CAN PARTIAL METHYLATION EXPLAIN THE COMPLEX FRAGMENT PATTERNS OBSERVED WHEN PLANT MITOCHONDRIAL DNA IS CLEAVED WITH RESTRICTION ENDONUCLEASES?

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1. Introduction

It is widely accepted (e.g. [1,2]) that the mitochondrial DNA (mtDNA) of higher plants consists of a uniform population of 30 μm circular molecules, of mol. wt $\sim 70 \times 10^6$ [3,4]. However, restriction endonuclease patterns of plant mtDNA are considerably more complex than expected for a homogeneous population of this size [5–7]. In contrast, the simpler restriction patterns of chloroplast DNA [8] are compatible with the presence of a homogeneous population of molecules [9], even though studies [10–12] of higher plant chloroplast DNA have suggested that it is physically larger and kinetically more complex than the mtDNA of the same organisms (cf. [4]).

To rationalize this apparent discrepancy, it has been proposed [5] that plant mtDNA may actually be heterogeneous, consisting of several types of physically indistinguishable molecules having different sequence arrangements of the same genetic information. Alternatively, an unexpectedly complex restriction pattern could result from failure of a restriction endonuclease to cleave all potential sites in what appears to be a homogeneous DNA population. This could occur if each restriction site existed in either modified (e.g., methylated) or unmodified versions in different mtDNA molecules, with only the unmodified site being susceptible to cleavage by the endonuclease in question.

To test this latter possibility, we have examined the distribution of 5-methyldeoxycytidine (m^5C) in wheat mitochondrial as well as nuclear DNA, using the isoschizomers *Msp* I and *Hpa* II. These endonucleases both cleave the sequence –C–C–G–G–, but only *Msp* I will also hydrolyze the methylated analogue, –C–m^5C–G–G–. These two enzymes can therefore distinguish between methylated and non-methylated DNA [13–17], since DNA which contains –C–m^5C–G–G– sequences will give a different restriction pattern with each of the enzymes, while identical patterns will be generated if the DNA contains –C–C–G–G– but not –C–m^5C–G–G–.

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2. Experimental

Mitochondrial and nuclear DNAs were prepared from the appropriate subcellular fractions of homogenates of viable wheat embryos (*Triticum vulgare* Vill. [*Triticum aestivum* L.] var. Thatcher) germinated for 24 h in the dark. Crude mitochondrial fractions were treated with DNase I before further purification on discontinuous sucrose gradients, after which DNA was isolated from the recovered mitochondria according to [11]. For DNA–RNA hybridization experiments, probe RNAs were extracted either from separated mitoribosomal subunits (mitochondrial 26 S and 18 S rRNAs) or from the post-mitochondrial supernatant of an embryo homogenate (cytosol 26 S + 18 S rRNA). Further details of these preparative procedures are given in [7], which also describes conditions for agarose gel electrophoresis of restriction endonuclease digests and for detection of rRNA genes by Southern hybridization [18]. Hydrolysis of DNA

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with the various restriction endonucleases was carried out according to the recommendations of the suppliers, Boehringer Mannheim (*Eco* RI), New England Biolabs (*Xho* I, *Sal* I, *Sma* I, *Msp* I), and Bethesda Res. Labs. (*Hpa* II).

3. Results

3.1. *High degree of methylation of –C–G– in wheat nuclear DNA*

In eukaryotic nuclear DNA (nDNA), including that of wheat, the sequence –C–G– occurs with a frequency significantly lower than that of any other dinucleotide sequence [19]. Moreover, methylation of C residues in eukaryotic nDNA, including wheat nDNA, is largely confined to this same sequence [20–22]. Thus, in view of its high overall content of m^5C (~25% of total C) [23], it seemed possible that wheat nDNA might be exceptionally resistant to hydrolysis by restriction endonucleases (like *Hpa* II) whose cleavage sites contain –C–G–. If so, wheat nDNA would provide a suitable control for verifying the specificities of *Hpa* II and *Msp* I, prior to experiments with wheat mtDNA.

