Theor Appl Genet (1992) 85:9–14

THEORETICAL AND APPLIED GENETICS

Multiple patterns of mtDNA reorganization in plants regenerated from different in vitro cultured explants of a single wheat variety

M. C. Morère-Le Paven [1], J. De Buyser [2], Y. Henry [2], F. Corre [1], C. Hartmann [1] and A. Rode [1]

[1] Laboratoire de Biologie Moléculaire Végétale, URA CNRS 1128, Bâtiment 430, Université Paris XI, 91405, Orsay, France
[2] Laboratoire de Génétique Végétale, URA CNRS 115, Bâtiment 360, Université Paris XI, 91405, Orsay, France

Received December 12, 1991; February 26, 1992
Accepted by D. R. Pring

Summary. We have previously shown that tissue cultures derived from various explants of the wheat variety Chinese Spring exhibit organ/tissue-specific changes in the organization of their mitochondrial genome. The aim of this work was to study the influence of passage out of in-vitro culture, and subsequent plant regeneration, on the in vitro "induced" reorganization of this genome. In all cases but one, subgenomic configurations present in both the donor parent and the tissue culture were evident, in corresponding regenerated plants. The presence, in regenerated plants, of subgenomic configurations found in tissue culture but undetectable in the donor parent appeared to be both time- and organ/tissue-dependent. Moreover, when present, these novel organizations were not systematically found in all regenerated plants. Finally, novel subgenomic configurations were specifically detected after passage out of in-vitro culture. As these results were obtained from a single plant variety, they clearly confirm the extreme plasticity of mitochondrial genome structure in response to in-vitro culture.

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

Introduction

During the last decade, numerous reports have firmly established that in vitro-cultured plant cells and tissues display extensive genetic variability (for a review, see Lee and Phillips 1988), at the level of both nuclear and mitochondrial genomes. These changes in genomic organization, following transfer of a differentiated structure into culture medium (first transition), result principally from either variation (amplification or loss) in chromosome number (Bayliss 1980) or from rearrangements probably involving hot spot regions at which recombination events can occur (Roth et al. 1989).

The possibility of producing regenerated plants following the transfer of tissue cultures onto regeneration medium (second transition) raises the question as to whether genomic variability revealed by the first transition is conserved, either totally or partially, or reversed. In addition, the second transition could theoretically induce a novel genetic variability which, in turn, might or might not be transmitted to the progeny of regenerated plants.

As far as the mitochondrial genome is concerned, rearrangements have been found in plants regenerated (1) from immature embryo cultures of maize (Umbeck and Gengenbach 1983), barley (Breiman et al. 1987), and wheat (Hartmann et al. 1989), (2) from leaf mesophyll protoplast cultures of potato (Kemble and Shepard 1984) and alfalfa (Rose et al. 1986) and (3) from inflorescence cultures of sugarbeet (Brears et al. 1989). It must be stressed that these rearrangements have been characterized relative to the donor plant only. Under these conditions, if the organization of the mitochondrial genome in regenerated plants is found to be rearranged, it is not possible to determine whether or not the changes in structure arose at the first or the second transition. In contrast, if no changes in structure are detected in regenerated plants (Kemble et al. 1988), there is no possibility to check whether the second transition could have reversed rearrangements revealed by the first transition.

To-date, plants regenerated from tissue culture of a single explant have been studied for only one species. We have shown that the patterns of mtDNA variability in long-and very long-term-tissue cultures of hexaploid

Correspondence to: A. Rode

wheat were dependent both on the nature of the variety used as an explant donor (Rode et al. 1987; Hartmann et al. 1987) and on the nature of the explant itself (Morère-Le Paven et al. 1992). Thus, a worthwhile study of genomic variability in regenerated plants not only requires a knowledge of the molecular arrangement representative of each of the three steps of a complete culture cycle (explant – tissue culture – regenerated plant) but also needs to be performed with cultures established from different explants of the same variety.

In this paper we describe a study of the changes in structure affecting an apparently non-coding region of the mitochondrial genome of the wheat variety Chinese Spring in plants regenerated from the long-term culture of inflorescence, leaf base, and shoot meristem. In addition, two regions encompassing a known coding sequence (*atp*A and *cox*II genes) have also been checked.

