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Evidence for tissue-specific cytosine-methylation of plastid DNA from *Zea mays*

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Summary. Total DNA isolated from leaves, etiolated seedlings, roots, endosperm or embryos of *Zea mays* was digested separately with each of the restriction enzymes HpaII, MspI and HhaI, and the resulting fragment patterns, which were specific for the plastid rRNA operon, were analyzed by Southern hybridization. While most of the fragment patterns were consistent with previously established physical maps, the partial resistance shown by one HpaII site and one HhaI site, both of which reside in the 16S/23S rDNA spacer region, was observed in DNA isolated from embryo, root tissue and endosperm. The partially resistant HpaII site was susceptible to cleavage with restriction enzyme MspI. From this and from the known inhibition of restriction enzyme HhaI at methylated HhaI sites, we conclude that the partial resistance of the two sites is caused by C-specific methylation of plastid DNA in the respective tissues. The tissue specificity of this DNA methylation is likely to reflect a differential expression of plastome encoded genes.

Key words: DNA methylation – Plastid DNA – *Zea mays* – rRNA operon – Tissue specificity

Introduction

DNA methylation has been studied extensively in both prokaryotic and eukaryotic systems (for reviews see Doerfler 1983; Trautner 1984; Taylor 1984; Razin et al. 1984). In bacteria its main function is thought to be that of providing protection against restriction endonucleases (Arber 1974); in eukaryotic cells, gene inactivation (Bird 1986).

The methylation of organelle DNA, on the other hand, has for several years appeared to be restricted to chloroplast DNA from the algae *Chlamydomonas*, and it had been proposed that a restriction/methylation system was the basis of maternal inheritance (for reviews see Dyer 1982; Sager et al. 1984). Only recently has the methylation of DNA from non-photosynthetic plastids of cell lines of a higher plant, sycamore, been reported (Ngernprasirtsiri et al. 1988a). Differential methylation patterns have also been observed in DNA isolated from the plastids of tomato leaves and fruit (Ngernprasirtsiri et al. 1988b).

We had also initiated a search for the existence of methylated sites in the plastid DNA of higher plants by systematically screening regions of the plastid rRNA operon of *Zea mays* that contains CCGG and CGCG sites resistant to cleavage by the restriction endonucleases HpaII, MspI and HhaI. The data presented in this publication indicate that no such resistant sites could be detected in DNA isolated from green leaves and etiolated seedlings. We did observe, however, two separate sites that were partially resistant to either HpaII or HhaI digestion in plastid DNA isolated from embryo, root and endosperm tissues. From this we conclude that (1) a DNA methylation system also exists for plastid DNA of higher plants by which distinct C-positions can be methylated in vivo and (2) this methylation is specific for DNA of non-photosynthetic plastids and therefore may function as a mechanism involved in the differential regulation of plastome-encoded genes.

Materials and methods

Restriction endonucleases, T4 polynucleotide kinase and DNA polymerase I were obtained from commercial sources (Boehringer Mannheim, Bethesda Research Laboratories, Amersham, Pharmacia). The enzyme reactions were carried out as recommended by the suppliers.

