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Organ/tissue-specific changes in the mitochondrial genome organization of in-vitro cultures derived from different explants of a single wheat variety

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Summary. We have previously shown that the mitochondrial genome of long-term tissue cultures prepared from immature embryos of several varieties of cultivated wheat underwent variety-specific rearrangements resulting from either changes in the relative amounts of subgenomic components or from the appearance of novel genomic configurations. In the present work, both categories of rearrangements were studied in long-term tissue cultures initiated from other explants (shoot meristem, young leaf base, young root tip, immature inflorescence) of the same wheat variety (Chinese Spring) and were compared to those previously obtained with immature embryo cultures. Two main patterns of reorganization were found in a region of the mitochondrial genome known to be hypervariable in structure. In addition, some of the novel subgenomic configurations were obviously organ/tissue-specific whereas others were present in more than one type of organ. In several instances, the age of culture was found to determine the degree of mitochondrial DNA rearrangement. The data presented in this study strengthen the hypothesis of an association between a particular organization of the mitochondrial genome in tissue culture and its regeneration capacity.

Key words: Wheat – Somatic tissue culture – Mitochondrial DNA – Chondriome variability

Introduction

The mitochondrial genome of higher plants, known to be large and heterogeneous (Ward et al. 1981; Newton 1988), can be generally considered as a "master" chromosome containing all the sequence information. In every case but two (Palmer and Harbon 1987; Ohyama et al. 1991), this genome is characterized by the presence of either one (Palmer and Shields 1984) or several (Lonsdale et al. 1984) sets of repeated sequences which probably permit the generation of a collection of circular subgenomic chromosomes.

It is now widely documented that the passage of plant tissues through an in-vitro culture can induce changes in the molecular organization of the mitochondrial genome. These changes are characterized either by some variation in the stoichiometry of restriction fragments already present in the parental plant or by the detection of novel fragments. A survey of the literature concerning the variability, revealed through in-vitro culture, of the plant mitochondrial genome shows that a number of different organs have been used to initiate the tissue cultures: namely, leaves (Dörfel et al. 1989; Saleh et al. 1990), mature seed scutellum (Saleh et al. 1990), inflorescences (Brears et al. 1989), hypocotyls (Shirzadagan et al. 1989, 1991), stems (Grayburn and Bendich 1987), roots (Morgens et al. 1984), and embryos (Negruk et al. 1986; De Buyser et al. 1988). However, in the case of tissue cultures derived from a given explant which have been initiated from different plant species, there is only one example (Saleh et al. 1990) in which in-vitro cultures were prepared from more than one organ of the same plant variety (leaf base and mature seed scutellum). Thus, for a given variety, and with this one exception, mtDNA variability has been defined in terms of comparisons between tissue cultures prepared from a given organ and the source parent plant.

In a previous paper, we studied the organization of the mitochondrial genome in tissue cultures initiated from immature embryos of several isoplasmic wheat varieties (Rode et al. 1987; Hartmann et al. 1987). Surpris-

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ingly, the changes we detected were shown to be variety-specific. From these results, the question was asked whether the reorganization of the mitochondrial DNA could also be organ/tissue-specific. Such a study requires the use of tissue cultures initiated from different explants belonging to the same plant variety.

In this paper we decribe an analysis of the reorganization of a well-characterized hypervariable region of the mitochondrial genome in long-term tissue cultures prepared from four different explants of the wheat variety Chinese Spring (shoot meristem, young leaf base, immature inflorescence, young root tip).

We have previously shown an association between the regeneration capacity of tissue cultures and the precise organization of their mitochondrial genome (Rode et al. 1988). The validity of such an association in tissue cultures prepared from different organs of the same variety is discussed.

Materials and methods

Somatic tissue culture

Glasshouse-grown plants of *Triticum aestivum*, variety Chinese Spring, were used as explant donor. Immature inflorescences (14-day old) were surface-sterilized and excised. Shoot meristems, basal segments of young leaves, and root tips, were excised from aseptically-germinated mature seeds. Explants were plated ten per 9-cm Petri dish on a modified Murashige and Skoog (1962) medium containing 2 mg/l of 2,4-dichlorophenoxyacetic acid, as described earlier (Rode et al. 1987). Calli were subcultured at 2 month-intervals onto the same medium. Cultures were maintained in a growth chamber at 27 ± 1 °C under very low illumination ($10\ \mu E.m^{-2}.s^{-1}$) with a 16-h day length. Callus samples were recovered for the study of their mitochondrial DNA (mtDNA) organization after 8 and 52 months for inflorescence cultures, 26 and 52 months for shoot meristem and young leaf base cultures, and 22 and 52 months for root tip cultures.