Materials and methods

Plant regeneration

Tissue cultures were established from immature inflorescences, from shoot meristems, from young root tips and from basal segments of young leaves as described previously (Morère-Le Paven et al. 1992). To induce shoot regeneration, pieces of callus were cultured on Murashige and Skoog (1962) medium devoid of 2,4-dichlorophenoxyacetic acid. Fertile regenerated plants, obtained from leaf base and inflorescence cultures, were self-pollinated. Shoot meristem cultures regenerated into sterile plants. Cultures derived from root tips were unable to regenerate into green plants, in agreement with Morrish et al. (1987). From four to six plants of each series were used to study the organization of their mitochondrial genome.

Isolation, restriction and electrophoresis of total cellular DNA

Total cellular DNA of Chinese Spring and of single plants regenerated from long-term culture of inflorescence, shoot meristem and leaf base was prepared as described previously (Hartmann et al. 1989). DNA samples were digested with *Sal*I and fractionated by electrophoresis in vertical 0.8% agarose gel slabs.

Molecular probes

*Sal*I – 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. Two probes were also used to check for rearrangements involving known coding sequences (Fig. 1): (1) a wheat *Sal*I-*Hae*III mtDNA fragment, 546 bp long, comprising the last 400 bp of the 3′ end of *cox*II gene and 146 bp downstream from the 3′ end (a generous gift of B. Lejeune) and located within the *Sal*I fragment H_2; (2) a maize *Eco*RI – *Sst*I mtDNA fragment, 1073 bp long, internal to the *atp*A gene (a generous gift of C. J. Leaver). In a wheat *Sal*I library, this *Eco*RI – *Sst*I fragment is found in two arrangements, due to the fact that it is located at the vicinity of a recombinogenic repeated sequence. As this fragment contains a *Sal*I site, its use as a probe against a *Sal*I digest of wheat mtDNA allows three *Sal*I restriction fragments to be detected (Fig. 1): fragments E_2, I and P, according to the nomenclature of Quétier et al. (1985).

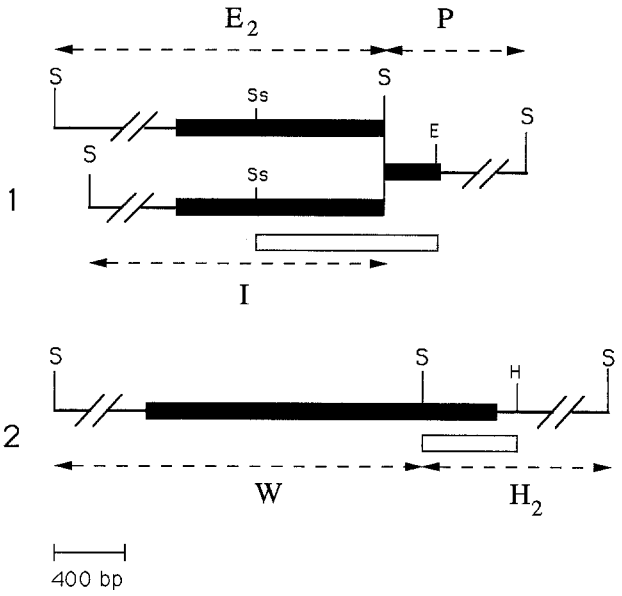

Fig. 1. Location of the genes for (*1*) the alpha subunit of F1 ATPase (*atpA*) and (*2*) the subunit II of cytochrome oxidase (*coxII*) in wheat mitochondrial DNA. ▬, *atpA* and *coxII* genes. *S*, *Ss*, *E*, *H*: *Sal*I, *Sst*I, *Eco*RI and *Hae*III restriction sites respectively. E_2, *P*, *I*, *W*, H_2: *Sal*I restriction fragments encompassing the *atpA* and *coxII* genes. ▭, regions covered by probes. *1*: a *Sst*I-*Eco*RI fragment, 1073 bp long; *2*: a *Sal*I-*Hae*III fragment, 546 bp long