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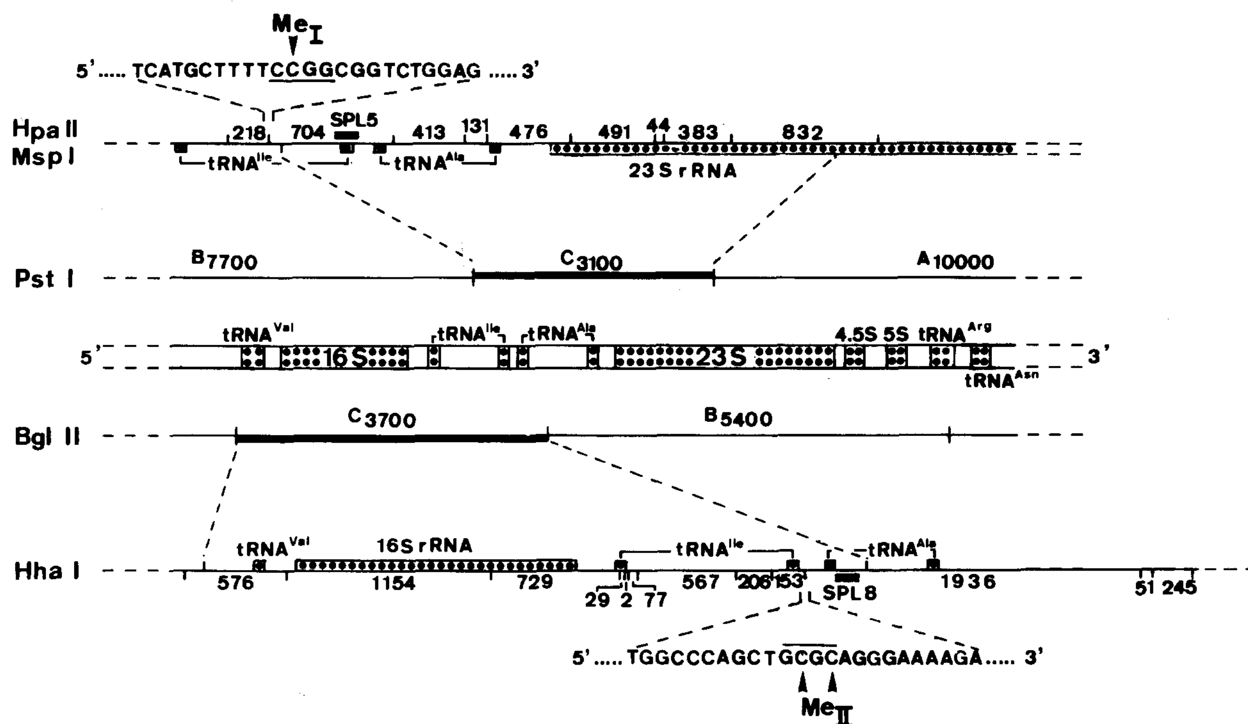


Fig. 1. Physical map and position of DNA probes used for screening methylated sites within the rRNA operon of *Zea mays* chloroplasts. The *middle part* shows the relative positions of the rRNA and tRNA genes of the operon as analyzed previously (Schwarz and Kössel 1980; Schwarz et al. 1981; Koch et al. 1981; Edwards and Kössel 1981) and the positions of fragments BglII · $C_{3,700}$ and PstI · $C_{3,100}$, which were used as hybridization probes. The *top line* shows the HpaII/MspI fragments covered by the PstI probe and the position of the partially resistant HpaII site (Me_I) together with its flanking sequences. The *numbering* refers to the lengths of the HpaII/MspI fragments in bp. The *bottom line* shows the HhaI fragments included in the BglII probe and the position of the partially resistant HhaI site (Me_{II}), again with flanking sequences. The regions complementary to oligodeoxynucleotide probes SPL5 and SPL8 are marked by *solid bars*