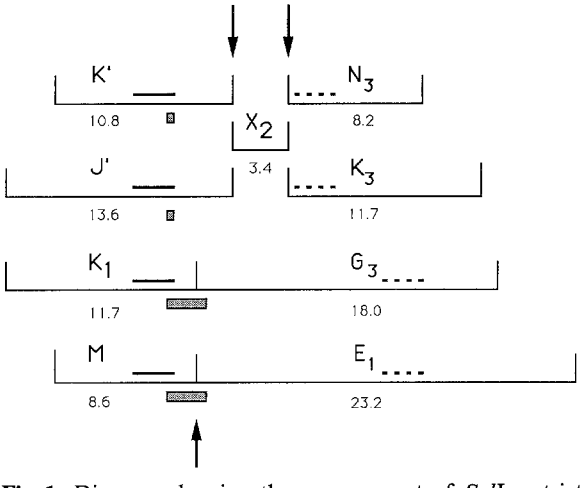

Fig. 1. Diagram showing the arrangement of *SalI* restriction fragments in a region of the wheat mitochondrial genome known to be hypervariable in structure in immature embryo culture. The nomenclature of the restriction fragments is that of Quétier et al. (1985). *Vertical bar*, *SalI* site; ——, - - -, location of recombinationally active repeated sequences (RS5 and RS 10 respectively), each of them generating four different sequence arrangements. *Vertical arrows*, location of *SalI* sites where multiple possibilities of fragment arrangements can occur (Falconet et al. 1985; Lejeune, personal communication). ▒, location of the 26 *s* ribosomal RNA gene (Falconet et al. 1985). Fragment size (in kb) is indicated beneath each restriction fragment

Isolation, restriction and electrophoresis of total cellular DNA

Total cellular DNA of inflorescences, shoot meristem, leaf bases and root tips, and of corresponding tissue cultures, was prepared as described previously (Hartmann et al. 1989). DNA samples were digested with *SalI* and fractionated by electrophoresis in vertical 0.8% agarose-gel slabs.

Molecular probes

SalI-cloned wheat mtDNA fragments (a generous gift of B. Lejeune), which are known to reveal genomic variability induced by in-vitro culture of immature embryos [fragments K', X_2, K_3 and N_3, according to the nomenclature adopted by Quétier et al. (1985)], were used as labelled probes.

DNA-DNA hybridizations

Southern blots of *SalI*-restricted cellular DNA were prepared and probed as described previously (Rode et al. 1987).

Results

Some variety-specific changes occur in the mitochondrial genome organization of tissue cultures initiated from immature embryos of several isoplasmic varieties of cultivated wheat (Hartmann et al. 1987; Rode et al. 1987, 1988) and are principally located within a particular fraction of the genome (the "hypervariable region", see Fig. 1). Two main types of genomic variability can be distinguished. They are called the "Chinese Spring-type" and the "Aquila-type" since the most characteristic differential changes were obtained from these two varieties. The main features of these changes are as follows (Fig. 1): (1) two *SalI* restriction fragments (E_1 and K_3) are lost in both types of culture; the presence of these fragments in restriction patterns is thus typical of the organized plant; (2) four *SalI* restriction fragments (J', K', X_2 and N_3) are either lost or are considerably diminished in "Aquila-type" cultures specifically. In addition, novel hybridization signals – representative of novel molecular organizations – are specifically detected in Chinese Spring cultures: a 9.8 kb fragment (lettered "f") when probing with a labelled K_3 fragment, and an 8.2 kb fragment (lettered "b") when probing with a labelled K' fragment (Hartmann et al. 1987).

The data we present below, carried out with tissue cultures derived from different explants of the variety Chinese Spring, utilize these results as standards of variability.