DNA-DNA hybridizations

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

Results

Variability in plants regenerated from an immature inflorescence culture

Total DNA was prepared from the self-pollinated progeny of five plants regenerated from an 8-month old culture, restricted with *Sal*I and probed with the labelled fragments K′ (Fig. 2, A), X_2 (Fig. 2, B) and K_3 (Fig. 2, C). As far as the hypervariable region is concerned, all the DNA samples had the same organization as that of cultures from which they arose. In contrast, the additional "b" and "f" fragments were not detected in the regenerated plants, as was the case with plants regenerated from immature embryo cultures of Chinese Spring (Hartmann et al. 1989). Also, no additional hybridization signals specific to a given regenerated plant were detected, contrary to what was obtained previously with plants regenerated from long-term cultures of Chinese Spring immature embryos (Hartmann et al. 1989). However, due to the small number of individuals studied, it was not possible to conclude that the second transition cannot generate any new subgenomic structures in inflorescence

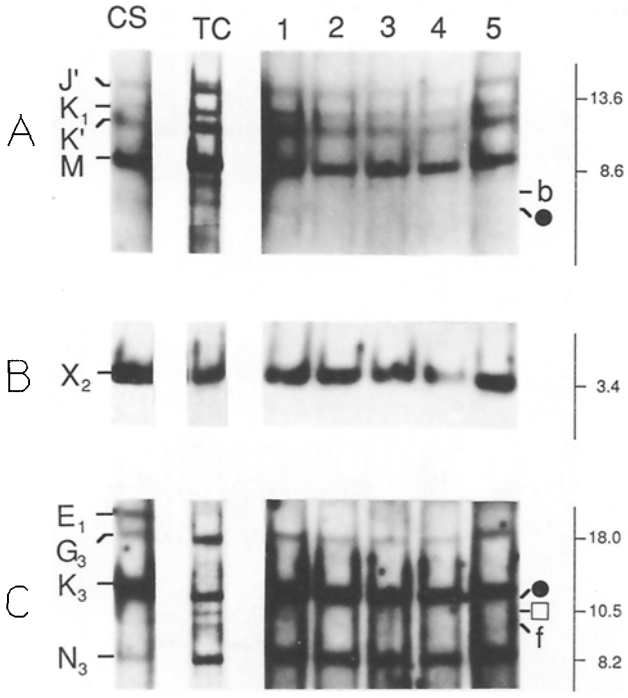

Fig. 2A–C. Southern blot analysis of DNA prepared from the self-pollinated progeny of plants regenerated from an 8-month old culture of an immature inflorescence. Total cellular DNA prepared from a control parental variety (CS), from an 8-month old tissue culture (*TC*), and from five individuals (*numbered 1–5*), was restricted by *Sal*I, blotted onto nitrocellulose and probed with the cloned wheat mtDNA fragments K′ (**A**), X_2 (**B**) and K_3 (**C**). The identity of hybridizing fragments is indicated on the left of each panel. The symbols "*b*", "*f*" and "□" (10.5 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

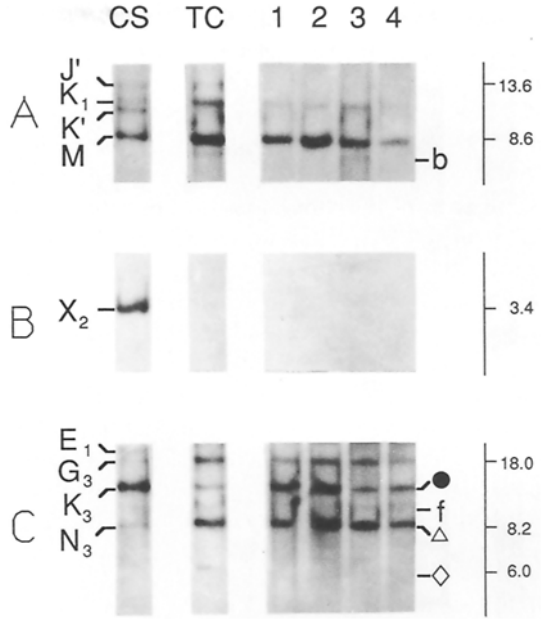

Fig. 3A–C. Southern blot analysis of DNA prepared from plants regenerated from a 52-month old culture of shoot meristem. Total cellular DNA prepared from a control parental variety (*CS*), from a 52-month old tissue culture (*TC*), and from four regenerated plants (*numbered 1–4*), was processed as described in Fig. 2. The symbols "△" (8.2 kb) and "◇" (6.0 kb), on the right of the panels, identify hybridizing fragments from novel mtDNA configurations. Other notations as in Fig. 2

cultures (or else amplify some underepresented subgenomic molecules already present in tissue culture but in amounts too low to be detected).

