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# Changes in methylation of tissue cultured soybean cells detected by digestion with the restriction enzymes HpaII and MspI *

**Hector Quemada **, E. Jill Roth, and Karl G. Lark**

University of Utah, Department of Biology, Salt Lake City, UT 84112, USA

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Abstract

Each of the tandemly arranged 5S RNA genes of soybean contain two CCGG sites which, if unmethylated, can be digested by both MspI and HpaII. Methylation of the internal cytosine (CmeCGG) prevents digestion by HpaII but allows digestions by MspI.

Suspension cultures were prepared from soybean plants and the DNA from these cultures was examined for the susceptibility of 5S RNA genes to digestion by MspI and HpaII. 5S genes from DNA extracted from intact plants can be partially digested with MspI but not at all by HpaII. In contrast, shortly after cells were cultured the 5S RNA could be hydrolyzed by both HpaII and MspI. After prolonged cell culture, the 5S genes from some cell lines were found to have become partially or even completely resistant to HpaII digestion. The results suggest that lack of methylation can occur when cells are cultured and that such methylation may play a role in the heritable changes observed in cell culture.

## Introduction

The 5S RNA of soybean is encoded by a set of tandemly arranged genes, each ca. 330 nucleotides long. The copy number of these genes varies from as low as 5,000 copies/genome to as high as 400,000 copies/genome in different cultivars. In two cultivars (Minsoy and Noir 1) these genes have been sequenced and shown to contain two CCGG restriction sites (figure 1) (Quemada 1986), one in the non-transcribed spacer and the other in the coding region. The restriction nucleases MspI and HpaII digest DNA at the specific sequence CCGG. Methylation of cytosine prevents digestion: CmeCGG can be digested by MspI but not HpaII; meCCGG and meCmeCGG cannot be digested by either enzyme (Busslinger et al. 1983, Keshet and Cedar 1983). Thus, restriction by HpaII or MspI can detect different degrees of methylation. Depending upon the frequency at which tandemly arranged sites are methylated, fragments of different sizes will be generated from the tandem array of 5S RNA genes. Frequently methylated sequences will generate larger fragments upon digestion with HpaII than the fragments generated when the same sequences are not methylated.

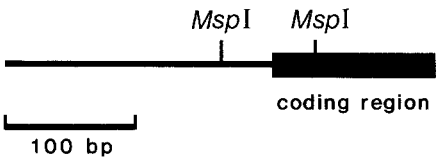


Fig. 1. CCGG sites in the 5S RNA gene of soybean are indicated as MspI.

We report here variation in digestion by HpaII (presumably methylation of CCGG) which results from culturing soybean cells. Our findings are similar to results reported for cultured rabbit cells (Waalwijk and Flavell 1978) or human cells (Ehrlich et al. 1982; Reis and Goldstein 1982). Recent reports have demonstrated that quantitative as well as qualitative changes occur in nucleotide sequences when plant tissues are cultured (Chaleff 1981, Larkin and Scowcroft 1983, Orton 1984, Scrowcroft et al. 1985, Frazier et al. 1986). The results reported below suggest that heritable changes in the pattern of DNA methylation occur when plant cells are cultured.

## Materials and Methods

### Plant Material

Two soybean cultivars, Noir 1 (PI 290136), Minsoy and an F1 sexual hybrid were used in these studies. Seeds were provided by R. Palmer (Iowa State University). Tissue cultures were derived from the root tissue of these plants (Roth et al. 1982). Tissue culture lines 2 and 5 were derived from the F1 line after treatment with CIPC (Roth and Lark 1984). Tissue culture cells were frozen and stored in liquid nitrogen, and later thawed according to the procedure of Weber et al. (1983).

Tissue culture lines were grown in Gamborg's B5 medium + 2g/liter casein (Gamborg et al. 1968). Callus was grown on "feeder plates" (Weber and Lark 1979). Minsoy, Noir 1 and the F1 hybrids were grown in pots in a greenhouse at temperatures of 21-27° C during the day and 10-15° C at night. Daylengths were 14 hours of light, 10 hours of darkness.

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** *Present address:* The Upjohn Company, Kalamazoo, Michigan, USA
*Offprint requests to:* H. Quemada

DNA Isolation

DNA from suspension cultures (figure 3), callus cultures (figure 4) and plant tissues (figure 2) was isolated by a modification of the procedure of Dellaporta et al. (1983). The extraction buffer for plant tissue and for suspension cultures contained 50 mM Tris pH 8.0, 100 mM EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, 1% (w/v) SDS, and 5 mM 1,10 phenanthroline. The extraction buffer for callus cultures contained, in addition, 1% polyvinylpyrrolidone (PVP-40) and 50 mg/ml Proteinase K. 10 mls of extraction buffer was added for every 1 gram of plant material. The material from suspension cultures and plant tissue was heated at 65°C for 20 minutes. Callus culture material was heated for 1 hour. Suspension culture and plant tissue DNA was further purified by centrifugation to equilibrium on CsCl gradients. The callus culture DNA was not purified on CsCl gradients, but was extracted with phenol and chloroform.

