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Organelle genome stability in anther-derived doubled haploids of wheat (*Triticum aestivum* L., cv. 'Moisson')

A. Rode [1,*], C. Hartmann [1], M. Dron [1], E. Picard [2] and F. Ouetier [1]

[1] Laboratoire de Biologie Moléculaire Végétale, Bâtiment 430, Université Paris XI, F-91405 Orsay, France
[2] Groupement d'Intérêt Scientifique du Moulon, La Ferme du Moulon, F-91190 Gif-sur-Yvette, France

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Summary. Chloroplast and mitochondrial compartments of a parental line of wheat (*Triticum aestivum* L., cv. 'Moisson') and its anther-derived doubled haploid lines have been analyzed and compared on the basis of their DNA restriction patterns. The results obtained show that no noticeable difference can be detected between doubled haploid lines and parental line at the level of ctDNA and mtDNA organization. It may be concluded that in vitro culture by itself does not systematically generate a cytoplasmic variation in germ cells.

Key words: Wheat – Anther-derived doubled haploids – Chloroplast DNA – Mitochondrial DNA – Restriction patterns

Introduction

Studies dealing with chloroplast (ct) and mitochondrial (mt) DNA variations have been conducted only during the last few years. In vitro somatic tissue or cell cultures as well as the procurement of cybrids by protoplast fusion have proved to be a reliable tool for generating mt DNA variation (Gengenbach et al. 1981; Kemble et al. 1982; McNay et al. 1984; Belliard et al. 1979). Indeed, these data and several others based upon DNA restriction pattern analyses demonstrate that mt DNA can undergo more and less important variation due either to recombination phenomena or to the presence or absence of small mt DNA molecules. Tissue culture of somatic cells is able, by itself, to generate certain mt DNA variations whereas ct DNA has not been shown, until now, to be affected by somaclonal variation.

One of us succeeded in the production of embryoids from the anthers of *Triticum aestivum* (Picard and de Buyser 1973). The chromosome complement of regenerated plants obtained from haploid embryos is artificially doubled by colchicine treatment or spontaneously doubled during the culture, resulting in the subsequent production of fertile homozygous doubled haploid (DH) plants. These DH exhibit considerable changes in their morphological and agronomical properties, such as height, date of heading, yield (Picard et al. 1978; Picard 1984) or in their response to some diseases (Parisi and Picard 1984) when compared to the homozygous parental line.

Considering the plasticity of the mtDNA structure and, to a lesser extent, of the ctDNA one, it is tempting to study the possible effects of in vitro androgenesis on the arrangement of ctDNA and mtDNA from regenerated DH plants (gametoclonal variation).

We report here the first analysis of cytoplasmic DNA in some DH fertile wheat lines obtained by anther culture of the cultivar 'Moisson'.

Material and methods

Development of DH plants

The doubled haploid plants used throughout this work were derived from anther culture of a winter wheat, *Triticum aestivum*, cv. 'Moisson' (Picard and de Buyser 1977; Picard et al. 1978). The three first cycle DH studied here were obtained either by colchicine treatment (DH M13 and DH M30) or by spontaneous diploidisation (DH M17). Regenerated plants were maintained by selfing and analyzed here at their fifth generation of selfing.

Isolation of ctDNA

Plantlets, grown in a greenhouse at 22 °C under intermittent light, were harvested when about 8–10 cm long. Roots were discarded and the material was surface-sterilized with sodium hypochlorite (5%, v/v), rinsed with distilled water, cooled at 2 °C and homogeneised with a Waring Blendor homogeneizer (2 × 1–2 s, low speed) in buffer A (Kolodner and Tewari 1975).