As indicated by the restriction profiles shown in fig.1A, wheat nDNA did indeed prove to be highly resistant to hydrolysis by *Hpa* II (–C↓C–G–G–; track b), *Sal* I (–G↓T–C–G–A–C–; track g), and *Xho* I (–C↓T–C–G–A–G; track f). In contrast, wheat nDNA was readily hydrolysed by *Eco* RI (tracks h,i), whose cleavage site (–G↓A–A–T–T–C–) does not contain –C–G–, and by *Msp* I (tracks, c,d), whose activity is not blocked by the presence of $-m^5C-G-$ in the restriction site [13]. Densitometer tracings (fig.2) of these gel profiles further emphasized

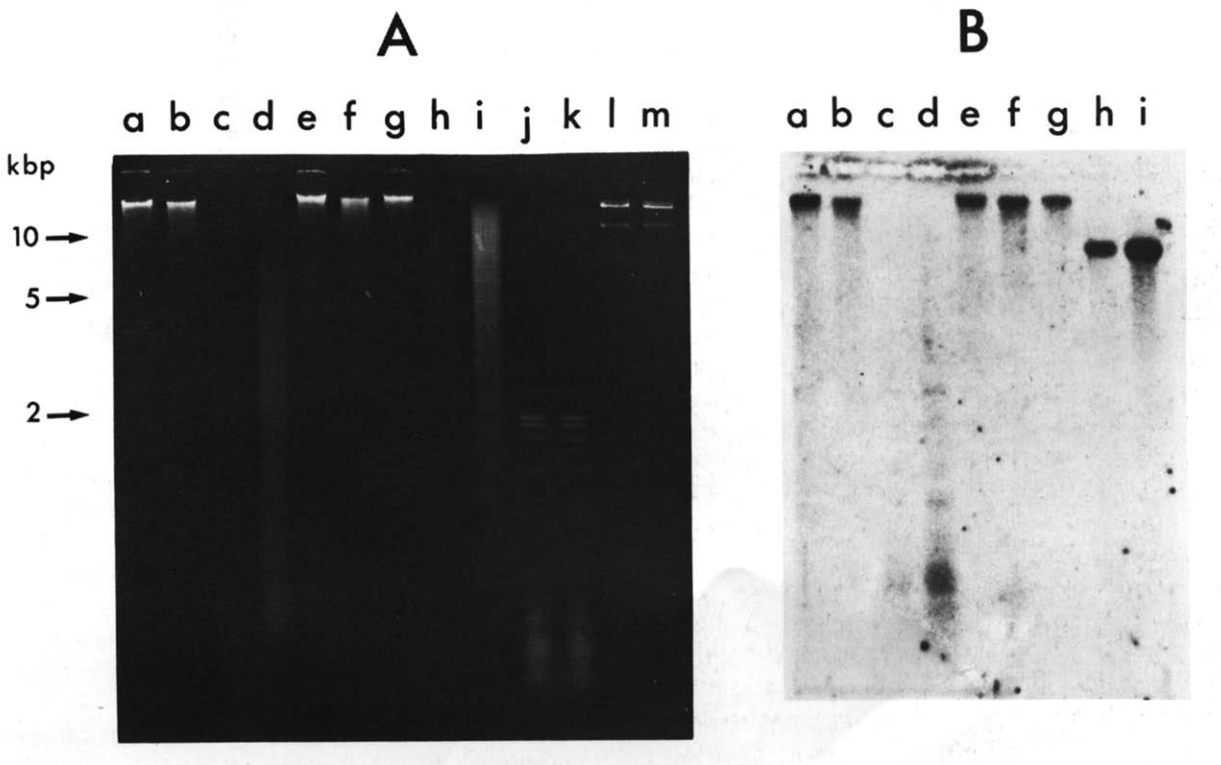

Fig.1. (A) Ultraviolet photograph showing the resolution of restriction endonuclease hydrolysis products of wheat nDNA (tracks a–i) and λ DNA (tracks j–m), after electrophoresis in a 1% agarose gel. DNA fragments were visualized by staining with ethidium bromide. The arrows indicate molecular size in kilobasepairs (kbp), as determined by reference to *Eco* RI restriction fragments of λ DNA. (a) nDNA, 1 μg, no enzyme; (b) nDNA, 1 μg, *Hpa* II; (c) nDNA, 1 μg, *Msp* I; (d) nDNA, 3 μg, *Msp* I; (e) nDNA, 1 μg, no enzyme; (f) nDNA, 1 μg, *Xho* I; (g) nDNA, 1 μg, *Sal* I; (h) nDNA, 1 μg, *Eco* RI; (i) nDNA, 3 μg, *Eco* RI; (j) λ DNA, *Hpa* II; (k) λ DNA, *Msp* I; (l) λ DNA, *Xho* I; (m) λ DNA, *Sal* I. (B) Autoradiogram of nitrocellulose-bound restriction fragments from (A) after hybridization with ^{32}P-labeled wheat cytosol 26 S + 18 S rRNA.