Enzymes and Chemicals

Restriction enzymes HpaII and MspI were obtained from Bethesda Research Laboratories (Gaithersberg Maryland). E coli DNA polymerase I for nick translations was purchased from Bethesda Research Laboratories or the New England Nuclear Company (Boston, Mass.). DNase I, Pancreatic RNase, agarose, 1,10 phenanthroline and PVP-40 were purchased from Sigma Chemical Company. Proteinase K was purchased from Boehringer Mannheim. Radioisotopes were purchased from New England Nuclear. M13 hybridization probe primer was purchased from Bethesda Research Laboratories.

Cloning

The probes used were 5S RNA clones isolated from Minsoy tissue culture DNA and cloned into a pUC9 vector or into an M-13 vector (Quemada 1986).

DNA Restriction Analysis and Southern Transfers

After digestion with restriction enzymes, DNA fragments were resolved by agarose gel electrophoresis through 1.2% agarose gels and transferred to a nitrocellulose membrane as described by Southern (1975). pUC9 hybridization probes (used in figures 2 and 3) were radioactively labeled with $^{32}$p dCTP by nick translation (Rigby et al. 1977). The M-13 hybridization probe (used in figure 4) was radioactively labeled with $^{32}$pdCTP according to the procedure of Messing (1983); see also BRL focus (1983).

Results:

Restriction of the tandem array of 5S genes in plant DNA with MspI (figure 2) generates a ladder of fragments varying in size by units of 330bp which only can result when one of the 2 CCGG sites in figure 1 is digested and the other is not. Restriction mapping of the digested site demonstrated that only the site in the coding region (see figure 1) was being cut. In contrast to MspI, HpaII did not digest the 5S genes from plant DNA. The lack of digestion by HpaII and the pattern of fragments generated by MspI (figure 2) was characteristic of 5S genes from plants of both cultivars, Minsoy and Noir, as well as their F1 hybrid (obtained by a sexual cross between these two parental plants). This was true of the 5S genes in different tissues: leaves, roots, embryos or root tips. In a control experiment in which lambda phage DNA was added to extracts of plant DNA material, the phage DNA was cut to completion by HpaII, indicating the absence of inhibitors of HpaII in the plant DNA extracts.

In contrast, 5S genes from tissue cultures recently prepared from these cultivars could be digested by HpaII to yield a ladder of fragments similar to that observed with MspI. Figure 3A shows examples of such digests,using DNA extracted from tissue cultures prepared from the Minsoy and hybrid cell line as well as from two tissue culture lines (2 and 5) derived form the hybrid line.

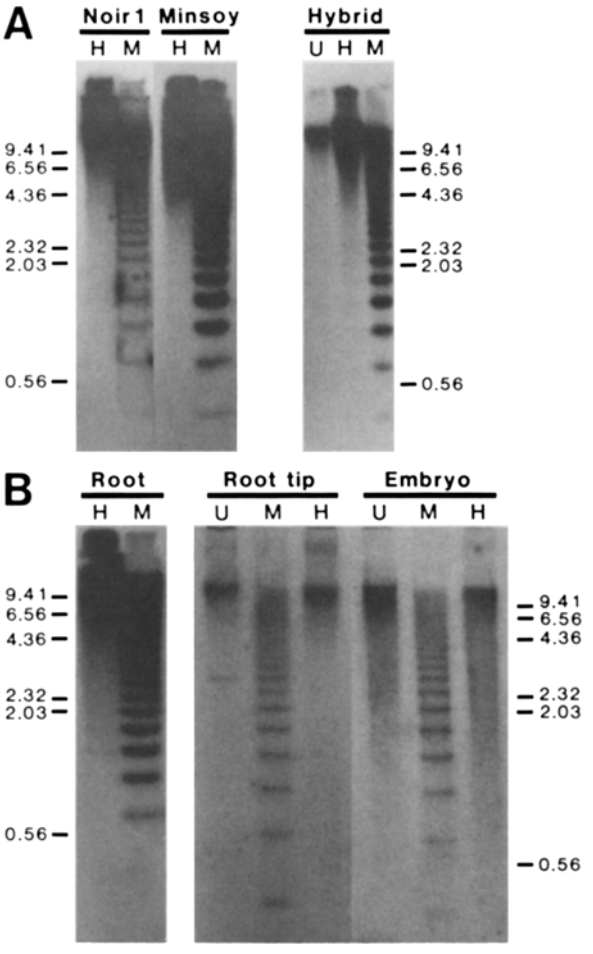


Fig. 2. HpaII and MspI digestion of 5S RNA genes, using DNA isolated from plant tissue.