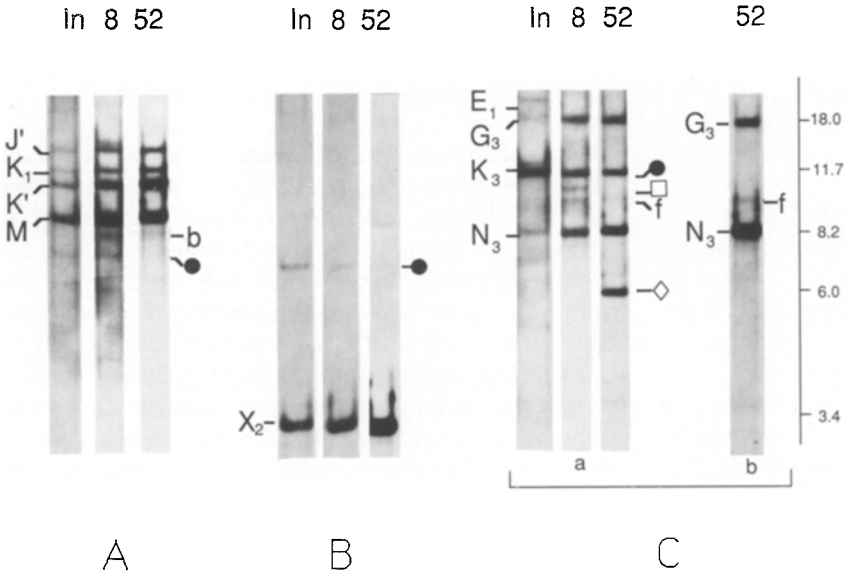

Fig. 2 A–C. Southern blot analysis of DNA prepared from immature inflorescences (*In*) and from corresponding 8-month old (*8*) and 52-month old (*52*) cultures. Total cellular DNA that had been *Sal*I-restricted was fractionated by agarose gel electrophoresis and blotted onto nitrocellulose. It was then probed with four cloned wheat mtDNA fragments (**A** fragment K′; **B** fragment X_2; **Ca** fragment K_3; **Cb** fragment N_3) belonging to the hypervariable region of the genome. The identity of hybridizing fragments is indicated on the left of each panel. The symbols "*b*", "*f*", "□" (10.5 kb), "◇" (6.0 kb), on the right of panels, identify hybridizing fragments from novel mtDNA configurations. The symbol "●" corresponds to a chloroplast DNA fragment with homology to the mitochondrial probe used. Fragment size (in kb) is indicated on the right

Variability in cultures initiated from the immature inflorescence

The hybridization patterns displayed by the DNA fragments belonging to the hypervariable region in 8-month old cultures of the immature inflorescence (Fig. 2, A, B, C) were typically those found in long-term cultures derived from immature embryos of Chinese Spring (loss of fragments E_1 and K_3 only) (Fig. 2, Ca). Furthermore, hybridization signals corresponding to the additional fragments "b" (Fig. 2, A) and "f" (Fig. 2, Ca), previously found in immature embryo cultures of Chinese Spring, were also detected. This suggested that the appearance of the new molecular configurations encompassing these fragments depends on the presence of a Chinese Spring background rather than on the nature of the explant. Lengthening the duration of in-vitro culture leads to the detection of a novel hybridization signal (6.0 kb) with homology to fragment K_3 (Fig. 2, Ca). This 6.0 kb fragment does not hybridize to N_3 (Fig. 2, Cb). Thus, sequence homology is located in the fraction of fragment K_3 between the right border of repeated sequence no 10 and the righthand *Sal*I site (see Fig. 1). Fragments "b" and "f" were also still present in these very long-term cultures.

Variability in cultures initiated from the young root tip

Surprisingly, the organization of the hypervariable region in a 22-month old culture of the root tip (Fig. 3, A, B, C) was similar to that obtained from the long-term culture of immature Aquila embryos [loss, or marked stoichiometric decrease, of fragments J′ and K′ (A); X_2 (B); E_1 and K_3 (Ca); N_3 (Cb)]. The study carried out on 52-month old cultures in fact indicated that the faint hybridization signal detected after probing with fragment K_3, at a position close to that expected for fragment N_3 (Fig. 3, Ca), did not correspond to fragment N_3. Indeed, this signal was not evident when fragment N_3 itself was used as a probe (Fig. 3, Cb). Novel arrangements were detected in 22-month old cultures and were still present in 52-month old cultures, as shown by an approximately 25 kb hybridizing band when probing with fragment K_3 (Fig. 3, Ca). This band was found to be specific for root tip cultures. This was not the case for a novel 6.0 kb fragment, found only in 52-month old cultures. Indeed, this fragment, carrying homology to K_3 (Fig. 3, Ca) but unrelated to N_3 (Fig. 3, Cb), had already been detected in 52-month old cultures of inflorescences. It must be emphasised that this 6.0 kb fragment was undetectable in both 8-month old inflorescence cultures and 22-month old cultures of root tips. Thus, the molecular mechanism which governs the appearance of a new genomic organization may be initiated a long time after the start of an in-vitro culture.