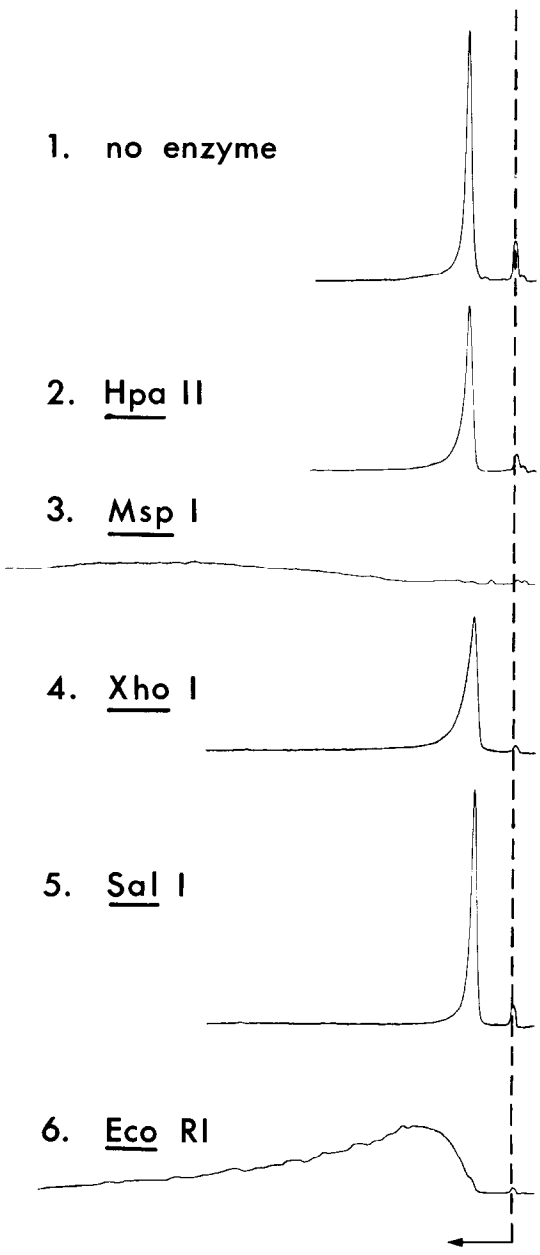

Fig.2. Densitometer tracings illustrating the mobility of wheat nDNA after treatment with *Hpa* II (2), *Msp* I (3), *Xho* I (4), *Sal* I (5), and *Eco* RI (6). The position of control (incubated, no enzyme) nDNA is shown in (1). The negatives of ultraviolet photographs of ethidium bromide-stained gels (fig.1A) were scanned with a microdensitometer (Joyce, Loebl and Co.). The vertical dashed line marks the position of the origin and the arrow indicates the direction of electrophoresis.

the very different susceptibilities of wheat nDNA to hydrolysis by *Hpa* II (track 2) and *Msp* I (track 3), as well as the almost total resistance of this DNA to cleavage by *Xho* I (track 4), and *Sal* I (track 5). Under the same hydrolysis conditions, λ DNA was cleaved as expected (fig.1A), not only by *Eco* RI (not shown) and *Msp* I (track k) but also by *Hpa* II (track j), *Xho* I (track l) and *Sal* I (track m). Moreover, the cleavage patterns of λ DNA with *Hpa* II and *Msp* I (tracks j,k) were identical.