* To whom correspondence should be addressed

The homogenate was filtered through three layers of "Miracloth" and a 50 µm nylon net, then centrifuged at 350 g and 2 °C for 10 min. The supernatant was centrifuged again at 350 g, then at 1,500 g for 10 min. The crude chloroplast pellet was dispersed with a smooth brush in buffer A and treated with DNAse I (50 µg/ml) for 30 min at 2 °C. The reaction was stopped by adding three volumes of buffer B (Kolodner and Tewari 1975). The chloroplast pellet obtained after a centrifugation at 1,500 g for 10 min was washed two times with buffer B and centrifuged under the same conditions. The resulting chloroplast pellet was lysed in 6 ml of buffer C (50 mM Tris-HCl, pH 8, 20 mM EDTA, 1% (w/v) sodium sarkosynate) in the presence of 100 µg/ml of Proteinase K and 0.26 g/ml of CsCl. After gentle stirring for 1 h at room temperature, the lysate was kept overnight at 2 °C. 8.6 g of CsCl and 300 µl of ethidium bromide (10 mg/ml) were added to the lysate and the ultracentrifugation (40,000 rpm, 20 °C, 40 h) was performed in a Beckman 50 Ti rotor. The fluorescent ctDNA band was recovered and ethidium bromide was removed by several butanol treatments. ctDNA was precipited by addition of three volumes of 70% (v/v) ethanol (2 h at −20 °C). DNA was pelleted at 8,000 g for 10 min, washed with 70% ethanol, briefly dried in a vacuum dessicator and dissolved in the required volume of buffer.

Isolation of mtDNA

Plantlets were grown seven days in darkness at 22 °C. Coleoptiles were harvested and washed as described for ctDNA isolation and homogeneised with a Waring blendor (3×5 s, maximum speed) in buffer A. The homogenate was filtered through a 50 µm nylon net and centrifuged at 1,500 g for 10 min. The supernatant was centrifuged at 12,000 g for 12–15 min. The crude mitochondrial pellet was dispersed with a brush and then with a Potter homogeneiser and centrifuged again at 12,000 g. The resulting mitochondrial pellet was dispersed in buffer A (10 ml/100 g fresh material) and treated with DNase I (100 µg/ml) for 1 h at 4 °C. The reaction was stopped by adding three volumes of buffer B. After a centrifugation at 12,000 g for 12–15 min, the pellet was resuspended in buffer B, loaded on a 30–45% (w/v) sucrose gradient in buffer B and centrifuged at 22,000 rpm for 35 min in a SW27-0 Beckman rotor. The mitochondrial band was recovered, slowly diluted with buffer B and pelleted. DNA was isolated as described for ctDNA.

DNA restriction and agarose gel electrophoresis

One to 3 µg of ctDNA or mtDNA were digested to completion by different restriction endonucleases (Sal I, Bam HI, EcoR I, Hind III) in a total volume of 25 µl. The restriction fragments were separated by electrophoresis on 0.8% agarose vertical or horizontal slab gels. After electrophoresis, gels were stained with ethidium bromide (1 µg/ml) and photographed under UV light. The negatives of the photographs were scanned on a microdensitometer (Joyce-Loebl double-beam microdensitometer).

Results

ct DNA cleavage patterns

Samples of ctDNA prepared from *T. aestivum* cv. 'Moisson' and from three DH of this cultivar obtained by in vitro androgenesis (M13, M17 and M30) were digested by several different restriction endonucleases and electrophoresed. As it can be seen on Fig. 1, the Sal I restriction patterns as well as the microdensitometer tracings corresponding to the parent and to the three androgenetic lines are qualitatively and quantitatively identical to each other and agree with the data reported by Bowman et al. (1981) for *T. aestivum* cv. 'Mardler'. Cleavage and ctDNA by certain restriction endonucleases such as Bam HI, EcoR I and Hind III (Fig. 2) and numerous other ones (not shown here) did not reveal any change in the doubled haploid ctDNA structure. In the same way, similar experiments carried out with *T. aestivum* cv. 'Cesar' and 7 DH obtained by in vitro androgenesis (one first cycle and six second cycle DH) did not exhibit any change in DH ctDNA structure (not shown here).

