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Nuclear genes control changes in the organization of the mitochondrial genome in tissue cultures derived from immature embryos of wheat

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Summary. Although the mitochondrial genomes of the Chinese Spring and Aquila varieties of wheat are normally similar in organization, this is not so in tissue cultures initiated from their immature embryos where the mitochondrial genomes of both are rearranged and in different, characteristic, ways. However, the mitochondrial genomes of tissue cultures of reciprocal F_1 crosses between these varieties were almost identical to one another, showing that nuclear genes control the rearrangement processes. These rearrangements are either due to the appearance of new structures or else result from changes in the relative amounts of subgenomic components. The severe reduction in the amount of certain molecular configurations in tissue cultures from reciprocal crosses is probably due to the presence of dominant information in the Aquila nuclear genome. Data obtained from tissue cultures initiated from F_2 embryos of the cross Aquila × Chinese Spring suggest that at least two complementary genes are involved in this control. In contrast, the presence of new molecular arrangements appears to be under the control of a dominant allelic form of a Chinese Spring gene or genes. Thus, this study demonstrates that at least two sets of nuclear genes control the reorganization of the mitochondrial genome which occurs when tissue cultures are initiated from the immature embryos of wheat.

Key words: Nuclear-cytoplasmic interactions – Mitochondrial genome – Chondriome variability – *Triticum aestivum*

Introduction

The nature of nucleo-mitochondrial interactions during mitochondrial biogenesis has been particularly studied in yeast (Darnell 1989) because of the availability of large collections of nuclear and mitochondrial mutants affecting the process in this organism (Dujon 1981). These studies have shown that the nuclear genome provides most of the genetic information essential for both the biogenesis and the correct working of the mitochondrial machinery. Most of the proteins located in the mitochondria are in fact nuclear-encoded, synthesized in the cytosol, and imported into mitochondria. For example, products of nuclear genes are required for the priming of mtDNA synthesis and for transcription (Winckley et al. 1985; Kelly and Lehman 1986; Greenleaf et al. 1986), for processing of the mtRNA precursors (Dieckmann et al. 1984), for splicing of some transcripts with introns (McGraw and Tzagaloff 1983), and for translation of mature mRNAs (Fox 1986).

According to Tzagaloff and Myers (1986), the deletion of segments of yeast mtDNA leading to the formation of "petite mutants" could be a consequence of a decrease in the translational activity of the mitochondria. Usually, petite mutants are characterized by random deletions. However, Zaviani et al. (1989) recently showed that the mitochondrial genome of some members of a human family with mitochondrial myopathy had multiple deletions, each of which involved the same fraction of the genome. In addition, genetic analysis indicated that these well-defined structural rearrangements of the mitochondrial genome were inherited in a Mendelian rather than a maternal fashion.

In higher plants, nucleo-cytoplasmic interactions have been shown to influence a number of quantitative traits (Iwagana et al. 1978; Rao and Fleming 1978; Robertson and Frey 1984; Beavis and Frey 1987; Cooper et al. 1990). Despite this, only limited data are available concerning the role of nucleo-cytoplasmic interactions in influencing the structural organization of plant organelle DNA. There are two main reasons why this is so for the mtDNA. Firstly, the plant mitochondrial genome is large and heterogenous, thus difficult to study (Newton 1988); and secondly, the only types of mitochondrial variants being studied extensively at present with respect to nucleo-cytoplasmic interactions are those of cytoplasmic male-

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sterile plants and their fertile revertants. Cytoplasmic male-sterility is a maternally-inherited phenomenon which results from nuclear-cytoplasmic incompatibility (Hanson and Conde 1985). It is generally accompanied by some defined modification in the organization of the mitochondrial genome (Levings and Pring 1976) which affects its transcription (Kennell et al. 1987) and/or the translation products (Forde et al. 1978).