On the other hand, fragments "b" (Fig. 3, A) and "f" (Fig. 3, C), undetectable in long-term cultures of Aquila immature embryos (Rode et al. 1987), were easily detected, confirming the hypothesis of a correlation between their presence and a Chinese Spring nuclear background.

Variability in cultures initiated from the shoot meristem

No significant qualitative variation was observed in the organization of the hypervariable region of 26-month old tissue cultures (Fig. 4, A, B, C). In contrast, results obtained from 52-month old cultures showed marked rearrangements comparable with those previously seen in cultures derived from Aquila immature embryos: loss of fragments J′ and K′ (Fig. 4, A); X_2 (Fig. 4, B); E_1 and K_3 (Fig. 4, Ca); N_3 (Fig. 4, Cb). Thus, when compared to the

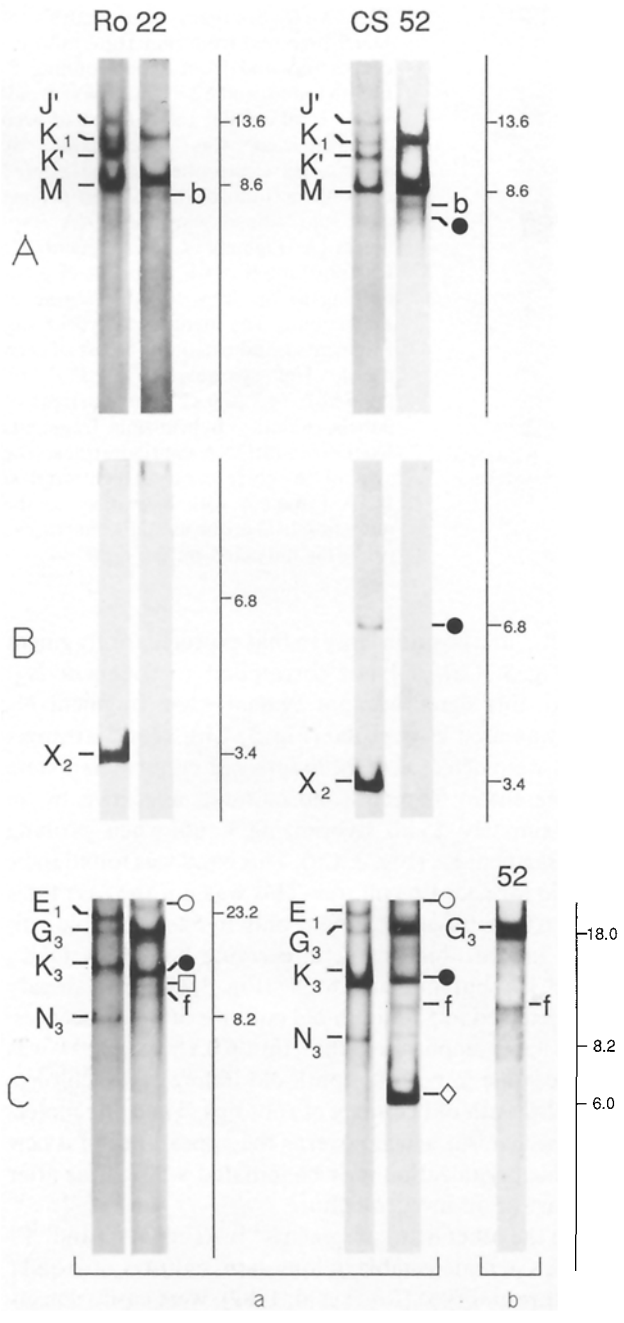

Fig. 3A–C. Southern blot analysis of DNA prepared from young root tips (*Ro*), from the whole plant (*CS*) and from 22-month old (*22*) and 52-month old (*52*) cultures derived from young root tips. Total cellular DNA, processed as described in Fig 2, was probed with the cloned wheat mtDNA fragments K′ (**A**), X_2 (**B**) K_3 (**Ca**) and N_3 (**Cb**). The symbol "o" (approximately 25 kb) identifies a hybridizing fragment from a novel configuration. Other notations as in Fig. 2