Variability in plants regenerated from a shoot meristem culture

Four individuals regenerated from 52-month old cultures were randomly chosen and their *Sal*I-restricted DNA was probed with the labelled fragments K′ (Fig. 3, A), X_2 (Fig. 3, B) and K_3 (Fig. 3, C). The organization of their hypervariable region proved to be the same as that of the cultures they came from, a feature already found with plants regenerated from inflorescence cultures. Whereas the additional "f" fragment was still present, although in low amounts (Fig. 3, C), the additional "b" fragment was not detected in regenerated plants (Fig. 3, A). This suggests that the expression of nuclear genes which control the appearance and disappearance of the novel molecular configurations associated with these fragments (Hartmann et al. 1992) could be organ/tissue-specific. Fragments "b" and "f" were also not detected in plants regenerated from inflorescence cultures. A 6 kb fragment, present in tissue culture, is absent from plants regenerated from shoot meristem cultures (Fig. 3, C). Finally, no specific novel organizations were detected for any regenerated plant.

Variability in plants regenereated from a leaf base culture

Hypervariable region and related additional fragments. As the reorganization of the mitochondrial genome of leaf base cultures appeared to evolve extensively as a function of time in culture, analyses were performed using the self-pollinated progeny of individuals regenerated from both 26- and 52-month old cultures. Once more, the reorganization of the hypervariable region of the mitochondrial genome in plants regenerated from 26-month old culture (Fig. 4, A, B, C) was a reflection of that found in corresponding tissue cultures. Moreover, the approximately 14 kb and 3.95 kb fragments were not detected but an additional 5.7 kb hybridizing fragment was present in individuals no. 3 and 4 (Fig. 4, C). The appearance of a new hybridizing fragment following the second transition has already been described in plants regenerated from a long-term culture of Chinese Spring immature embryos (Hartmann et al. 1989).

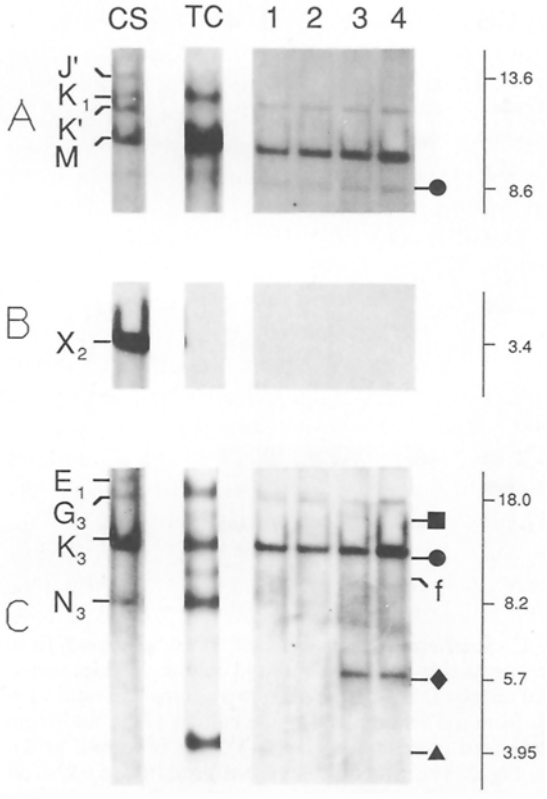

Fig. 4A–C. Southern blot analysis of DNA prepared from the self-pollinated progeny of plants regenerated from a 26-month old culture of a young leaf base. Total cellular DNA prepared from a control parental variety (*CS*), from a 26-month old tissue culture (*TC*), and from four individuals (*numbered 1–4*), was processed as described in Fig. 2. The symbols "■" (14 kb), "◆" (5.7 kb) and "▲" (3.95 kb) identify hybridizing fragments from novel configurations. Other notations as in Fig. 2