A notable property of some plant cell and tissue cultures is their capacity to regenerate into normal plants when grown in a suitable culture medium under appropriate environmental conditions. It has already been suggested that the capacity to regenerate is affected by nucleocytoplasmic interactions (Mathias et al. 1986). Recently, mtDNA of tissue cultures prepared from immature embryos of isocytoplasmic varieties of wheat has been shown to exhibit a unique feature: two main patterns of reorganization were found, each being correlated with either the ability or the inability of cells of these cultures to regenerate plants (Hartmann et al. 1987; Rode et al. 1987, 1988). This reorganization is in fact mainly characterized by a loss, or at least a large quantitative decrease, of a defined fraction of the genome in non-embryonic cultures and by the appearance of new subgenomic structures specific to embryonic cultures. Thus, two varieties which were apparently isocytoplasmic but with different nuclear backgrounds initiated alloplasmic tissue cultures. This provided us with a biological system to determine whether the differential reorganization was controlled by nuclear information.

In this paper we describe a study of the mitochondrial genome organization in somatic tissue cultures initiated from F_1 embryos of the reciprocal crosses between wheat varieties generating embryonic (Chinese Spring) and non-embryonic (Aquilla) tissue cultures and from F_2 embryos obtained by selfing F_1 plants. The results are consistent with the view that the changed organization of tissue culture mtDNA is inherited as a Mendelian trait.

Materials and methods

Preparation of tissue cultures. Glasshouse-grown wheat plants of varieties "Aquila" (kindly provided to us by Nickerson RPB Ltd., Rothwell, UK) and "Chinese Spring" were used as starting material. Reciprocal crosses were made between both varieties. Some F_1 plants obtained from the cross Aquila × Chinese Spring were selfed to produce F_2 embryos. Tissue cultures were prepared from both F_1 and F_2 embryos as follows. About 14 days after anthesis, the immature seeds were harvested and surface-sterilized (4% sodium hypochlorite for 1–2 min). The young embryos were excised under a dissecting microscope and then placed, ten per 9-cm petri dish, on agarose (6 g/l) medium with the scutellum exposed and the meristem and the epiblast embedded in the medium. The culture medium contained the Murashige and Skoog (1962) inorganic salts and vitamins and was supplemented with 20 g/l sucrose and 2×10^{-3} g/l of 2–4 dichlorophenoxyacetic acid. The pH was adjusted to 5.8 before autoclaving. Cultures were maintained at 27 ± 1 °C under low illumination with a 16-h day length and subcultured at 2-month intervals onto the same medium. The nomenclature adopted for tissue cultures takes into account the number (x) of subcultures (SC) preceding their study and is denoted as SC^x. SC^5 cultures, initiated from immature embryos of the varieties Chinese Spring and Aquila, were used as controls. SC^2 and SC^5 cultures were prepared from immature embryos of the F_1 generation of reciprocal crosses. SC^1 cultures were prepared from immature F_2 embryos of the cross Aquila × Chinese Spring.

DNA isolation, digestion and electrophoresis. Total DNA was prepared from epicotyls of the varieties Chinese Spring and Aquila and from tissue cultures as described by Dellaporta et al. (1983) with modifications including an exhaustive RNase treatment and a removal of the degraded RNA. Chloroplast DNA was prepared as previously described (Rode et al. 1985) from 10-day old plantlets. Five µg of total DNA was digested by *SalI* in the presence of 4 mM spermidine and in a total volume of 30 µl. DNA fragments were separated by electrophoresis on 0.8% agarose gel slabs in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA, pH 8). Chloroplast DNA digested by *SalI* (about 0.5 µg) was loaded on each gel to permit the detection of chloroplast DNA restriction fragments with homology to the mtDNA probes. After electrophoresis, gels were stained with ethidium bromide (1 µg/ml) and photographed under UV light.

Mitochondrial DNA probes and DNA-DNA hybridizations. *SalI*-cloned wheat mtDNA fragments [K′, X_2, K_3, N_3 and P, according to the nomenclature of Quétier et al. (1985)] were used as labelled probes. Gels were treated as described by Southern (1975) and DNA was transferred onto nitrocellulose membranes (Hybond-C extra, Amersham, UK). Membranes were preincubated in a 2× SSC, 1× Denhardt solution and allowed to hybridize at 42 °C for 14–16 h in a 2× SSC, 45% formamide (v/v), and 100 µg/ml of carrier DNA mixture containing the random primed mtDNA probe. After hybridization, filters were washed in 2× SSC (6 × 10 min at room temperature) then in 0.1× SSC, 0.1% SDS (2 × 30 min at 42 °C) and dried. Autoradiography was carried out at −80 °C for 1–10 days, using X-Omat AR5 Kodak film and intensifying screens. When quantitative data were needed, hybridization signals were scanned with a computerized DESAGA densitometer CD60.