A) DNA was prepared from leaves of Noir, Minsoy and a hybrid plant (Noir x Minsoy) and digested with HpaII (H) and MspI (M). DNA which has not been digested (uncut (U)) is also shown. Molecular weight standards (in kb) are indicated.

B) DNA was prepared from different tissues (root, root tip, and embryo) of Minsoy plants and digested with HpaII (H), MspI (M) or not digested (i.e., uncut (U)). Molecular weight standards (in kb) are shown.

Similar results were obtained for a number of other tissue culture lines examined under similar conditions (Quemada 1986). In each case HpaII digested the DNA to produce a ladder of fragments similar to those observed after MspI digestion.

The HpaII digests also contained a small proportion of fragments of a different size which suggests that digestion at an additional site had occurred. The size of this fragment does not correspond to a fragment generated by digestion at the second Msp/Hpa site, nor is it generated by MspI. This suggests that the additional site represents a polymorphism of the 5S gene in which an additional site occurs which is particularly sensitive to HpaII. DNA from plants is not digested by HpaII, so we conclude that in the plant this additional site is not susceptible to the enzyme.

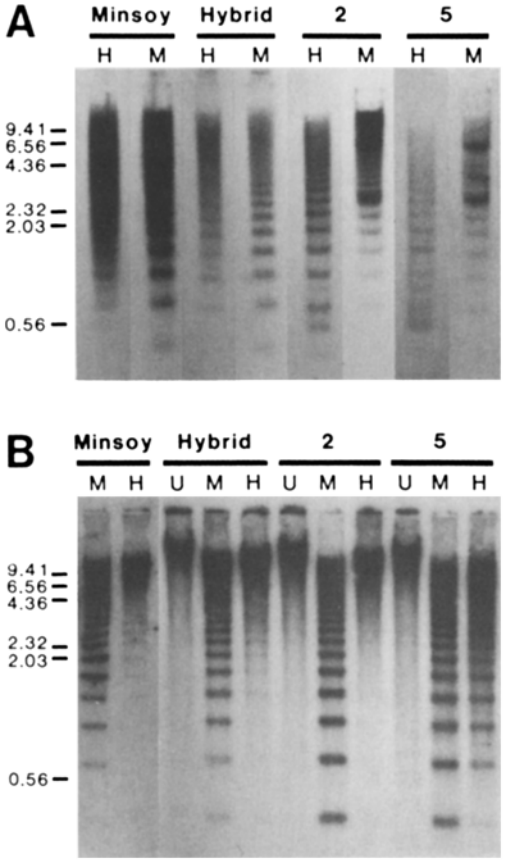


Fig. 3. HpaII and MspI digestion of 5S RNA genes in DNA extracted from soybean suspension cultures.

A) DNA extracted 8 months after suspension cultures were prepared from plant tissue.

B) DNA extracted from suspension cultures after more than 4 years of continuous growth in culture.

Digests of DNA from Minsoy, hybrid and two cell line cultures, 2 and 5 derived from the hybrid line (see materials and methods section) are shown. H = HpaII, M = MspI, U = no enzyme (uncut). Molecular weight standards are in kb.

The preparations shown in figure 3A were examined 8 months after being placed in culture. After a total period of 4 yrs, corresponding to ca. 1300 generations of growth, these tissue cultures were again examined. The results of digesting DNA from these cultures with HpaII and MspI are shown in figure 3B. Digestion by HpaII was reduced in some of the tissue culture lines. This is particularly clear in line 2 and can be seen in the Minsoy line as well. The original hybrid line shows reduced HpaII digestion whereas line 5 remains susceptible to HpaII digestion.

During the 4 year period between the early and late tissue culture samples (figures 3A and B), samples of cells had been frozen and stored in liquid nitrogen (Weber et al. 1983). Samples of line 2 were thawed, grown as callus and DNA prepared. These DNA samples represent stages in the growth of the cultures during which susceptibility to HpaII was lost. Figure 4 presents the pattern of restriction fragments obtained after digesting these with MspI and HpaII. It can be seen that with increasing time of culture the ladder of fragments disappear and the DNA becomes progressively more difficult to digest with HpaII.

Discussion:

The majority of CCGG sequences in the soybean 5S genes cannot be hydrolyzed by the enzyme MspI or HpaII. However a minor fraction of these sequences can be cut by MspI resulting in a "ladder" of fragment size separated by increments of 330bp. In DNA from plants, these sites could not be cut by HpaII. This was true for DNA from different plants and different tissues. In contrast, DNA from recently cultured tissue was hydrolyzed by HpaII to a similar extent as by MspI.