Cleavage of wheat nDNA by *Eco* RI and *Msp* I but not by *Hpa* II, *Xho* I, or *Sal* I was further confirmed by Southern hybridization experiments, using a mixture of ^{32}P-labeled wheat cytosol 26 S and 18 S rRNAs as probe. As shown in fig.1B, the distribution of rRNA genes paralleled the distribution of DNA in the case of control nDNA (no enzyme; tracks a,e) or nDNA incubated with *Hpa* II (track b), *Xho* I (track f), or *Sal* I (track g). With *Eco* RI, however, rRNA genes were found in a single, narrowly-defined region of the gel profile, corresponding to (a) fragment(s) about 10 kilobasepairs in size. In the case of *Msp* I, hybridization was not as discrete, with the bulk of the labeling occurring rather diffusely in a region of relatively small DNA fragments (<500 basepairs).

Taken together, these results not only clearly demonstrate that the *Hpa* II and *Msp* I used in this study were indeed distinct enzymes having the expected specificities, but also suggest that the sequence –C–G– must be almost fully in the methylated form (–m^{5}C–G–) in wheat nDNA.

3.2. *Lack of methylated –C–C–G–G– sequences in wheat mitochondrial DNA*

In contrast to the nDNA, wheat mtDNA is readily hydrolyzed by *Sal* I and *Xho* I [7], suggesting that the organelle DNA is not highly methylated. However, since the restriction patterns generated by these two enzymes with wheat mtDNA are more complex than expected, it seemed possible that there could still be partial rather than complete methylation of various restriction sites.

To investigate this possibility, wheat mtDNA was incubated with *Msp* I and *Hpa* II. No differences, either qualitative or quantitative, could be detected in the resulting restriction patterns, either by visual inspection of the ultraviolet photographs of ethidium bromide-stained gels (fig.3B) or by examination of densitometer tracings made from the negatives of these photographs (fig.3A). Moreover, when separated mito-