Results

All but one (Palmer and Herbon 1987) of the plant mitochondrial genomes which have been mapped appear to be composed of a collection of minichromosomes (or subgenomic molecules). In theory these can be derived from a "master molecule" due to the presence of sets of recombinogenic repeated sequences. These repeats differ in number according to the genus. For example, there is one set in *B. oleracea* (Palmer and Shields 1984) and at least ten in *T. aestivum* (Quétier et al. 1985). Thus, provided that they have a replication origin, a large number of subgenomic molecules are probably present in *T. aestivum*, their relative amounts perhaps depending on how often each set of repeated sequences recombines. Most of the in vitro-induced rearrangements take place in a well-defined region of the genome encompassing two of the ten major sets of repeated sequences (Hartmann et al. 1987; Rode et al. 1987). In Fig. 1, a schematic diagram of this variable region is presented and shows the changes which occur in the genome of tissue cultures prepared from immature embryos of varieties Chinese Spring and Aquila.

Certain *SalI* fragments derived from the variable region can be used as reliable molecular probes for studying changes in the organization of the mitochondrial genome in tissue cultures. As shown in Fig. 1, the loss – or almost complete loss – in Aquila cultures of fragments J′ and K′ (encompassing RS5), X_2 (a unique sequence) and N_3 (en-

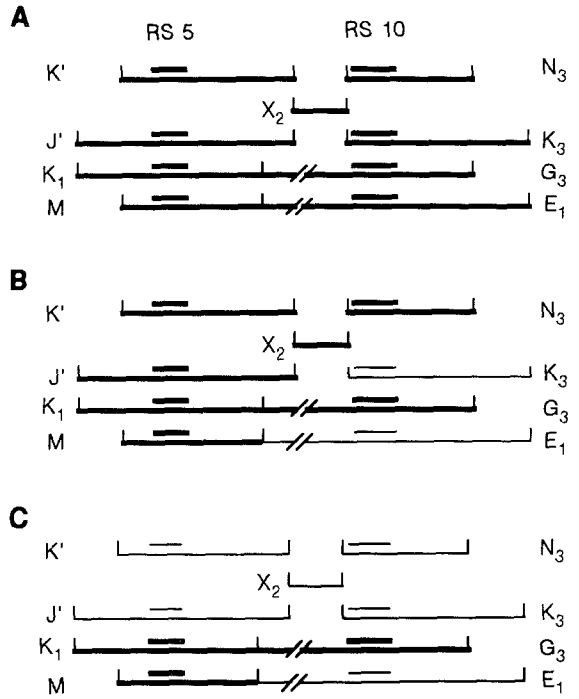

Fig. 1 A–C. Arrangement of the *SalI* restriction fragments in a variable region of the mitochondrial genome. **A** cultivated plants of the isoplasmic varieties Chinese Spring and Aquilla. **B** tissue cultures initiated from immature embryos of the variety Chinese Spring. **C** tissue cultures initiated from immature embryos of the variety Aquila. Four different sequence arrangements occur due to the recombination between the repeated sequences RS5 and RS10, the positions of which are shown by *bars* above the lines representing individual fragments (**A**). The nomenclature of the restriction fragments is that of Quétier et al. (1985). The restriction fragments were aligned with respect to the homologous repeated sequences they contain and shortened versions (*II*) of the fragments G_3 and E_1 are shown. The *thin lines* indicate the location of fragments which are lost or are considerably diminished in amount in tissue culture

compassing RS10) differentiates Aquila cultures from Chinese Spring cultures in which these fragments are always present and even amplified.

