlaps a CAG trinucleotide (results not shown). Thus although we have shown that, in transient expression assays, both promoters are sensitive to CG methylation, and the rbcS2 promoter is sensitive to CGW methylation, we have not found a situation where the endogenous bean rbcS2 promoter is methylated, despite examining tissues that show a wide range of expression. This is in contrast with the ＞90% methylation of PstI sites (CTGCAG) and HpaII sites (CCGG) in total pea DNA [5].

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