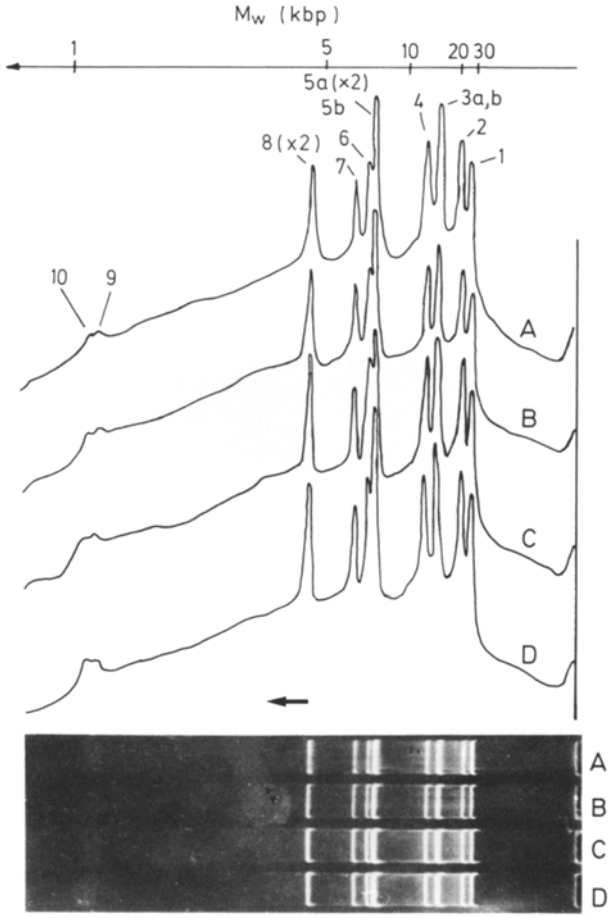


Fig. 1. Agarose gel electrophoretic patterns of Sal I digests of wheat ctDNA and corresponding microdensitometer tracings. *A*: cv. 'Moisson'; *B, C* and *D:* DH M13, M17 and M30 respectively. Electrophoresis on 0.8% agarose was run at 1.4 mA/cm for 12 h in TAE buffer (0.04 M Tris-acetate, pH 8, 0.002 M EDTA) in the direction indicated by the *arrow.* Hind III digest of DNA was used as a molecular weight marker

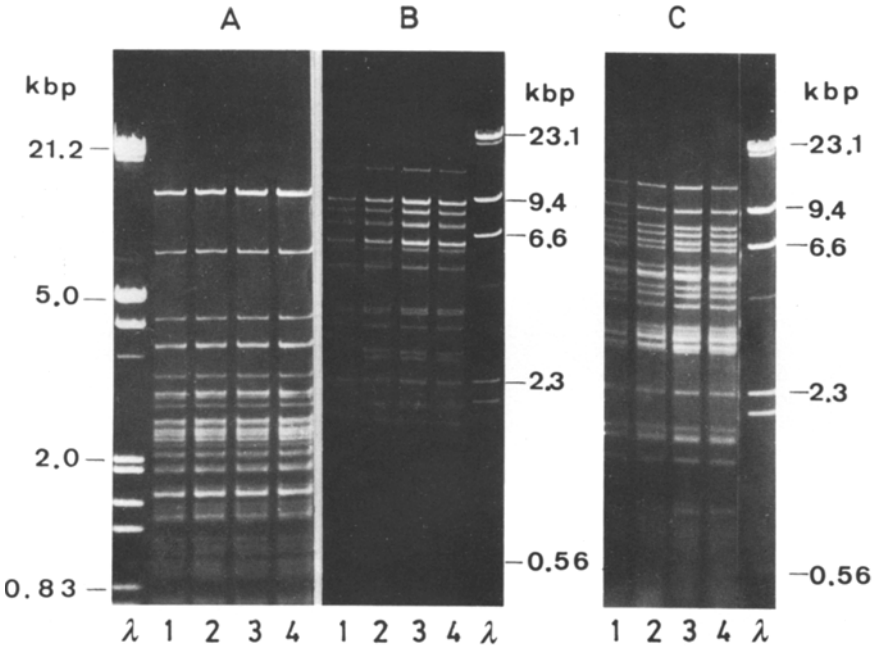
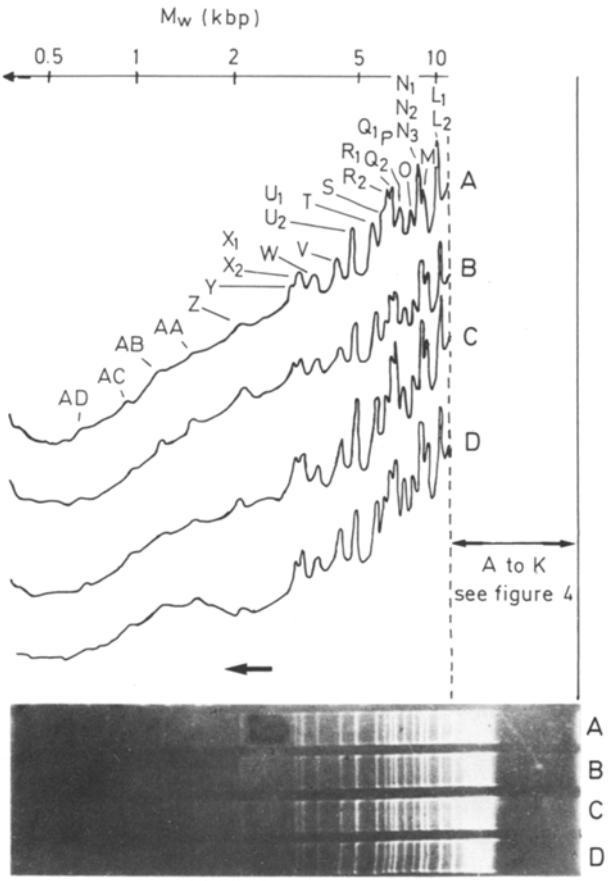