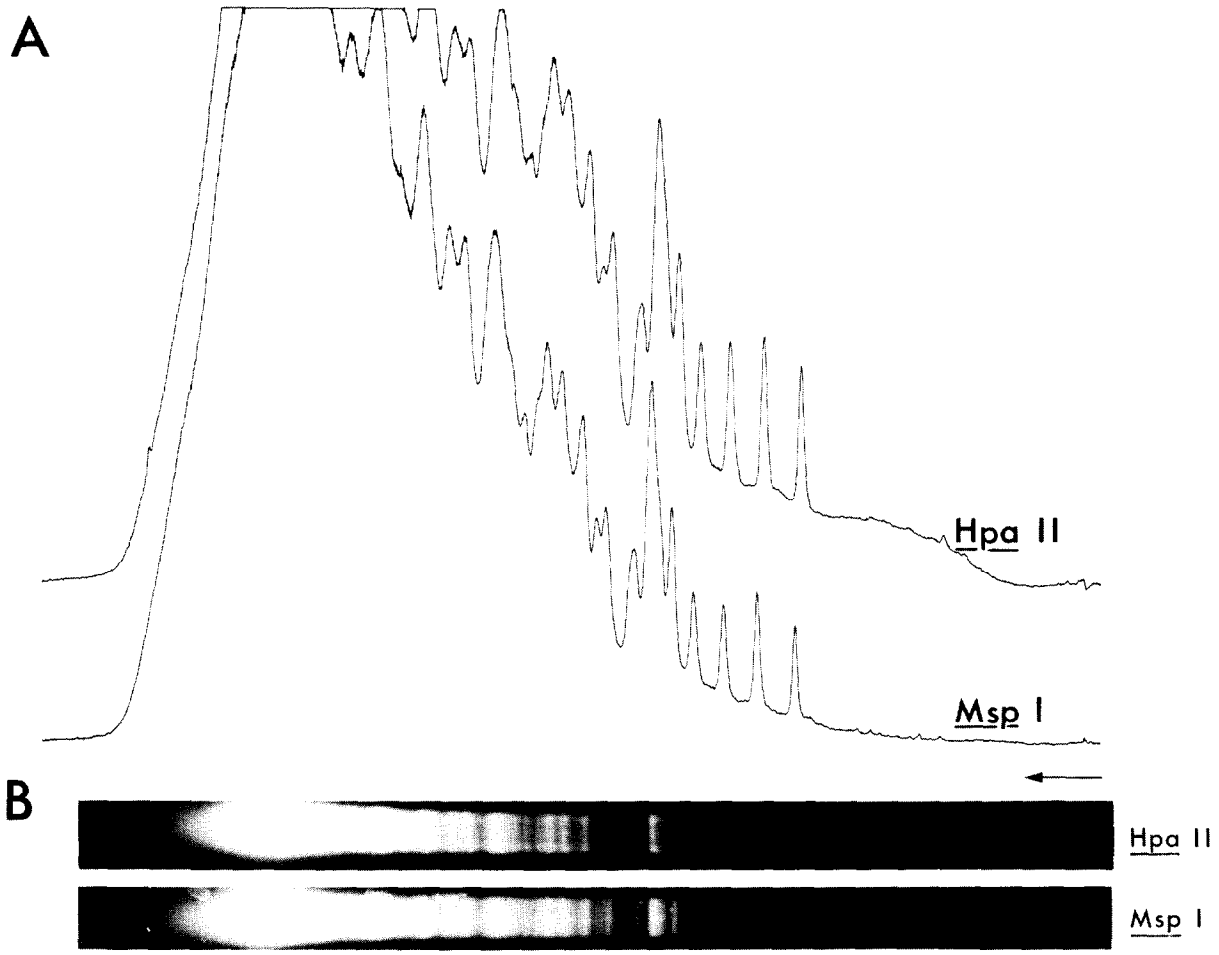

Fig.3. (A) Densitometer tracings showing the restriction profiles of wheat mtDNA after cleavage with *Hpa* II and *Msp* I. (B) Ultraviolet photographs of ethidium bromide-stained *Hpa* II and *Msp* I restriction fragments of wheat mtDNA, separated by electrophoresis in a 1% agarose gel. Electrophoresis was carried out in the direction indicated by the arrow until the bromophenol blue marker had migrated 8 cm. The negatives of these photographs were used to produce the densitometer scans in (A) as in fig.2.

chondrial rRNAs (26 S and 18 S) were used as probes in Southern hybridization experiments, the resulting labeling patterns were identical for the two enzymes (fig.4). As observed for wheat mtDNA hydrolyzed by other restriction endonucleases [7], mitochondrial 26 S and 18 S rRNAs each hybridized to several restriction fragments in each digest, with the fragments labeled by 26 S r[^{32}P]RNA being different from those labeled by 18 S r[^{32}P]RNA.

These results indicate that wheat mtDNA contains exclusively –C–C–G–G– rather than –C–m^{5}C–G–G–, a conclusion also reached in [24] for mtDNAs from yeast, *Neurospora*, rat and calf. However, these observations alone do not exclude the possibility that other methylated nucleosides, such as m^{6}A (N^6-methyldeoxyadenosine), may be contributing to the observed complexity of *Eco* RI, *Xho* I, and *Sal* I digests of wheat mtDNA. If this is so, one would expect that enzymes whose recognition sites contain only C and G would generate less complex fragment patterns, with a lower additive molecular weight, than enzymes (such as *Eco* RI, *Xho* I, *Sal* I) whose cleavage sites contain A and T as well as C and G. That this is not the case is indicated by the data in fig.5, which shows the restriction pattern of wheat mtDNA hydrolyzed to completion with *Sma* I, whose cleavage site