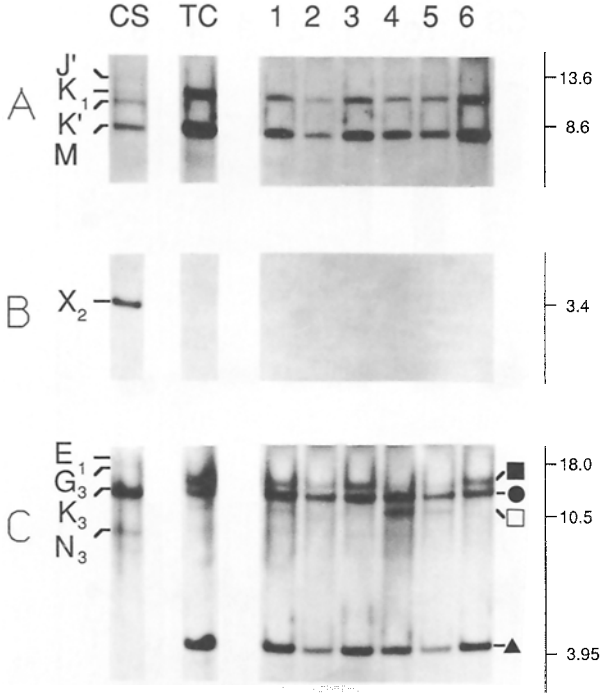

Fig. 5A–C. Southern blot analysis of DNA prepared from the self-pollinated progeny of plants regenerated from a 52-month old culture of young leaf base. Total cellular DNA prepared from a control parental variety (*CS*), from a 52-month old tissue culture (*TC*), and from six individuals (numbered 1–6), was processed as described in Fig. 2. Other notations as in Figs. 2 and 4

The study of mitochondrial genome organization in plants regenerated from 52-month old cultures (Fig. 5, A, B, and C) showed extensive changes when compared to those regenerated from 26-month old culture. Firstly, fragment G_3 was lost in all six individuals tested (Fig. 5, C), thus resulting in the complete disappearance of the four major *Sal*I restriction fragments which hybridize to K_3 in normal mtDNA. According to the arrangement of the nine *Sal*I fragments composing the hypervariable region (Morère-Le Paven et al. 1992), it was not theoretically possible that fragments K_1 and M could both be present if fragments E_1 and K_3 were both absent. Fragments G_3 and E_1 enclose the 3′ end of the gene encoding the 26*s* rRNA gene, whereas fragments K_1 and M enclose the corresponding 5′ end. Falconet et al. (1985) have shown that the wheat mitochondrial fragments C_2 (present in tissue cultures and corresponding regenerated plants, data not shown) G_3 and E_1, share at least a 2.4 kbp common sequence from their leftward *Sal*I site. The fraction of the 3′ end of the 26*s* rRNA gene enclosed in fragments E_1 and G_3 is considerably less than 2.4 kbp. As the arrangements M-C_2 and K_1-C_2 have effectively been found in a cosmid wheat mtDNA library (Lejeune, personal communication) it follows that a complete 26*s* rRNA gene must be present in two configurations in these regenerated plants. In contrast, the approximately 14 kb and 3.95 kb fragments, undetectable in plants regenerated from a 26-month old culture (Fig. 4, C), were present as major bands in plants regenerated from a 52-month old culture (Fig. 5, C). In addition, three of them (plants 1, 4, and 5) had the 10.5 kb additional fragment seen as a minor band in a 52-month old culture (Fig. 5, C). It thus appears, in plants regenerated from 52-month old cultures, that part of the genome sharing homology with fragment K_3 has been nearly completely reorganized.

atpA and coxII genes. The *Sal*I – restricted DNA of very long-term cultures established from immature inflorescences, young root tips, shoot meristems, and leaf bases, was probed with an internal part of the *atp*A and *cox*II genes. Major extra hybridizing fragments (7.2 and 13.5 kb long, with homology to the *cox*II and *atp*A probes respectively) were present in the leaf base culture (Fig. 6, A, B). Cultures derived from the other explants did not show any detectable rearrangement (data not