Fig. 2. Agarose gel electrophoresis patterns of restriction endonuclease digests of ct DNA from cv. 'Moisson' and its three DH lines. ct DNA was digested by Eco RI (*A*), Hind III (*B*) and Bam HI (*C*). *Lane 1:* cv. 'Moisson'; *lane 2:* DH line M13; *lane 3:* DH line M17; *lane 4:* DH line M30λ: double digest (Hind III and Eco RI) or Hind III digest of phage λ DNA were used as molecular weight markers. Electrophoresis was in 0.8% agarose at 1.4 mA/cm for 11–13 h in TAE buffer



mtDNA cleavage patterns

As already shown several years ago (Quétier and Vedel 1977) wheat mtDNA displays complex restriction patterns. The best situation, i.e. the smallest number of restriction fragments, has been shown to occur with the endonuclease Sal I. The Sal I physical map of wheat (*T. aestivum*, cv. 'Capitole') mtDNA has been established in our laboratory (Quétier et al. 1984). It comprises 52 different fragments ranging from 29 kbp to 0.4 kbp, which have been lettered A to Z and then AA to AG. This nomenclature has been adopted in this paper as the Sal I restriction pattern obtained with *T. aestivum* cv. 'Capitole' is identical to that obtained with *T. aestivum* cv. 'Moisson'. As for ctDNA, the Sal I restriction patterns and the resulting microdensitometer tracings of mtDNA isolated from the DH M13, M17 and M30 are identical to that of the parental line: On Fig. 3 one sees the cleavage patterns, and microdensitometer tracings of fragments L to AD whereas Fig. 4 shows the microdensitometer tracings obtained from

Fig. 3. Agarose gel electrophoretic patterns of Sal I digests of wheat mtDNA and corresponding microdensitometer tracings. *A:* cv. 'Moisson'; *B, C* and *D:* DH M13, M17 and M30, respectively. Electrophoresis was run on 0.8% agarose at 1.4 mA/cm for 11 h in TAE buffer. For each restricted DNA sample, only the portion of the negative corresponding to bands L to AD has been scanned

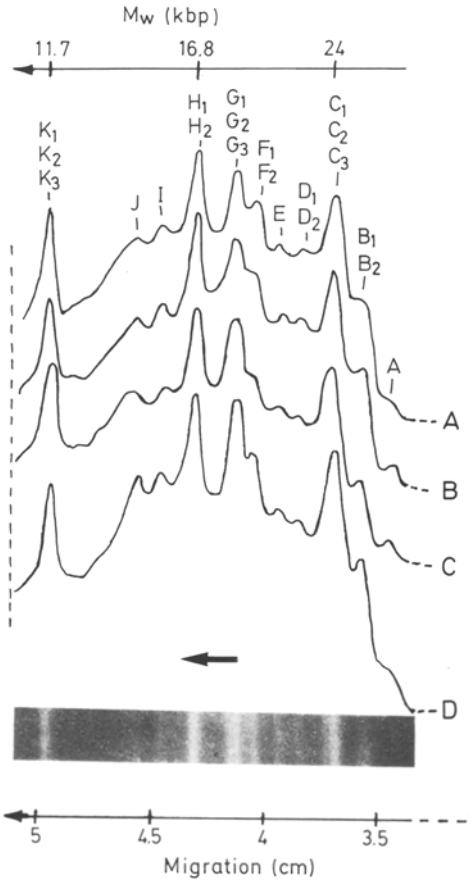


Fig. 4. Microdensitometer tracings (*bands A to K*) of Sal I digests of mtDNA. *A:* cv. 'Moisson'; *B, C* and *D*: DH M13, M17 and M30, respectively. Electrophoresis was run on 0.8% agarose for 20 h at 1.4 mA/cm in TAE buffer. The direction of electrophoresis is indicated by the arrow. A typical photograph of this region of the gel is shown