This was true for all culture lines, and included DNA prepared from suspension cultures as well as DNA prepared from callus obtained from cells preserved by freezing. We believe that these differences in restriction patterns are the result of differences in methylation patterns, since DNA preparation showed the same patterns regardless of the method of preparation or whether tissue was in the form of callus or was in suspension. Moreover, control experiments demonstrated that other DNA was completely hydrolyzed under these conditions. Presumably the DNA from tissue culture lacked methylation (CCGG) found in DNA from intact plants, (CmeCGG). Two explanations for this observation may be proposed: a) the conditions (hormone treatment etc.) for culturing cells prevents methylation of these sites, resulting in susceptibility to HpaII or b) a small proportion of cells, within the intact plant, lack these methyl groups and these cells are selected during cell culture. No data has been obtained to distinguish between these possibilities.

Upon prolonged culture, some cell lines again can become resistant to HpaII suggesting remethylation of the internal cytosine of the CCGG tetra nucleotide. This process appears to be infrequent, since after 3.5 years, only one cell line (#2) was completely resistant to HpaII whereas another cell line (#5) was almost as susceptible to digestion as when it was initially isolated. Other cell lines (Quemada 1986) had become partially, but not completly resistant to this enzyme.

These differences may be due to differences in the genetic background of these different culture lines, since (for example) lines #2 and #5 previously had been treated to induce chromosome loss (Roth and Lark 1984). Despite this reservation, the results suggest that at the population level CmeCGG sites replace CCGG sites slowly, either by remethylation, or by unequal crossing over in which genes with CmeCGG replace those with CCGG. We do not know whether cells with CmeCGG are selectively at an advantage or at a disadvantage in cell culture.

Previous studies on plants in which T-DNA expression is suppressed (Amasino et al. 1984) demonstrated that treatment with 5-azacytidine to reduce methylation altered the expression of T-DNA in these plants and allowed tumorous growth. Earlier experiments had shown similar effects of 5-azacytidine in tissue culture (Hepburn et al. 1983).

In our studies we have observed changes which suggest that a reduction in methylation can accompany the culturing of plant cells. Our observation may be relevant to the habituation of tobacco pith cells described by Meins and Lutz (1980). Methylation presents one mechanism which could explain habituation. Lack of methylation could activate genetic material resulting in the ability of cells to be induced to grow without hormones in cell culture. Upon regeneration in plants, remethylation could again inactivate the genes involved.

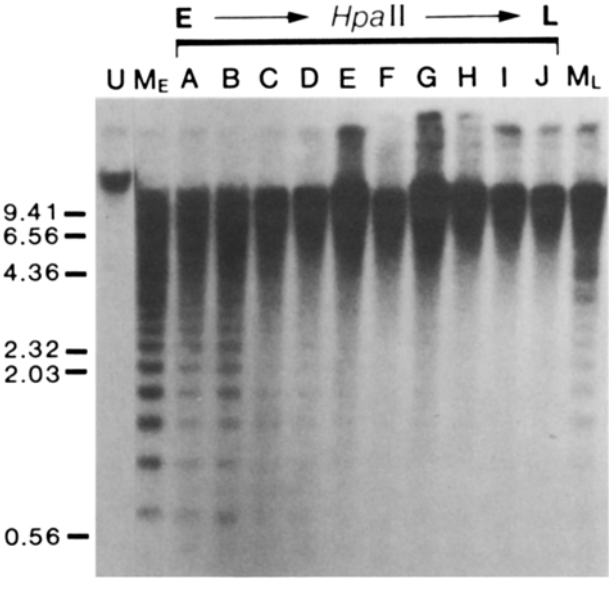


Fig. 4. HpaII digestion of samples from tissue culture line #2 frozen and stored after different periods of continuous growth. DNA was isolated from callus cultures prepared from thawed cells.

U, uncut DNA. ME - MspI digest of an early (8 month) soybean; ML - Msp digest of a late (4 yr) sample. HpaII digests of early (E) to late (L) samples are arranged from left to right. A, 8 mo, B, 14 mo, C, 19 mo, D, 26 mo, E, 33 mo, F, 39 mo, G, 46 mo, H, 46 mo, I, 52 mo, J, 52 mo. Samples A,B C D E, G & I were grown in B5C medium. Samples F H & J were grown in 5BC supplemented with 100 mg/L sequestrene. Molecular weight standards in kb are shown.

The recently developed ability to routinely regenerate soybean plants from cultured immature embryos (Lazzeri et al. 1985; Ranch et al. 1985) presents the opportunity to investigate the level of methylation of the 5S genes and other sequences during the regeneration process in soybean.

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