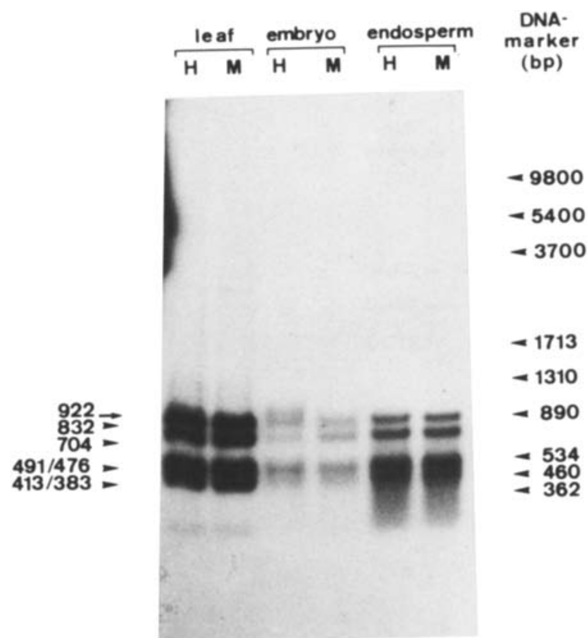
[γ-^{32}P]-ATP (> 5,000 Ci/mmol) for the 5'-terminal labelling of oligodeoxynucleotides and [α-^{32}P]-dGTP (3,000 Ci/mmol) for the internal labelling of restriction fragments BglII · $C_{3,700}$ and PstI · $C_{3,100}$ and the labelling of pBR329 DNA by nick translation (Rigby et al. 1977) or random priming (Feinberg and Vogelstein 1983) were purchased from Amersham (UK). The oligodeoxynucleotides SPL5 (TGGGCCATCCTGGACTTGAACCAGAGACCTC-GCCCG) and SPL8 (ATCATTACCGCCTGGACAATTAGAC) were synthesized in our laboratory by Dr. G. Igloi with an automated DNA-synthesizer from Applied Biosystems. The oligodeoxynucleotides are complementary to positions 1287 to 1322 (SPL5) and 1452 to 1476 (SPL8) of the spacer rDNA from *Zea mays* chloroplasts (Koch et al. 1981) and are, therefore, specific probes for fragments $HpaII_{704}$ and $HhaI_{1,936}$, which are derived from this region (see Fig. 1). The *E. coli* clone pZmc134, which harbours a plasmid containing one complete maize chloroplast rRNA operon (Schwarz and Kössel 1980; Schwarz et al. 1981; Edwards and Kössel 1981) was used for preparing fragments BglII · $C_{3,700}$ and PstI · $C_{3,100}$. DNA size-markers were obtained by digesting plasmid pZmc134 DNA with enzyme BglII to yield large size-markers of about 9,800, 5,400 and 3,700 bp (the latter being identical to fragment BglII · $C_{3,700}$) and by digesting λdv1DNA with enzyme HaeIII to yield the fragment series below 1,713 bp (Lusky and Hobom 1979).

Tissue-specific nucleic acid containing DNA and RNA from *Zea mays* was prepared as described by Dellaporta et al. (1983). Green leaves and roots were harvested from 10-day-old seedlings grown under a 12 h light/12 h dark regime. Etiolated leaves were obtained from 10-day-old seedlings grown in the dark. Embryo and endosperm tissue was from maize kernels soaked in water for 2 h. About 1 mg of nucleic acid fraction was obtained from 13 g green leaves, 7.5 g etiolated seedlings or 15 g roots. For the preparation of about 1 mg nucleic acid fraction from embryo and endosperm, 35 and 150 kernels, respectively, were taken and dissected manually into embryo and endosperm. No attempts were made to separate the nucleic acid fractions thus obtained into DNA and RNA or into nuclear and organellar fractions.

The DNA fragments were digested with restriction enzymes, separated on agarose gels and blotted onto nitrocellulose or nylon (hybond N) membranes according to standard procedures (Southern 1975; Maniatis et al. 1982). The hybridization procedure was performed under stringent conditions at 65°C and in 3 × SSPE (30 m*M* NaH_2PO_4, 450 m*M* NaCl, 3 m*M* EDTA, pH 7.4) when using nitrocellulose or in 6 × SSC (900 m*M* NaCl, 90 m*M* Na-citrate) when using nylon membranes.

Results

The restriction fragments PstI · $C_{3,100}$ and BglII · $C_{3,700}$ were used as specific probes for hybridization with the HpaII/MspI and HhaI fragments, respectively, of the rRNA operon of *Zea mays* chloroplast (Fig. 1). The physical maps of these HpaII/MspI and HhaI fragments (top and bottom line of Fig. 1) were obtained from published sequences of this operon (Schwarz



and Kössel 1980; Koch et al. 1981; Schwarz et al. 1981; Edwards and Kössel 1981). When PstI $C_{3,100}$ was used to probe DNA isolated from leaf and endosperm tissue, the resulting restriction pattern of the HpaII/MspI bands was consistent with this physical map (Fig. 2) and there were no differences in the patterns obtained with the two isoschizomeric enzymes. How-