Mitochondrial genome organization in tissue cultures prepared from F_1 embryos of reciprocal crosses: effect of nuclear complement on mtDNA configuration

The banding patterns obtained with DNA from the parental varieties (Fig. 2, lanes 1 a and 1 b) were identical to each other demonstrating their isoplasmicity. However, the arrangement of mtDNA in tissue cultures derived from these two varieties (lanes 2 a and 2 b) was different and therefore alloplasmic. In contrast, the mtDNAs in tissue cultures derived from the embryos of F_1 crosses between these varieties were virtually the same as one another (lanes 3 a, 3 b and 4 a, 4 b) and as that in cultures derived from Aquilla (lane 2 b). For example, fragments, J', K' (panel A), X_2 (panel B) and N_3 (panel C) are absent, or considerably diminished in amount, in comparison with Chinese Spring. These results suggest that the severe reduction in the amount of certain molecular configurations is due to the presence in a dominant form of Aquila nuclear gene(s) which either suppress or else do not promote the replication of certain molecular species in tissue cultures. As common wheat is allohexaploid (genome formula: AABBDD) it will be interesting to see whether the dominant Aquila gene(s) is (are) located on one or more of the homoeologous chromosomes. Concurrent with the loss of structures containing certain fragments, new hybridizing fragments appeared (Fig. 2, lane 2 a) in Chinese Spring tissue cultures. These new fragments ("b" and "f") are also found in the mtDNA of SC^2 tissue cultures derived from the embryos of F_1 crosses (lanes 3 a

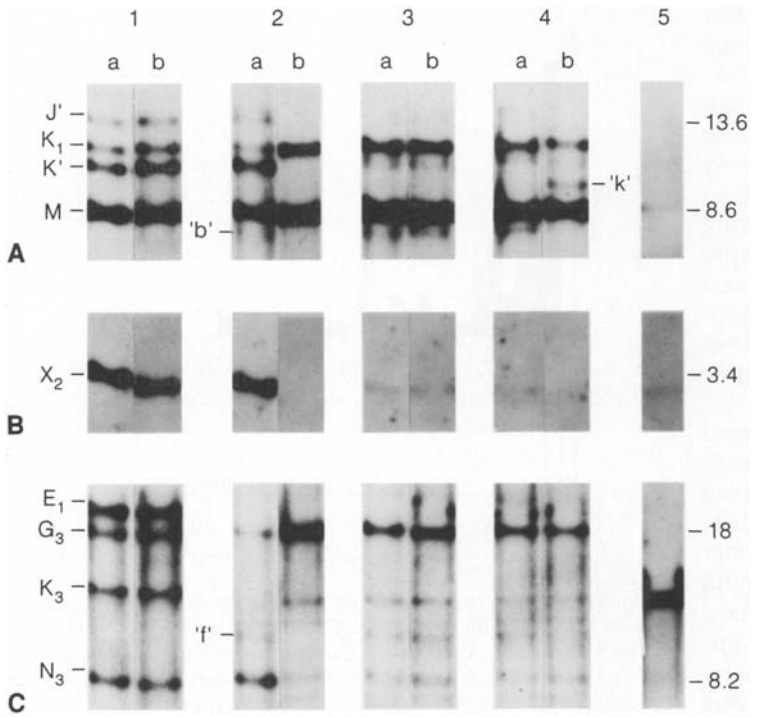

Fig. 2 A–C. DNA gel blot analysis of *SalI*-digested total cellular DNA of tissue cultures prepared from F_1 embryos of the reciprocal crosses between varieties Chinese Spring and Aquila. DNA was probed with a series of cloned mtDNA fragments (**A**, probe K'; **B**, probe X_2; **C**, probe K_3). The identity of hybridizing fragments is indicated on the left and fragment size (in kb) on the right. The symbols "*b*", "*f*" and "*k*" identify hybridizing fragments from novel mtDNA configurations (see text). *Lanes 1 a and 1 b* contain DNA from the parental Chinese Spring and Aquila lines respectively. *Lanes 2 a and 2 b* have DNA from corresponding SC^5 tissue cultures. In *lanes 3 a and 3 b* there is DNA from SC^2 tissue cultures derived from the crosses Chinese Spring (♀) × Aquila (♂) and Aquila (♀) × Chinese Spring (♂) respectively. *Lanes 4 a and 4 b* contain DNA from the corresponding SC^5 cultures. *Lane 5* contains purified chloroplast DNA to permit identification of any fragment with homology to the mitochondrial probes used

and 3b). Thus, new molecular organizations in tissue cultures can also occur due to the presence of a gene or genes in dominant form in the Chinese Spring background. In contrast to fragment "f", fragment "b" had almost completely disappeared in the SC^5 culture of the Aquila × Chinese Spring cross (lane 4b). In these, a new fragment ("k") became apparent. It is thus likely that increasing time in culture favours the generation of new recombination products.