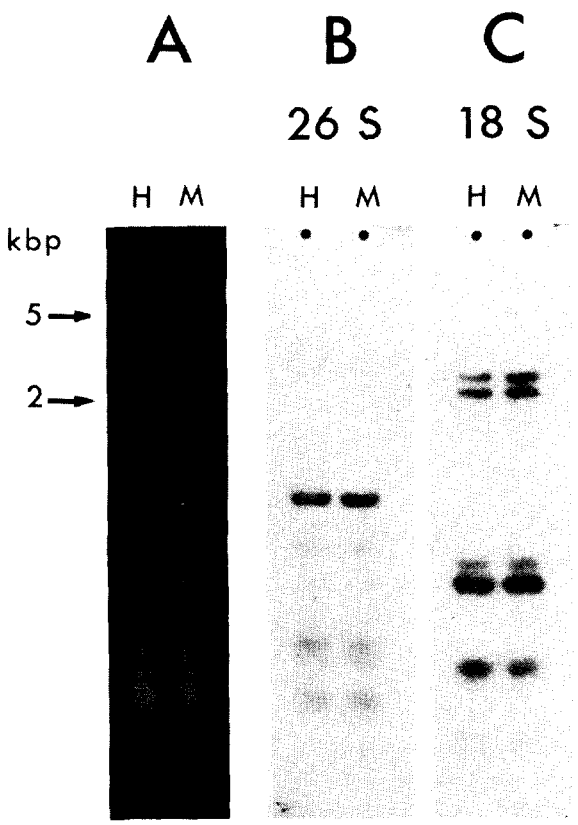

Fig.4. (A) Ultraviolet photograph showing the *Hpa* II (H) and *Msp* I (M) restriction profiles of wheat mtDNA (hydrolysis products were separated in a 1.4% agarose gel). After transfer to nitrocellulose filters, restriction fragments were probed with purified wheat mitochondrial 26 S (B) or 18 S (C) rRNA, and the hybridizing bands visualized by autoradiography.

(–C–C–C↓G–G–G–) contains only C and G. Both qualitatively and quantitatively, the complexity of *Sma* I digests is as great as that of *Eco* RI, *Xho* I, and *Sal* I digests [7] (the latter 3 endonucleases, like *Sma* I, having a 6-nucleotide cleavage site). Disregarding possible multiplicities of some bands, the additive molecular weight of *Sma* I fragments of wheat mtDNA is ~165 × 10^6, about the same as that of *Eco* RI fragments [7]. (Similar calculations could not be done for *Hpa* II and *Msp* I digests, where limited resolution of lower molecular weight fragments (fig.3) precluded a reliable estimate of the additive molecular weight.)

4. Discussion

The results reported here make it unlikely that partial methylation of restriction sites could be contributing significantly to the observed complexity of the restriction digests of higher plant mtDNA. Although *Hpa* II and *Msp* I together can probe only a proportion of the total –C–G– sequences in any DNA, it seems improbable that individual restriction sites for some enzymes (e.g., *Sal* I, *Xho* I) would be present in both methylated (containing –m^5C–G–) and unmethylated (containing–C–G–) forms, while all restriction sites for other enzymes (such as *Hpa* II) would be completely devoid of –m^5C–G–.

While the present study has focused primarily on methylation of C in the sequence –C–C–G–G–, our data (fig.5) also appear to exclude the possibility that methylation of residues other than C contributes to the complexity of restriction digests of wheat mtDNA. Presumably, confirmation of this conclusion could be obtained with other pairs of endonucleases which can distinguish between methylated and non-methylated forms of the same nucleotide in a particular restriction site. In this connection, *Dpn* I (↓G–m^6A–T–C–) and *Dpn* II (↓G–A–T–C–) were used [25] to show that the mtDNA from *Paramecium aurelia* does not contain m^6A in the sequence –G–A–T–C–.

Accordingly, other explanations for the complexity of plant mtDNA restriction patterns must be sought, and our results (this paper and [7]) suggest that primary consideration should be given to possible heterogeneity of plant mtDNAs, as originally suggested [5]. It should be stressed that in spite of the widespread view that plant mtDNA consists of a homogeneous population of 30 μm circular molecules, there is in fact no general agreement in the literature as to the molecular form of mtDNA in higher plants and the size of the plant mitochondrial genome (cf. [26]). Depending on the plant system and the methods of analysis, plant mitochondria have yielded both circular [3,4,27] and linear [28–30] DNA forms, in varying proportions and length distributions. Maximum molecular weight estimates are 60–120 × 10^6, based on electron microscopy [3,27,28] or 70–140 × 10^6, based on renaturation kinetics [3,30,31], whereas restriction analysis yields minimum molecular weight estimates which are usually appreciably higher than these values [5–7]. While some of these discrepancies undoubtedly have their origin in preparative artifacts and differences in the analytical techniques, the possibility remains that real differences in the organization of the mitochondrial genome may exist in different higher plants. It seems, therefore, that it will be neces-