other culture types, the time-course of reorganization of the hypervariable region is considerably slowed down in shoot meristem cultures, especially during the first 2 years of culture. This could result from the molecular mechanisms which function at the origin of the reorganization of the hypervariable region becoming qualitatively and quantitatively disturbed by the loss of six of the 42 chromosomes during in-vitro culture (data not shown).

The additional 6.0 kb fragment, already detected in 52-month old cultures of both the inflorescence and the root tip, was also present in this type of culture (Fig. 4, Ca) whereas fragment "f", usually found in 8- and 22-month old cultures, was present only in 52-month old cultures (Fig. 4, Ca, Cb), strengthening the conclusion that the mechanism of genome reorganization could be considerably slowed down in shoot meristem culture. Finally, two hybridizing fragments of lengths 6.8 kb and approximately 14 kb were detected after probing with N_3 (Fig. 4, Cb). The approximately 14 kb fragment was also detected when K_3 was used as a probe (Fig. 4, Ca).

Variability in cultures initiated from the young leaf base

The banding patterns of the hypervariable region of mtDNA from leaf base cultures were globally "Aquila-type" (Fig. 5). Indeed, in 26- and 52-month old cultures, fragments J′ and K′ (A), X_2 (B), E_1 and K_3 (Ca) and N_3 (Cb) had completely (or almost completely) disappeared. An 8.2 kb hybridizing signal (at a position corresponding to that of fragment N_3), was detected when probing with fragment K_3. In addition, an approximately 8.2 kb weak signal was also present after probing with fragment X_2 (Fig. 5, B). This result suggests that the 8.2 kb fragment, undetectable after probing with N_3 (Fig. 4, Cb), could belong to a novel subgenomic organization generated by a recombination event involving fragments K_3 and X_2.

The region of the mitochondrial genome encompassing the recombinogenic repeated sequence no 10 appears to undergo extensive rearrangements in leaf base cultures characterized by the appearance of new organizations specific to these cultures. For example, hybridization of *Sal*I-restricted DNA of 26-month old cultures with K_3 (Fig. 5, Ca) reveals two additional hybridizing fragments of respective lengths approximately 14 kb and 3.95 kb. The latter (specific for the leaf base culture) was also detected after probing with K′ (Fig. 5, A). This fragment could result from the induction or the amplification, consequent upon the transfer of the explant into the culture medium, of a subgenomic structure generated by a recombination event involving fragment K_3 and, in the present case, fragment K′. Increasing the time in culture obviously favours the relative amplification of the subgenomic structures encompassing both the approximately 14 kb and 3.95 kb fragments (Fig. 5, A, Ca, Cb). It must be stressed that both probes, K_3 and N_3, share homology with the approximately 14 kb fragment, whereas the 3.95 kb fragment was detected only after probing with K_3 (Fig. 5, Ca, Cb). Thus, the approximately 14 kb fragment has homology to the fraction of fragments K_3 and