From the results, it may, therefore, be concluded that there are nuclear genes which control changes in the organization of the mitochondrial genome in tissue cultures.

Mitochondrial genome organization in tissue cultures prepared from F_2 embryos obtained by selfing the Aquila × Chinese Spring cross: evidence for Mendelian inheritance of controlling elements

DNA samples (78) were prepared from individual cultures of F_2 embryos and the *Sal*I-restricted DNAs were blotted onto nitrocellulose. The same blot was probed with fragment X_2 (Fig. 3) and, after dehybridization, with fragment P (data not shown) which belongs to a region of the genome unaffected by tissue culture. A visual examination of the results shown on Fig. 3 suggests that three groups of fragment intensities exist. Samples B8, F9, G4, G7, G8, and G9 gave a strong hybridization signal implying that in these fragment X_2 was at approximately the same concentration as in Chinese Spring tissue cultures.

About 40 samples had only a small amount, or none, of this fragment as shown by a weak or undetectable hybridization signal. This was also found with Aquila tissue cultures (Fig. 2b). The remaining 30 samples gave a hybridization signal of intermediate intensity. However, the division of hybridization intensity into three visual groups can be considered as somewhat subjective. For this reason, the autoradiograms obtained after probing with fragments X_2 and P were scanned with a computerized densitometer. Then, the X_2/P intensity ratio was calculated for each DNA sample. The resulting histograms (F_2 and parental genotypes) are shown in Fig. 4. If only a single dominant or recessive nuclear gene was involved in the regulation of the amount of the X_2-hybridizing fragment then a 3:1 ratio in the different phenotypes would be expected. A chi^2 analysis showed that the ratio observed fits a theoretical 1:6:9 expected for the inheritance of two independent complementary nuclear genes involved in regulating the amount of the subgenomic molecules containing fragment X_2.

The results obtained from tissue cultures prepared from embryos of the F_1 progeny of reciprocal crosses thus suggest that two different sets of genes are involved in controlling the rearrangements of the mitochondrial genome. The first set is concerned with the copy number of some molecular species already present in parental plants while the second set affects the appearance of new molecular organizations. It seemed possible to distinguish unambiguously between these two types of genes by probing digests of DNA samples prepared from tissue

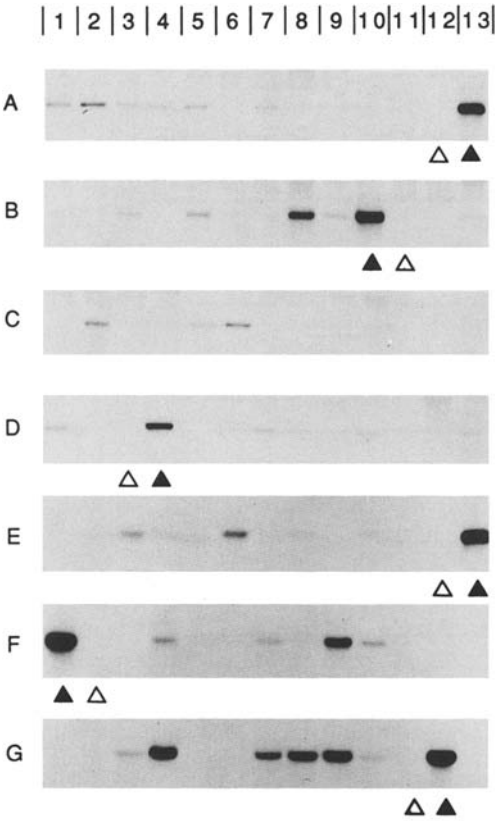

Fig. 3. DNA gel blot analysis of *Sal*I-digested total cellular DNA of tissue cultures prepared from F_2 embryos of selfed F_1 plants. Five µg of DNA prepared from 78 individual cultures was probed with the cloned mtDNA fragment X_2 which is specifically lost in Aquila tissue cultures. Control samples derived from Chinese Spring and Aquila are shown by (▲) and (△) respectively. DNA samples are indexed with *numbered horizontal lines (1–13)* and *lettered vertical lines (A–G)*. There is no DNA sample in G13