Fig. 2. Southern blot analysis of fragments obtained from total maize DNA of leaves, embryos and endosperm tissue after digestion with restriction enzymes HpaII (*lanes H*) or MspI (*lanes M*) and subsequent probing with radioactive fragment PstI · $C_{3,100}$ (see Fig. 1). The fragments were separated by electrophoresis in 1% agarose gel. The positions and sizes of the marker DNAs are given (in bp) along the *right-hand side.* Strong hybridization was observed with fragments BglII · $B_{5,400}$ and BglII · $C_{3,700}$ (see Fig. 1), which were included in the marker series (not shown). The positions of the expected major HpaII/MspI fragments overlapping with the probe DNA are given alongside the *left lane.* The position of the 922 bp fragment resulting from embryo-specific resistance of the HpaII site between the neighbouring fragments of 218 and 704 bp is indicated by an *arrow*

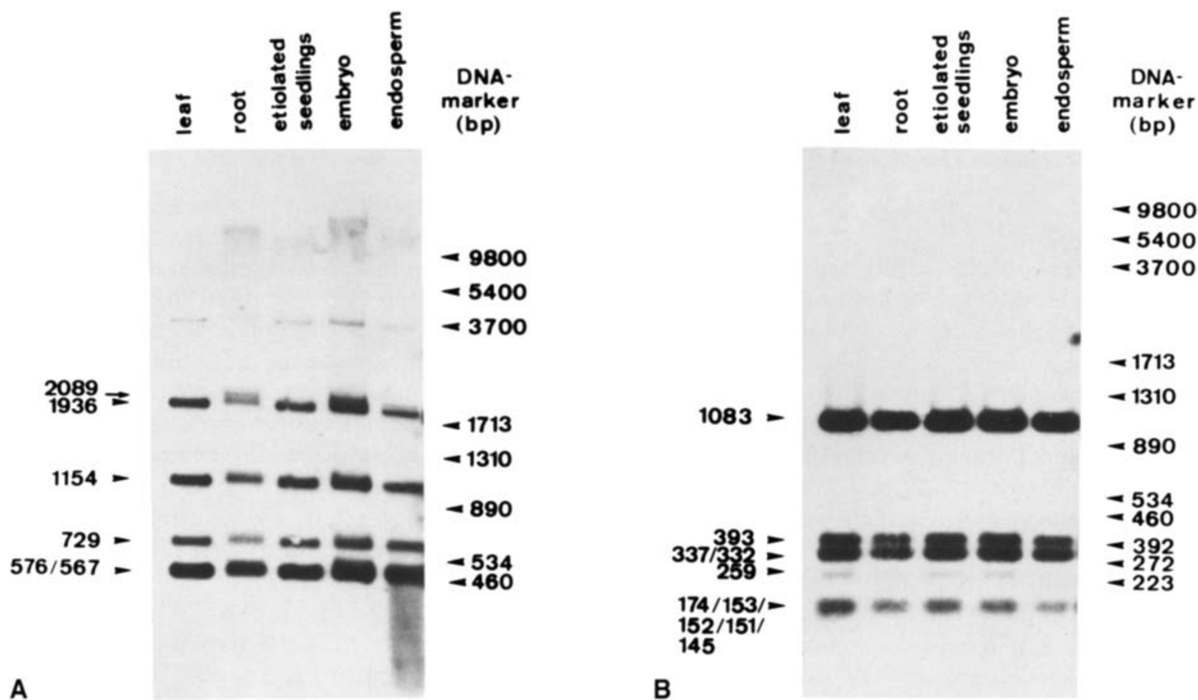


Fig. 3. **A** Southern blot analysis of fragments obtained from total maize DNA of leaves, roots, etiolated seedlings, embryo and endosperm tissue after digestion with restriction enzyme HhaI and probing with radioactive fragment BglII · $C_{3,700}$ (see Fig. 1). Fragments were separated by electrophoresis in 1.5% agarose gel. Positions and sizes of the expected major HhaI fragments overlapping with the probe DNA are given in bp along the *left-hand side.* The position of the 2,189 bp fragment resulting from root, embryo and endosperm specific resistance of the HhaI site between the neighbouring fragments of 153 and 1,936 bp is marked by an *arrow.* **B** Hybridization of the same fragment mixtures as in **A** but with radioactive pBR329 DNA as the probe. The positions and sizes of the major HhaI fragments derived from pBR329 DNA (Covarrubias and Bolivar 1982) are given on the *left.* The position and sizes of the markers (in bp) are given alongside the rightmost lanes of both parts (**A** and **B**) of the figure. It should be noted that the electrophoresis conditions were not identical for the two blots, as is evident from the differences in the marker positions

