An organ-specific hypomethylation of cotyledon genomic rDNA in *Pisum sativum* L.

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Received 18 September 1986; revised version received 21 October 1986

The CCGG and GCGC sequences in pea genomic rDNA were found to be hypomethylated relative to the leaf in the developing cotyledon. No rRNA gene copies were detected with only unmethylated CCGG sequences and the majority appeared to contain a mixture of doubly methylated ($^{m}C^{m}CGG$) and partially methylated ($C^{m}CGG$) sites. Some CCGG sequences were shown to have a greater probability of being undermethylated. Seed development did not appear to act as a stimulus for de novo methylation.

(Pisum sativum *L.*) *DNA methylation* *rRNA gene* *Gene expression* *Development*

1. INTRODUCTION

The post-replicative methyl-modification of some cytosine residues (5-mC) in eukaryotic DNA is well documented [1–3]. The usual site of cytosine methylation is the dinucleotide CpG. This dinucleotide is more frequent in plant DNA (3–4%) than in animal DNA (0.5–1%) [4], and analyses of base composition have indicated that the level of 5-mC in plants is substantially higher than that found in animals [5,6].

The ribosomal RNA (rRNA) genes are generally arranged as tandem arrays of repeating units [7–9] and the extent of ribosomal DNA (rDNA) methylation is known to vary in different organisms. In invertebrates (e.g. *Drosophila*), rDNA is unmethylated [10] but in amphibia, somatic rDNA is highly methylated whilst amplified rDNA was found to contain many unmethylated sites [11]. In other vertebrates, for example, the mouse and rat, a combination of methylated and unmethylated rDNA was found [12,13]. Hypomethylation has been related to transcriptional activity of the rRNA genes [14,15].

In this paper the cytosine methylation of pea rDNA was assessed using the restriction endonuclease isoschizomers *Msp*I and *Hpa*II, which cleave at CCGG sequences; however, fully methylated sequences ($^{m}C^{m}CGG$) are resistant to cleavage by both enzymes, while partially methylated sequences ($C^{m}CGG$) are resistant to *Hpa*II only. An organ-specific difference in the extent of methylation of the ribosomal genes in pea is reported and discussed.

2. MATERIALS AND METHODS

The growth of *Pisum sativum* L. var. 'Feltham First' (Sutton Seeds Ltd) and extraction of DNA are described elsewhere [15,17,18]. Genomic DNA was digested to completion with a 15-fold excess of enzyme, in the buffer recommended by the manufacturers. In some cases an internal control, 0.1 μg SV40 DNA, was mixed with the genomic DNA prior to enzyme digestion. The digested DNA fragments were separated on a 0.65% agarose gel by overnight electrophoresis and were transferred to nitrocellulose filters by the method of Southern [19].

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

The filter was prehybridized at 62°C for 2–4 h in 5 × SSC, 100 μg/ml denatured herring sperm DNA, 5 × Denhardt's solution [20 × SSC is 3 M NaCl, 0.3 M sodium citrate buffer, pH 7.0, and 1 × Denhardt's is 0.02% (w/v) bovine serum albumin, 0.02% (w/v) Ficoll (Pharmacia Fine Chemicals), 0.02% (w/v) polyvinylpyrrolidone]. Hybridization was at 62°C for 30–40 h in 5 × SSC, 1.5 × Denhardt's solution, 100 μg/ml denatured herring sperm DNA. After hybridization the filter was washed (at the temperature indicated in the figure legend) as follows: 2 × 15 min, 2 × SSC, 0.1% (w/v) SDS, then 2 × 15 min, 0.1 × SSC, 0.1% (w/v) SDS, air-dried and exposed to X-ray film at −80°C.

The hybridization probe used (pHA 1) was a pea ribosomal repeat unit cloned into pACYC 184, and was a gift from R. Cuellar (Plant Breeding Institute, Cambridge). Radioactive probes were [^{32}P]dCTP labelled by nick-translation [20].

3. RESULTS

Fig.1a and b shows the results obtained when restricted fragments of pea genomic DNA were blotted and hybridized with labelled pea ribosomal repeat unit (clone pHA 1). The restriction enzymes used had four nucleotide recognition sequences as follows: *Hae*III, 5′-GGCC-3′; *Hha*I, 5′-GCGC-3′; *Msp*I and *Hpa*II, 5′-CCGG-3′. *Hae*III cleaved pea genomic DNA into fragments with an average size of less than 2 kb (fig.1a, lanes c, g, k, o and s), whereas after digestion by both *Hha*I and *Hpa*II a considerable amount of DNA remained as fragments > 25 kb (fig.1a, lanes b, e, f, i, j, m, n, q, r and u).

The pHA 1 probe hybridized to a wide size range of restriction fragments (fig.1b). Following *Hha*I digestion of cotyledon DNA (lanes b, f, j and n), major bands of hybridization were observed at 2.69, 3.39, 3.85, 4.84, 6.73 and 10.00 kb. The smallest *Hha*I fragments to hybridize to the probe were 1.00 and 1.27 kb and these gave only a weak signal. The basic pattern of hybridization remained the same in the leaf DNA (lane r) but the relative intensities of the signals for the 2.69, 3.39, 3.85, 4.84 and 6.73 kb bands were reduced.