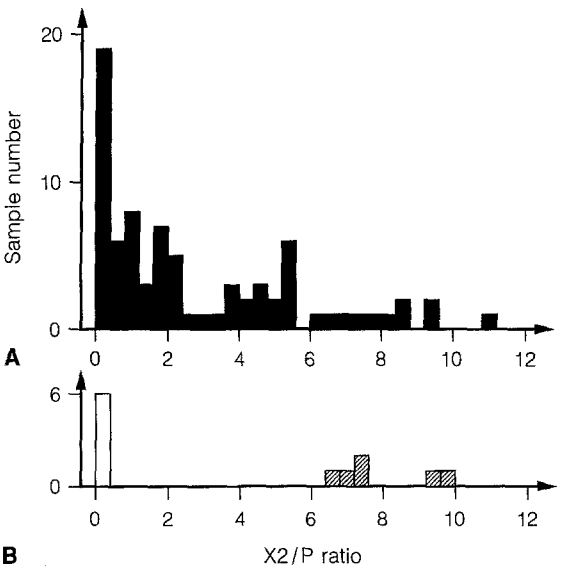

Fig. 4A, B. Histograms showing distribution of the X_2/P intensity ratio from hybridization of *Sal*I-restricted total DNA with fragments X_2 and P. **A** individual cultures prepared from F_2 embryos (*black bars*). **B** individual control cultures prepared from Aquila (*white bars*) and Chinese Spring (*dashed bars*) embryos

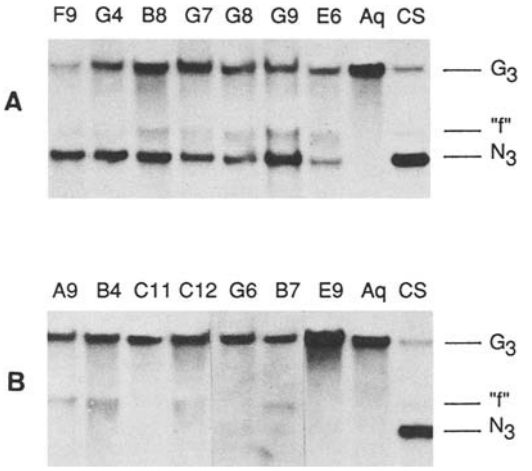

Fig. 5A, B. DNA gel blot analysis of selected DNA samples from tissue cultures of F_2 embryos in which the X_2 fragment was either present or undetectable. Five μg of DNA was probed with the cloned mtDNA fragment N_3. **A** DNA samples encompassing fragment X_2 (see Fig. 3). **B** DNA samples lacking fragment X_2 (see Fig. 3). The band marked "*f*" is the same as that identified in Fig. 2. *Aq*, control tissue cultures prepared from Aquila, *CS*, control tissue cultures prepared from Chinese Spring

cultures with fragment N_3. When this fragment is used as a probe, both fragments N_3 (present in parental plants but either lost or considerably diminished in tissue cultures initiated from Aquila) and "f" (absent in parental plants but present in tissue cultures initiated from Chinese Spring) can be detected. This was so for all the DNA samples giving a strong hybridization signal when probed with fragment X_2 (Fig. 5, panel A). In contrast, when the same experiment was carried out with some DNA samples in which fragment X_2 was undetectable (samples A9, B4, C11, C12, G6, B7, and E9, cf. Fig. 3), we were able to confirm (Fig. 5, panel B) the existence of two separate sets of genes. Indeed, some samples lacked both N_3 and "f" fragments (samples C11, G6, and E9) whereas other samples (A9, B4, C12, and B7) contained only fragment "f" thus showing that the genes controlling them assorted independently.