the portion of the negative corresponding to fragments A to K, for each of the samples. The same conclusion can be deduced from the restriction patterns obtained from the endonucleases Bam HI and Eco RI (not reported here).

Conclusion

This investigation of the ctDNA and mtDNA organization of anther-derived wheat DH leads to the conclusion that, for at least genetic material checked here, no differences can be observed when comparing the cleavage patterns of the original parent to those of the DH lines. It may be deduced from the stoechiometric data of the densitometer tracings that neither the ribosomal ctDNA (included in fragments 5 and 8, Bowman et al. 1981) nor the ribosomal mt DNA (including in fragments M and T, Quétier et al. 1984) from the DH lines have undergone any amplification or reduction of copy number. This fact has been confirmed by hybridization analyses (to be published) with nick-translated DNA probes of wheat ribosomal ct DNA (fragments 5 and 8, cloned by one of us) and ribosomal mt DNA (fragments M and T, gift of Dr. D. Falconet).

Plant cell culture by itself may sometimes generate genetic variability in cultured subclones as well as in regenerated plants (Larkin and Scowcroft 1981). For example, as far as cytoplasmic genomes are concerned, the induction of mitochondrial variability consecutive to tissue or cell cultures has already been described (Gengenbach et al. 1981; Kemble et al. 1982; Orton 1983; Mc Nay et al. 1984). As our experiments show, in vitro anther culture by itself does not involve systematically a cytoplasmic variability, it may thus be concluded that the induction of cytoplasmic variability consecutive to in vitro cultures is not a general phenomenon.

Somatic cell hybridization experiments have proved to be a powerful tool for studying extra-chromosomal inheritance in higher plants. For example, the study of cybrids of *N. tabacum* obtained by heteroplasmic protoplast fusion have shown that their progeny contained only one or the other parental ct DNA (Belliard et al. 1978) whereas mt DNA was different from that of the parents and from the mixture of the two (Belliard et al. 1979), indicating some rearrangements in mt DNA organization (Nagy et al. 1983). The problems raised by plants regenerated from in vitro culture of anthers are, indeed, quite different. Androgenesis involves the development of a haploid embryo from only the male gamete. The genetic information encoded into this embryo is then only dependent on the cytoplasmic organization of the male gamete. As a consequence, it seems difficult to apply strictly the concept of maternal inheritance for mitochondria and chloroplast of androgenetic DH. Nevertheless, as the genitors of the male parent possess the same cytoplasmic genome, one would expect to obtain DH wheat plants possessing – if no rearrangements occur – the same cytoplasmic genome as the male parent, and this is in agreement with our experimental results. However, a report dealing with ct DNA deletions associated with androgenetic wheat plants has appeared recently in literature (Day and Ellis 1984) and introduces an important contribution to the understanding of maternal inheritance of chloroplasts. The results obtained by these authors are concerned with albino DH wheat plants – which remain vegetative and do not flower – whereas our experiments have been carried out with fertile DH lines possessing, therefore, a normal chloroplastic compartment and being able to represent a reliable tool in the improvement of plant breeding. For instance, the DH line M17 presents a better yield than the original cv.

'Moisson'. It can be assumed nevertheless that anther culture does not bring special advantages to the enhancing of cytoplasmic variability when compared to somatic cell culture.

On the other hand, some preliminary results dealing with the organization of the nuclear genome of the same anther-derived DH plants are in good agreement with the results obtained by de Paepe et al. (1982) and Dhillon et al. (1983) with anther-derived DH of *Nicotiana sylvestris* and *Nicotiana tabacum* respectively, i.e. they demonstrate that some changes do occur into the nuclear DNA organization (Rode et al., in preparation).

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