Following *Hae*III digestion of leaf and cotyledon DNA (fig.1a, lanes c, g, k, o and s), pHA 1 hybridized to a series of fragments ranging in size from 1.04 to 0.21 kb which, apart from the 1.04 kb band, were all of a similar intensity.

pHA 1 also hybridized to a series of fragments in cotyledon DNA digested with *Msp*I (lanes d, h,

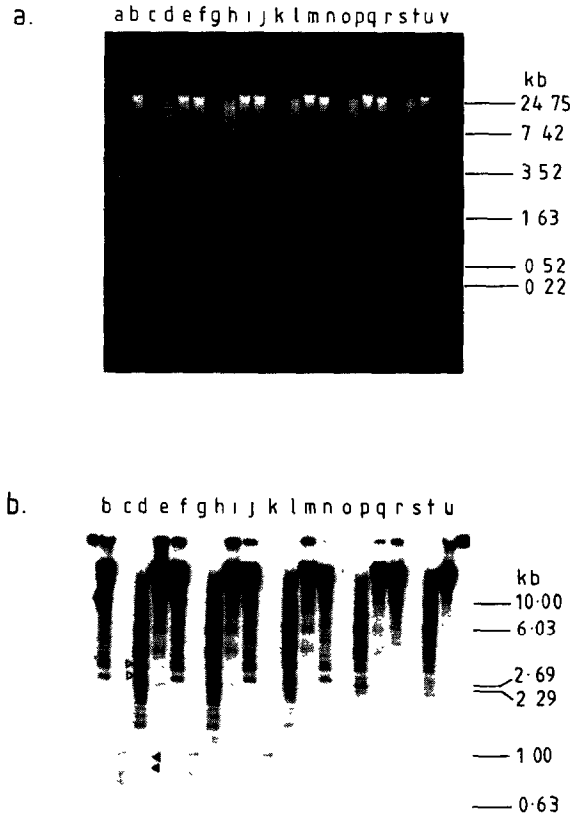

Fig.1. (a) Gel photograph of *Msp*I, *Hpa*II, *Hae*III and *Hha*I digested cotyledon and leaf DNA (lanes b–u: 2 μg DNA/lane). Lanes: b, c, d and e, 10 d.a.f. cotyledon DNA; f, g, h and i, 12 d.a.f. cotyledon DNA; j, k, l and m, 15 d.a.f. cotyledon DNA; n, o, p and q, 21 d.a.f. cotyledon DNA; r, s, t and u, leaf DNA. Lanes: b, f, j, n and r, *Hha*I; c, g, k, o and s, *Hae*III; d, h, l, p and t, *Msp*I; e, i, m, q and u, *Hpa*II. Lanes a and u contain marker DNAs. Lane a, NM258 + *Ava*I; pBR322 + *Hin*cII; pBR322 + *Taq*I. Lane u, NM258 + *Eco*RI; pBR322 + *Hin*fI. (b) Autoradiograph showing the hybridization of ^{32}P-pHA 1 to a Southern blot of the gel illustrated in a. The identities of the lanes are as indicated in a. The specific activity of the probe was 4.9×10^7 cpm/μg and the filter was washed at 50°C. (▷) Typical bands present in *Msp*I digests absent from *Hpa*II digests; (◁) weakly hybridizing bands of < 1.0 kb in *Msp*I digests.

l and p), but gave stronger signals with the larger fragments, which ranged in size from 10.00 to 2.29 kb. The signal intensity was reduced when pHA 1 hybridized to fragments of between 2.09 and 0.93 kb and was further reduced when the probe hybridized to fragments of less than 0.8 kb. Following *Msp*I digestion of leaf DNA (lane t), a similar pattern of hybridization was obtained, except that fragments of between 2.29 and 3.78 kb (which had hybridized very strongly in the cotyledon lanes) now had a reduced signal level, compared to the same bands in cotyledon DNA. On the other hand, hybridization to bands of >10 kb was increased compared to cotyledon DNA.

pHA 1 hybridized to fewer distinct bands in the *Hpa*II digested cotyledon DNA (lanes e, i, m and q) than in *Msp*I digested cotyledon DNA. Only a weak signal of hybridization was detected for bands in the range 2.09–0.93 kb, and a reduced signal strength was also observed for the hybridization of the probe to fragments greater than 2.29 kb. Two fragments noted in the higher molecular mass range of *Msp*I digested cotyledon DNA (3.45 and 2.83 kb), indicated in fig.1, were absent in the *Hpa*II cotyledon tracks. Hybridization of pHA 1 to fragments of <25 kb in *Hpa*II digested leaf DNA (lane u) was also far less than to cotyledon DNA. Even when the filter was overexposed, only a weak signal was observed for hybridization to the 10.00 kb fragment.