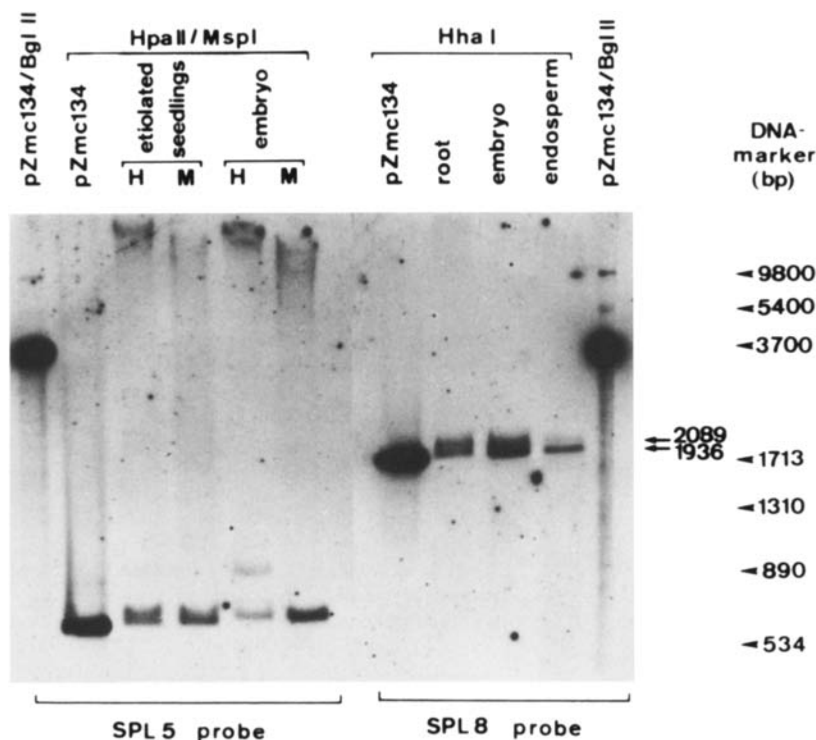


Fig. 4. Southern blot analysis of fragments obtained from total maize DNA isolated from roots, etiolated seedlings, embryo and endosperm tissue after digestion with restriction enzymes HpaII (*H*) or MspI (*M*), and HhaI with the radioactive oligodeoxynucleotides SPL5 and SPL8, respectively, as probes. The fragments were separated by electrophoresis in 1.5% agarose gels. Hybridizations of fragments obtained from plasmid pZmc134 DNA after digestion with the enzymes BglII and HpaII are shown as controls. The positions and sizes of the marker fragments are given along the *righthand side*; the positions and expected sizes (see Fig. 1) of the hybridizing fragments are indicated by *arrows*. As in the previous figure, fragments $HpaII_{922}$ and $HhaI_{2,089}$ result from resistance of the respective restriction sites caused by methylation

ever, when the same probe hybridized with DNA from embryo tissue, a new fragment positioned closely above the 832 bp fragment was observed among the HpaII fragments together with a concomitant weakening of the 704 bp fragment. This band was absent from the MspI digest. The position of this embryo-specific HpaII fragment corresponded to a fragment having a length of 922 bp, which is the sum of the 704 bp fragment and its neighbouring 218 bp fragment (see Fig. 1, top line). From this, we conclude that in the plastid DNA of maize embryo tissue the HpaII site separating these two fragments is partially resistant due to a modification in the second C residue of the CCGG sequence (Position Me_I of Fig. 1, top line). The complete cleavage of this site by the enzyme MspI excludes the possibility of a restriction fragment polymorphism caused by a mutation in the HpaII site.