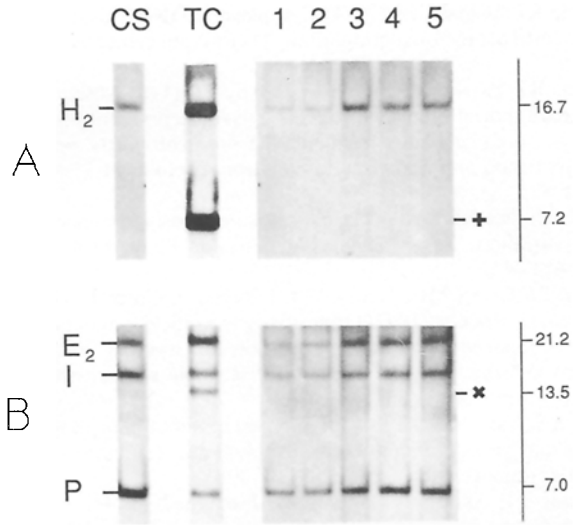

Fig. 6A, B. Hybridization between *SalI*-restricted DNA isolated from the parental variety (*CS*), from a 52-month old tissue culture of young leaf base (*TC*), and from the self-pollinated progeny of five regenerated plants (*numbered 1–5*), with an internal part of (**A**) the wheat *coxII* gene and (**B**) of the maize *atpA* gene (see Fig. 1). The identity of hybridizing fragments is indicated on the left of each panel. The symbols "+" (7.2 kb) and "×" (13.5 kb) identify hybridizing fragments from novel configurations found specifically in tissue culture. Fragment size (in kb) is indicated on the right

shown). Thus, only the DNA of plants regenerated from leaf base cultures was subsequently assayed. Neither additional fragment was detected (Fig. 6, A, B) clearly showing that the second transition had reversed the changes in structure revealed by the first transition. Finally, no novel arrangements specific to the second transition were found.

Discussion

Mitochondrial DNA variability has been studied in plants regenerated from long-term tissue cultures established form various explants of the same wheat variety. The regions of the genome we have checked correspond to both coding and apparently non-coding sequences. These experimental conditions have allowed us to demonstrate that all the possibilities of genomic variability consecutive to the second transition can occur.

In the donor parent, the so-called hypervariable region of the mitochondrial genome comprises nine *SalI* fragments (Morère-Le Paven et al. 1992). Some of these fragments are not detected in long-term cultures, defining a first type of organ/tissue-dependent variability (Morère-Le Paven et al. 1992). As far as regenerated plants are concerned, in all cases but one the organization of the hypervariable region was identical to that of the tissue culture from which they arose; however, fragment G_3, present in trace amounts in 52-month old leaf base cultures was not detected in corresponding regenerated plants. Thus, the submolecular configurations present in tissue culture, and encompassing some of the fragments belonging to the hypervariable region, are not systematically present in regenerated plants.

A second type of variability in tissue culture deals with the appearance of a novel mitochondrial molecular organization. The question was asked whether such novel organizations were still present in regenerated plants or were specific to the in vitro stage. In fact, both possibilities exist. Some fragments are present in all the regenerated plants derived from a single explant (for example, the 14 kb and 3.95 kb hybridizing fragments detected in 52-month old leaf base cultures). Other fragments are found only in some of the regenerated plants (for example, the 10.5 kb hybridizing fragment detected in 52-month old leaf base cultures). Finally, some fragments are lost in all the regenerated plants (for example, the 14 kb and 3.95 kb hybridizing fragments in 26-month old leaf base cultures). It is of interest to stress that the duration of the in vitro stage appears to be critical for the presence, in regenerated plants, of those hybridizing fragments detected in tissue cultures: they are lost in plants regenerated from 8-month old inflorescene cultures and 26-month old leaf base cultures but, in contrast, are still present, and even sometimes amplified, in plants regenerated from 52-month old cultures of both shoot meristem and leaf base.

Finally, as was the case in some plants regenerated from the long-term culture of the immature embryo (Hartmann et al. 1989), a novel hybridizing fragment was observed in two plants regenerated from a 26-month old culture of the leaf base, strengthening the conclusion that the second transition can trigger the appearance, in detectable amounts, of molecular configurations undetectable in both donor plant and tissue culture.

Our results also suggest that the different subgenomic molecules which compose the overall mitochondrial genome are not equally susceptible to in vitro induced variability. This is particularly evident as far as the regions of the genome encompassing the *atpA* and *coxII* genes are concerned. On the one hand, only 52-month old cultures of the leaf base exhibit RFLP while, on the other hand, plants regenerated from these cultures have reverted to the donor plant type, contrary to what we observed, for the same regenerated plants, in their hypervariable region.