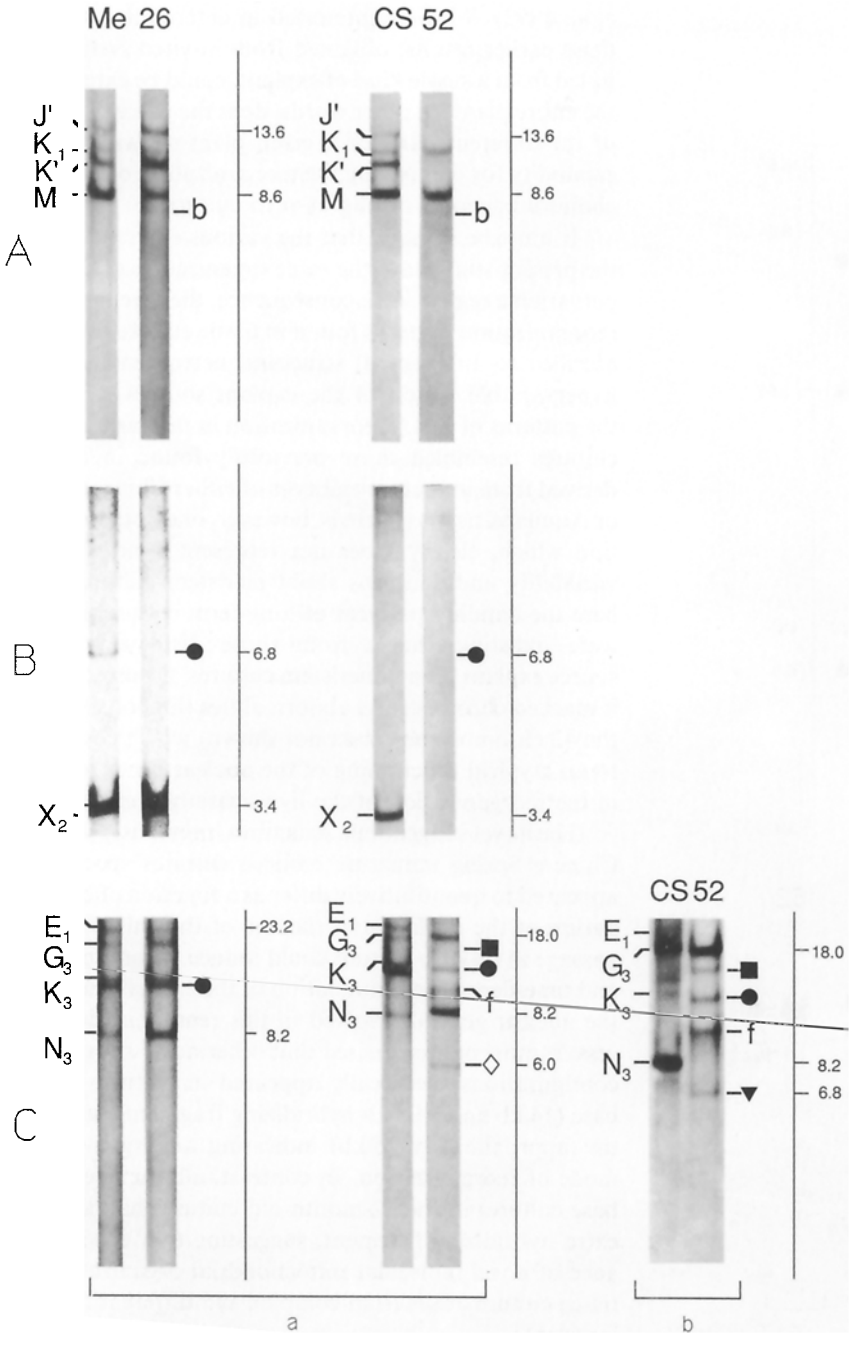

Fig. 4A–C. Southern blot analysis of DNA prepared shoot meristem (*Me*), from the whole plant (*CS*) and from corresponding 26-month old (*26*) and 52-month old (*52*) cultures. Total cellular DNA, processed as described in Fig. 2, was probed with the cloned wheat mtDNA fragments K′ (**A**), X_2 (**B**), K_3 (**Ca**) and N_3 (**Cb**). The symbols "■" (14 kb) and "▼" (6.8 kb) identify hybridizing fragments from novel configurations. Other notations as in Fig. 2

N_3 located between the right border of repeated sequence no 10 and their left hand *SalI* sites whereas the 3.95 kb fragment shares homology only with the portion of fragment K_3 downstream from the right border of repeated sequence no 10 (see Fig. 1). It must be emphasised that the additional fragment "f", present in 26-month old cultures, was not detected in 52-month old cultures (Fig. 5, C). Finally, a 10.5 kb hybridizing fragment was identified after probing the DNA of 52-month old cultures with fragments K_3 and N_3 (Fig. 5, Ca, Cb).