A similar screening with tissue-specific DNA fragmented with the enzyme HhaI and then probed with fragment $BglII \cdot C_{3,700}$ yielded the patterns shown in Fig. 3A. Using DNA obtained from leaves and etiolated seedlings, we obtained a fragment pattern identical with that of the known physical map of the respective HhaI fragments (see Fig. 1, bottom line). The DNA from root, embryo and endosperm tissues, however, contained a separate fragment that migrated slightly slower than the longest HhaI fragment (1,936 bp). Its position was consistent with that of a fragment having a length of 2,089 bp, which again corresponds to the sum of the two adjacent HhaI fragments of 1,936 and 153 bp. In order to exclude the possibility that this fragment resulted from an incomplete digest, pBR329 DNA was mixed with maize DNA samples prior to digestion with HhaI and then probed for completion of the digests. As shown in Fig. 3B, only those fragments expected from a complete digest of pBR329 DNA were observed when radioactive pBR329 DNA was used as the probe. From this we conclude that the partial resistance shown by the HhaI site separating the 1,936 and 153 bp fragments is caused by an intrinsic propery of the DNA such as DNA modification, rather than by insufficient enzyme activity. However, we are unable to explain the weak bands observed in the 3,700 bp region of Fig. 3A, which show about equal relative strength in all tissues.

The two sites were further characterized using the oligodeoxynucleotides SPL5 and SPL8 (see Fig. 1) as more specific hybridization probes and (in the case of the HpaII site) again by digestion with the isoschizomeric enzyme MspI. As shown in Fig. 4, both the relative positions and the tissue specificity of the two resistant sites could be confirmed: selective hybridization of the 704 bp and 922 bp HpaII/MspI bands was observed with the SPL5 probe, while selective hybridization of the 1,936 bp and 2,089 bp HhaI bands was obtained with the SPL8 probe. Again no 922 bp band was observed when enzyme MspI was used instead of HpaII. The specificity of the two oligodeoxynucleotides used as hybridization probes also excluded the possibility that the two partially resistant sites reside in other positions of the rRNA operon covered by the large fragments PstI · $C_{3,100}$ and BglII · $C_{3,700}$, which were our original hybridization probes.

The two sites thus identified are at positions 790 (HpaII) and 1,326 (HhaI) of the rDNA spacer region of the maize plastome (Koch et al. 1981). Whereas the HpaII site resides within the $tDNA^{Ile}$ intron region (see Fig. 1 top line), the HhaI site is directly adjacent to the right $tDNA^{Ile}$ exon within the $tDNA^{Ile}/tDNA^{Ala}$ intergenic region (see Fig. 1 bottom line).

Discussion

In view of the known inhibition of restriction enzymes HpaII and HhaI at methylated recognition sites (Kessler and Höltke 1986), it is reasonable to conclude that the partial resistance that we observed for single HpaII and HhaI sites within the rDNA spacer region of maize chloroplasts is caused by base modification. Since C- (and A-)methylations are the only naturally occurring DNA modifications known, it is also likely that the modifications causing the observed resistance are C-methylations (adenine residues are not present in the two recognition sites). Other types of modifications cannot, however, be strictly excluded. Therefore, the direct identification of modified bases in the DNA of non-photosynthetic plastids from maize by a careful analysis of the base composition is desirable.

If we assume a C-methylation, the methylated C-position can unambiguously be assigned to the second C of the HpaII site (methylation of the first C would render the site resistant to MspI and susceptible to HpaII), whereas the resistance of the HhaI site may be caused by methylation of either one of the C-positions. No conclusion can be drawn as to whether only one or both DNA strands are modified.

The sequences surrounding the identified modification sites (see Fig. 1) show no similarities to each other. They do, however, conain the C-G and C-X-G sequences that are preferential methylation sites in the nuclear DNA of higher plants (Gruenbaum et al. 1981). This characteristic and the known transfer of plastid DNA sequences to the nuclear genome (Timmis and Scott 1984) suggests the possibility that the DNA carrying the resistant sites represents a portion of the plastid rRNA operon residing in the nucleus, where it has been modified by the nuclear methylation system. We believe this possiblity to be highly unlikely for the following reasons:

1) The number of plastome sequences located in the nucleus is too low to account for the observed hybridization signals. The existence of ten plastome equivalents (approximately 1.4×10^6 bp) in the nuclear DNA of maize (about 10^{10} bp/diploid genome, Petersen et al. 1980) would account for no more than 0.014% of the total DNA, whereas the ratio of true plastid DNA to nuclear DNA within the total DNA can vary between 1% and 23% depending on the tissue (Bendich 1987; Lawrence and Possingham 1985). Thus, even the minimum value of this proportion renders the true plastid DNA in a 60- to 70-fold excess over the plastid DNA residing in the nucleus. Therefore, if all the cpDNA sites transferred to the nucleus were methylated, a maximum of 1%–2% resistance for each site could be expected. In the case of a more realistic 30% of nuclear DNA methylation, this value further decreases to about 0.3%–0.6%, which is far below the 10%–30% resistance level evident from Fig. 4.

2) It is difficult to imagine a function for the spacer rDNA sequences from chloroplasts that are found within the nuclear genome. Consequently, in the absence of a functional constraint, rapid divergence would be expected, and this should lead to apparent restriction fragment length polymorphisms. This is not the case: the fragment patterns observed in Figs. 2–4 correspond to the patterns predicted from corresponding chloroplast DNA sequences (with the exception of the bands indicating partial resistance).

The latter argument is weakened to some extent by the observation that sequences transferred from the maize plastome to the mitochondrial genome (Stern and Lonsdale 1982; Fejes et al. 1988) show strong sequence conservation in the absence of any recognizable functional constraint. This observation also suggests the remote possibility that the partial resistant sites observed in this study reside in plastid DNA sequences that have been transferred to the mitochondrial genome and modified by an hitherto unknown mitochondrial methylation system. However, the border of the 12 kb fragment (containing the 16S rDNA and part of the spacer rDNA, including the left exon of the split $tRNA^{Ala}$ gene; see Fig. 1) transferred from the plasto-

me to the mitochondrial genomes lies near the left end of this fragment (within the tDNA^{Ala} intron). Therefore, it would be expected that the 1,936 bp HhaI resistant fragment would have a different length. The existence of the 1,936 bp HhaI fragment as part of the 2,089 bp fragment carrying the resistant HhaI site excludes this possibility.

When the functions of a plastome-specific methylation are considered, several possibilities can be envisaged. A plastid restriction/methylation system – as has been postulated as a basis for maternal inheritance in *Chlamydomonas* gametes (Sager et al. 1984; Bolen et al. 1982; Dyer 1982) – appears unlikely in view of the many unmethylated HpaII and HhaI sites observed in this work and in the absence of a consensus sequence between the two modified sites. Other possible functions of plastome-specific methylation might be the differential expression of plastome-encoded genes, which would be analogous to the situation of nuclear genes, and/or a role in the replication of plastid DNA. The specificity for DNA methylation in non-photosynthetic plastids that we observed (DNA from embryo, endosperm and root tissue) is highly suggestive of a function in differential expression; this has just recently also been suggested for the DNA methylation of non-photosynthetic plastids from sycamore cell lines and tomatoes (Ngernprasirtsiri et al. 1988a, b). In contrast to the latter systems in which no methylation was detectable in the 16S rDNA region, the present work indicates that at least the spacer rDNA flanking the 16S rRNA gene does contain methylation sites.

With respect to the possible function of the DNA methylation sites observed in this work, it is interesting that an origin of replication has recently been located in the spacer rDNA of pea chloroplasts (Meeker et al. 1988) that correlates to both methylation sites we observed in the maize plastome. This coincidence is suggestive of a role of the methylation status in signaling plastid DNA replication.

As has already mentioned, the sequence of the two methylation sites identified in this work are consistent with the nuclear methylation sites CG and CXG (Gruenbaum et al. 1981). It is, therefore, tempting to speculate that the methylase(s) catalyzing plastid DNA methylation is a nuclear-encoded enzyme which, after being synthesized on cytosolic ribosomes, is differentially targeted to the nucleus or to non-photosynthetic plastids. This possibility would also indicate a new type of communication pathway between the nuclear genome and the plastome of higher plants.

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