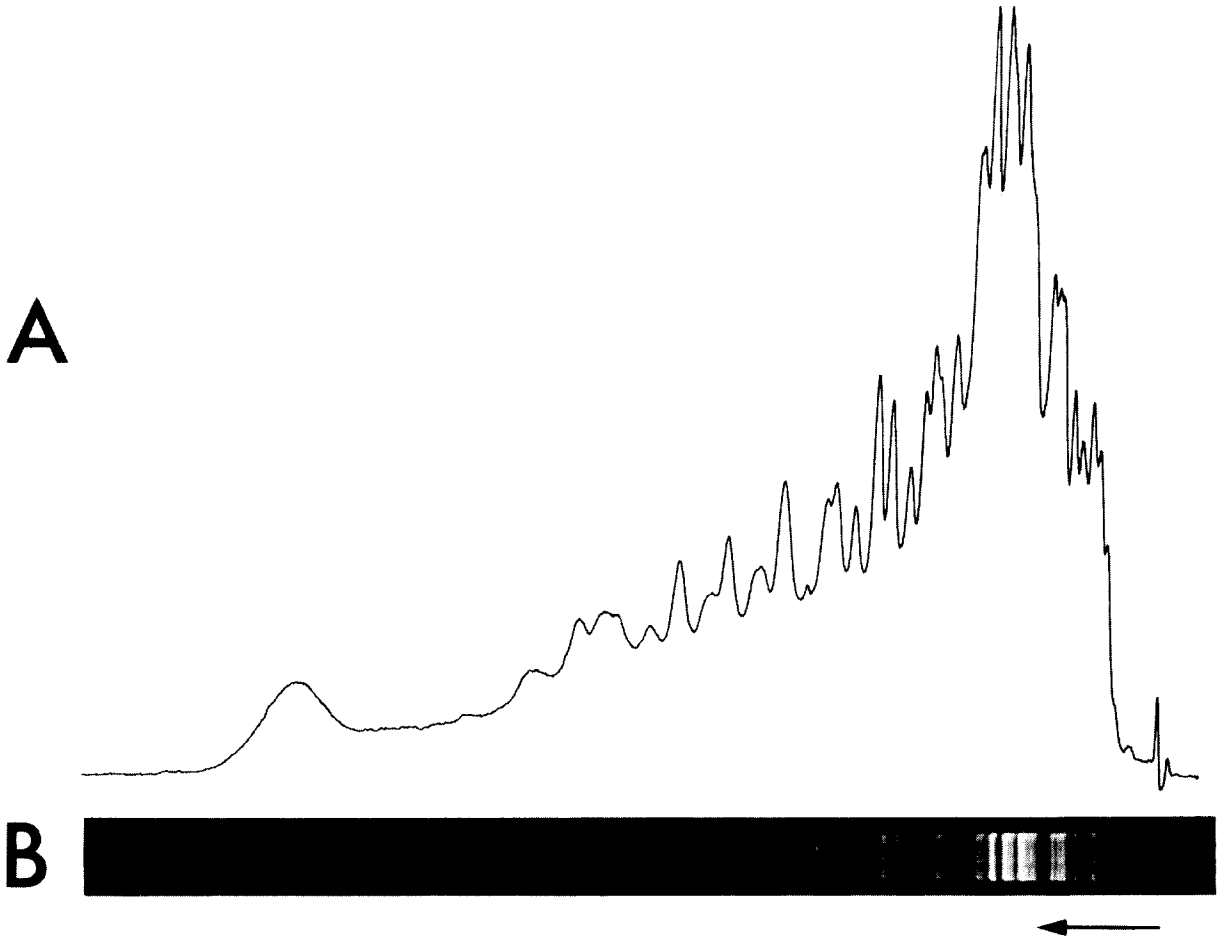

Fig.5. (A) Densitometer tracing showing the restriction profile of wheat mtDNA after cleavage with *Sma* I. (B) Ultraviolet photograph of ethidium bromide-stained *Sma* I restriction fragments of wheat mtDNA separated in a 1% agarose gel. Electrophoresis was carried out in the direction indicated by the arrow until the bromophenol blue marker had migrated 9 cm. The negative of this photograph was used to produce the densitometer scan in (A) as in fig.2.

sary to undertake additional studies involving a combination of electron microscopy, measurement of kinetic complexity, and restriction endonuclease analysis, applied to a wide variety of plant mtDNAs, before a clear picture of the potential information content of the plant mitochondrial genome finally emerges. As well, by using appropriate probes (e.g., cloned mtDNA fragments) to explore the basis of the unusual restriction patterns of plant mtDNA, it should be possible to gain additional insights into the organization of the mitochondrial genome in different plants.

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