Discussion

The structural changes occurring in the mitochondrial genome of tissue cultures initiated from immature embryos of several wheat varieties can be subdivided into two classes. The first affects the reorganization of a particular fraction of the genome (the hypervariable region); the second is concerned with the presence of novel subgenomic structures. Both kinds of structural change are controlled by different nuclear information (Hartmann

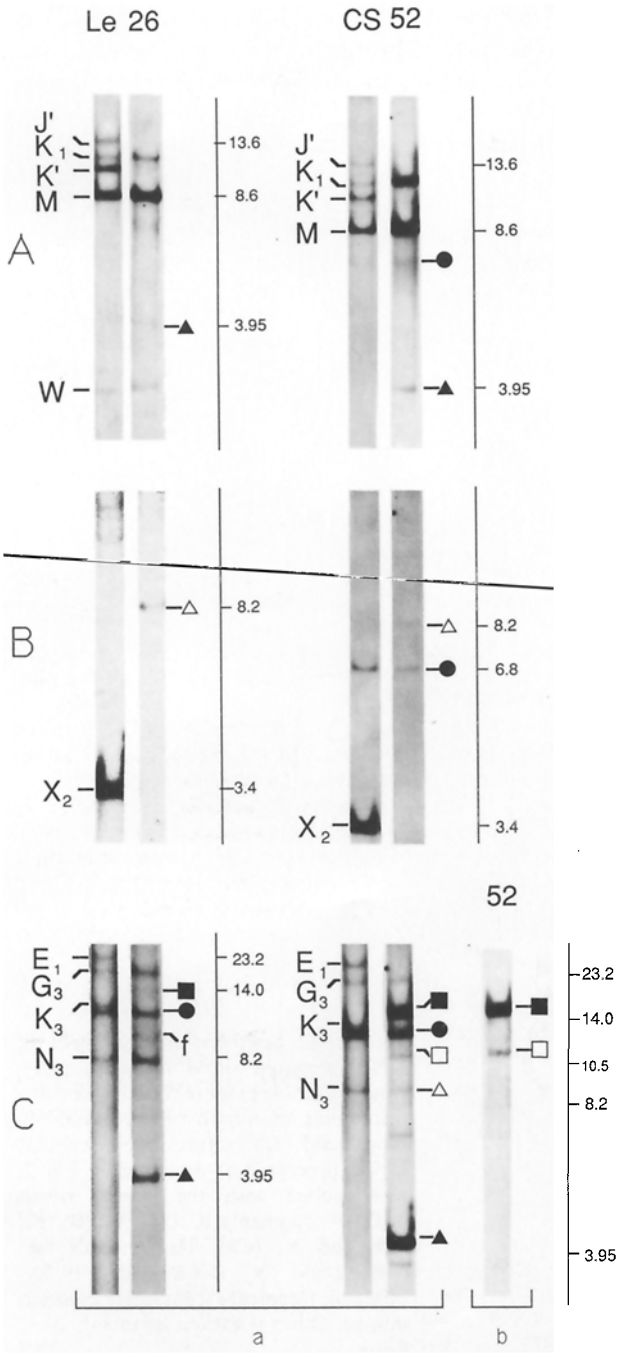

Fig. 5A–C. Southern blot analysis of DNA prepared from young leaf base (*Le*), from the whole plant (*CS*) and from corresponding 26-month old (*26*) and 52-month old (*52*) cultures. Total cellular DNA, processed as in Fig. 2, was probed with the cloned wheat mtDNA fragments K′ (**A**), X_2 (**B**), K_3 (**Ca**) and N_3 (**Cb**). The symbols "▲" (3.95 kb) and "△" (8.2 kb) identify hybridizing fragments from novel configurations. The *Sal*I restriction fragment *W* (3.6 kb) has a low homology to fragment K′, detectable only after a long exposure of the autoradiogram. Other notations as in Fig. 2

et al. 1992). We were interested in determining whether these earlier results, obtained from in-vitro systems initiated from a single kind of explant, could be extended to the entire plant. In other words, does the nuclear genome of the different parts of a given plant shows the same capability for controlling the reorganization of the mitochondrial genome during in-vitro culture.