From these results it is clear that both the first and the second transitions of a complete culture cycle can generate various types of genomic variability. The eventual utilization of this variability in plant breeding requires that it can be transmissible in sexual crosses. We are currently examining this problem by studying the organization of the mitochondrial genome of plants obtained

from reciprocal crosses between alloplasmic individuals regenerated from long-term tissue culture.

Acknowledgements. We are grateful to F. Quétier for helpful comments on the manuscript, to B. Lejeune and his team for the wheat mitochondrial clones, to C. J. Leaver for the maize *atp*A probe. We also thank S. Bagilet for her technical assistance. This work has been partially funded by the Commission of the European Communities (contracts no. 0014 and 0068).

References

Bayliss MW (1980) Chromosomal variation in plant tissue culture. Int Rev Cytol Suppl 11A:113–144

Brears T, Curtis GJ, Lonsdale DM (1989) A specific rearrangement of mitochondrial DNA induced by tissue culture. Theor Appl Genet 77:620–624

Breiman A, Rotem-Abarbanell D, Karp A, Shaskin H (1987) Heritable somaclonal variation in wild barley (*Hordeum spontanum*). Theor Appl Genet 74:104–112

Falconet D, Delorme S, Lejeune B, Sévignac M, Delcher E, Bazetoux S, Quétier F (1985) Wheat mitochondrial 26*s* ribosomal RNA gene has no intron and is present in multiple copies arising by recombination. Curr Genet 9:169–174

Hartmann C, De Buyser J, Henry Y, Falconet D, Lejeune B, Benslimane AA, Quétier F, Rode A (1987) Time-course of mitochondrial genome variation in wheat embryogenic somatic tissue cultures. Plant Sci 53:191–198

Hartmann C, Henry Y, De Buyser J, Aubry C, Rode A (1989) Identification of new mitochondrial genome organizations in wheat plants regenerated from somatic tissue cultures. Theor Appl Genet 77:169–175

Hartmann C, De Buyser J, Henry Y, Morère-Le Paven MC, Dyer TA, Rode A (1992) Nuclear genes control changes in the organization of the mitochondrial genome in tissue cultures derived from immature embryos of wheat. Curr Genet 21:515–520

Kemble RJ, Shepard JF (1984) Cytoplasmic DNA variation in a potato protoclonal population. Theor Appl Genet 69:211–216

Kemble RJ, Yarrow SA, Wu S-C, Barsby TL (198) Absence of mitochondrial and chloroplast DNA recombinations in *Brassica napus* plants regenerated from protoplasts, protoplast fusion and anther culture. Theor Appl Genet 75:875–881

Lee M, Phillips RL (1988) The chromosomal basis of somaclonal variation. Annu Rev Plant Physiol Plant Mol Biol 39:413–437

Morère-Le Paven MC, Henry Y, De Buyser J, Corre F, Hartmann C, Rode A (1992) Organ/tissue-specific changes in the mitochondrial genome organization of in-vitro cultures derived from different explants of a single wheat variety. Theor Appl Genet 85:1–8

Morrish F, Vasil V, Vasil IK (1987) Developmental morphogenesis and genetic manipulation in tissue and cell cultures of the gramineae. Adv Genet 24:431–499

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Quétier F, Lejeune B, Delorme S, Falconet D, Jubier MF (1985) Molecular form and function of the wheat mitochondrial genome. In: Groot G, Hall T (eds). Molecular form and function of plant genomes. Plenum Press, New York, pp 413–420

Rode A, Hartmann C, Falconet D, Lejeune B, Quétier F, Benslimane AA, Henry Y, De Buyser J (1987) Extensive mitochondrial DNA variation in somatic tissue cultures initiated from wheat immature embryos. Curr Genet 12:369–376

Rose RJ, Johnson LB, Kemble RJ (1986) Restriction endonuclease studies on the chloroplast and mitochondrial DNAs of alfalfa (*Medicago sativa* L.) protoclones. Plant Mol Biol 6:331–338

Roth EJ, Frazier BL, Apuya NR, Lark KG (1989) Genetic variation in an inbred plant: variation in tissue cultures of soybean [Glicyne max (L.) Merrill]. Genetics 121:359–368

Umbeck PF, Gengenbach BG (1983) Reversion of male-sterile T-cytoplasm maize to male fertility in tissue culture. Crop Sci 23:384–388