Discussion

From restriction enzyme mapping and cosmid-clone analysis, it has been proposed by several authors that plant mitochondrial genomes are circular in structure. It also seemed, from these analyses, that while some of the fragments produced by restriction enzyme digestion were from an intact "master" molecule containing all the unique genetic information, others might be from a population of smaller subgenomic circles, with interconnexion between the master and subgenomic forms occurring as a result of recombination between repeated sequences. However, no large circles of the predicted size for either the master or subgenomic molecules have yet been isolated from plant mitochondria (Bendich and Smith 1990). Most of the molecules which were extracted seemed to be in a 50–100 kb linear form, although some much larger linear molecules were also observed. In fact, a circular restriction map could be derived from the latter if they consisted of concatamers of monomer units. Furthermore, studies on yeast mitochondrial DNA show that there is extensive recombination between homologous sequences (Sena et al. 1986) so that several copies of the genome could be associated in a macromolecular complex.

Clearly then, there is still much to be discovered about the way in which the mtDNA of plants is organized and this complicates the interpretation of our results. What we have been able to show here is that the nuclear genome has an important role in determining the structure of the mitochondrial genome of wheat, with at least two sets of genes being involved. It was possible to demonstrate this because the mitochondrial genomes in tissue cultures prepared from embryos of the Chinese Spring and Aquila varieties were rearranged differently although their arrangements were apparently identical in the parental tissues. In tissue cultures initiated from the embryos of F_1 crosses between these two varieties, it was found that Aquila had dominant allelic forms of genes which caused the disappearance of some subgenomic structures which are present in both the parental varieties. It is of interest to stress the similarity between our results and the recent study of Belcour et al. (1991) which showed that a site-specific deletion in mtDNA of the filamentous fungus *Podospora anserina* is under the control of two nuclear genes.

Chinese Spring, on the other hand, has dominant allelic forms of a nuclear gene or genes which are involved in the appearance of new molecular organizations. The results of a further study carried out with tissue cultures initiated from the F_2 progeny obtained by selfing the Aquila × Chinese Spring cross demonstrated that there was Mendelian inheritance of these genes, and that differing alleles exist at the same locus.

How then do these nuclear genes exert their influence on the genome of this organelle? One possibility is that those genes which alter the relative stoichiometries of certain fragments regulate the replication of the subgenomic structures from which the fragments were derived. This could occur if the subgenomic structures each have their own origin of replication so that their relative amounts could be altered in characteristic ways in the tissue cultures of different varieties. It is not so easy to see how this would be possible if only a master molecule, from which all other subgenomic arrangements were derived by recombination, was replicated.

With respect to the Chinese Spring gene or genes which are involved in the appearance of new fragments, these could code for recombinases. As mentioned above, there is already evidence for abundant recombination in mitochondrial DNA. What we are seeing though is the formation of new configurations and this indicates some degree of recombinase specificity. Perhaps the gene for such an enzyme is activated in the Chinese Spring nuclei when tissue culture is initiated.

From these studies, therefore, we might expect to find changes in the type and amount of mitochondrial recom-

binases and replicases due to changes in the expression of the nuclear genome when tissue culture is initiated. An alternative explanation would be that this differential reorganization of the mitochondrial genome could be due to differing selection pressures on the mitochondria in the different cultures. In cultured cells, mitochondria are highly metabolically active. Rapid cell division and repeated subculturing could allow for the segregation of cells carrying mitochondrial genome variants. As the nuclear genomes of Chinese Spring and Aquila probably differ at a large number of loci, mtDNA variability could be the consequence of incidental pleiotropic effects on nuclear genes not directly involved in mtDNA structure.

To-date, evidence that nuclear genes interact with the plant mitochondrial genome to alter its conformation is exclusively derived from studies involving the conversion of cytoplasmic male-sterile lines to another nuclear background by recurrent backcrossing (Mackenzie et al. 1988; Escote-Carlson et al. 1990). Thus, somatic tissue culture could represent another attractive system to study further the role of nuclear-cytoplasmic interactions in the elaboration of the plant mitochondrial genome.

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