It must be stressed that the various explants used in the present study have the same organization of their hypervariable region. As a consequence, the diversity of the reorganization patterns found in tissue culture cannot be ascribed to an eventual structural heterogeneity in the hypervariable region of the explant sources. Globally, the patterns of DNA reorganization in the various tissue cultures resembled those previously found in cultures derived from immature embryos of either Chinese Spring or Aquila varieties. There is, however, one notable exception which, clearly, does not represent a new type of variability and concerns shoot meristem cultures only: here the banding patterns of long-term culture mtDNA were indistinguishable from those displayed by the source explant. Shoot meristem cultures, however, exhibit marked chromosomal abnormalities (loss of six out of the 42 chromosomes; data not shown) which could lead to an atypical functioning of the nuclear genes involved in the reorganization of the hypervariable region.

The novel subgenomic structures, previously found in Chinese Spring immature embryo cultures specifically, appeared to quantitatively differ as a function of both the nature of the explant and the age of the culture. Thus, passage in in-vitro culture could induce an organ/tissue- and time-dependent modulation of the expression rate of the nuclear gene(s) involved in this reorganization process. It must be emphasised that other novel subgenomic configurations specifically appeared in cultures of leaf base (14 kb and 3.95 kb hybridizing fragments) and root tip (approximately 25 kb) indicating an organ-specific mode of reorganization. In contrast, all but one (a leaf base culture) of the 52-month old cultures had the 6 kb extra hybridizing fragment, suggesting that the appearance of novel molecular mitochondrial organizations in tissue culture arises from complex and different molecular events.

Thus, the molecular mechanisms responsible for the two kinds of in vitro-induced rearrangements proceed along different pathways. Once the structural changes in the hypervariable region are completed, the resulting reorganized structure remains almost stable even if culture is continued for 5 years. Conversely, the appearance of novel subgenomic structures seem to obey more subtle laws. A plausible hypothesis would be that the expression of the genes controlling these rearrangements is more easily modulated by the constraints of in-vitro culture.

We have also previously drawn attention (Rode et al. 1988) to the existence of an association, the nature of

which remains to be determined, between mitochondrial genome organization and the capacity of immature embryo cultures to regenerate into green plants. The loss, or else the marked relative decrease, of fragments J', K', X_2 and N_3 was always associated with a loss of regenerative capacity. The results described in this paper allowed us to check whether this association could be extended to tissue cultures derived from various organs of the same variety. What we find is that tissue cultures derived from inflorescences do regenerate into green plants, whereas those derived from roots do not. In the former case, the reorganization of the hypervariable region was identical to that found in embryogenic cultures derived from Chinese Spring immature embryos; in the latter, this reorganization was similar to the one observed in non-embryogenic cultures derived from Aquila immature embryos (Rode et al. 1987).

The situation is not so easy to understand as far as leaf base cultures are concerned. Indeed, in most respects they are "Aquila-type" although they regenerate into green plants. However, they differ from the strict "Aquila-type" by the presence of an extra 8.2 kb hybridizing fragment with homology to both X_2 and K_3. We suggest, therefore, that only part of the hypervariable region is associated with a regeneration capacity. This particular DNA fraction would be present in the novel molecular structures encompassing the 8.2 kb extra hybridizing fragment detected in these cultures.

Tissue cultures initiated from the shoot meristem regenerate sterile plants possessing $2x=6n=36$ chromosomes (data not shown). As no change in the organization of their hypervariable region could be detected in 26-month old cultures, the association between the presence of fragments J', K', X_2 and N_3 and a regenerative capacity is apparently verified. However, this conclusion does not apply to 52-month old cultures. Indeed, they regenerate into sterile plants although the organization of their mitochondrial genome has undergone marked changes which cannot be associated with a regenerative capacity. Cultures derived from the shoot meristem are the only example in which the nuclear genome presents chromosomal abnormalities. It is possible, therefore, that nuclear effects involved in the molecular mechanisms responsible for a regenerative capacity are not operative in these cultures. Thus, it seems reasonable to conclude that, in cultivated wheat, the association previously observed between mitochondrial genome organization and the regeneration capability of immature embryo cultures can be extended to in-vitro cultures initiated from any plant organ possessing a normal chromosome complement.

Genomic variability detected through in-vitro culture raises the basic question as to whether the subgenomic organization found in tissue cultures, but undetectable in the explant, are either generated by de novo recombination events induced by in-vitro culture or are the result of a dramatic amplification of under-represented subgenomic fractions. We are currently addressing this issue through the use of Polymerase Chain Reaction technology.

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