When pHA 1 was digested with *Msp*I, bands of <1.0 kb were produced. Comparison of these *Msp*I fragments to those produced by digestion of genomic DNA and hybridizing to pHA 1 (not shown) showed that corresponding bands were present in both digests but were of very weak intensity in the genomic digests compared to the major hybridizing bands. No hybridizing bands of a corresponding size were detected in any of the *Hpa*II digests of genomic DNA.

4. DISCUSSION

The activity and specificity of action of the two restriction enzyme isoschizomers used as a methylation assay was confirmed using an unmethylated control DNA, SV40, incorporated with the genomic DNA prior to digestion [21]. Thus, any difference in the extent of digestion of genomic DNA by *Msp*I or *Hpa*II could not be due to incomplete digestion or contaminants.

The data of fig.1a (lanes e, i, m, q and u) show that pea genomic DNA comprises a major highly methylated fraction, m+ (i.e. the DNA in the very high molecular mass range, 25–30 kb, which was essentially resistant to cleavage by *Hpa*II), and a minor less highly methylated or 'unmethylated' fraction, m− (i.e. that which had been cleaved by *Hpa*II). This is in agreement with the presence of methylation 'domains' as suggested by Bird and Taggart [11].

rDNA was detected in both methylation domains (fig.1b, lanes e, i, m and q). Some sites in the ribosomal genes had a greater probability of being undermethylated, i.e. cut by *Hpa*II and *Msp*I in the repeats from the less highly methylated fraction, and partially methylated and therefore only cut by *Msp*I in the repeats from the highly methylated fraction (fig.1b, lanes d, e, h, i, l, m, p, q). Other workers (e.g. [14,22,23]) have demonstrated that in *Xenopus laevis* and *Xenopus borealis*, hypomethylated regions of the ribosomal repeat units are usually located in the non-transcribed spacer regions.

The low average size (<1 kb) of the fragments produced by digestion of the cloned ribosomal repeat pHA 1 with *Msp*I, compared to the length of the ribosomal repeat unit (9.3 kb), indicated that there were many sites for *Msp*I in the pea ribosomal repeat. It has been estimated that there should be 20–30 *Msp*I sites within an rRNA gene in cucurbits [24] and flax [25].

No completely unmethylated rRNA genes were detected in either cotyledon or leaf tissue by *Hpa*II digestion (fig.1b, lanes e, i, m, q and u). (The sensitivity of the system is sufficient to detect <10 gene copies per genome.) However, the weak hybridization of the probe to fragments of <1 kb in the *Msp*I lanes (fig.1b, lanes d, h, l, p and t) indicated that a few rRNA genes were detected which appeared to contain a mixture of unmethylated (CCGG) and partially methylated (C^mCGG) sites. The far greater hybridization of the probe to the larger *Msp*I fragments (1–10 kb) thus implied that the majority of the ribosomal repeat units contained a mixture of fully methylated ($^mC^mCGG$) and partially methylated (C^mCGG) sites, whilst a minority, i.e. those cleaved by *Hpa*II (fig.1b, lanes e, i, m, q and u) contained a mixture of partially

or fully methylated sites and some unmethylated sites. The results do not reflect significant inhibition of *Msp*I by 5′-CCG^{m}CGG-3′ sequences [26], since the smallness of the *Hae*III derived rRNA fragments, and the difference in the signal intensity of the *Msp*I and *Hpa*II hybridization bands, clearly indicate that the sequence 5′-GGC^{m}CGG-3′ is rare [26].

When the hybridization of the ribosomal probe to the cotyledon and leaf *Hpa*II digests was compared, there was distinct organ-specific difference in the extent of methylation of the ribosomal genes, with the rDNA from the developing cotyledons being hypomethylated with respect to that derived from the leaf. The rDNA hybridization pattern obtained for pea leaf plus *Hpa*II (fig.1b) was very similar to that described for marrow and pumpkin fruit tissue, turnip taproot [24] and mature tobacco leaves [27]; this relative hypomethylation of the pea cotyledon rDNA may reflect the specialised role of the pea cotyledon as the organ responsible for the synthesis of large amounts of storage proteins. A similar hypomethylation of pea cotyledon rDNA was also observed when a different subset of CpG dinucleotides was investigated, i.e. those contained in the *Hha*I recognition sequence, 5′-GCGC-3′, where cleavage by *Hha*I is prevented by methylation of either cytosine. Overall hybridization to discrete leaf rDNA *Hha*I fragments of <25 kb (fig.1b, lane r) was less than was observed for the cotyledon digests (fig.1b, lanes b, f, j and n). The similarity between this overall increased methylation of the *Hha*I sites in leaf rDNA, and that previously noted for the *Msp*I and *Hpa*II sites, supports the idea that the CpG dinucleotides monitored by these restriction enzymes are representative of the CpG dinucleotides in the surrounding DNA.

Interestingly, there was no change in the CpG hypomethylation observed during the development of the pea cotyledons (fig.1b, lanes b, f, j, n, e, i, m and q), despite protein synthesis being much reduced by the latest stage assayed, 21 d.a.f. [17]. This indicates that cessation of protein synthesis is not due to de novo methylation of rRNA genes.

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