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Rearrangements in sugar beet mitochondrial DNA induced by cell suspension, callus cultures and regeneration

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Abstract. Structural alterations in mitochondrial DNAs (mtDNAs) from a plant of a sterile sugar beet line, callus derived from it, suspension-cultured cells and plants regenerated from the callus were studied. *Bam*HI restriction analysis revealed that structural alterations between the mtDNAs of the callus and the control plant had occurred. Multiple rearrangements were also demonstrated in the mtDNA from the suspension culture, of which some were similar to those appearing in the callus, and others had arisen de novo. Rearrangements were also identified by means of blot hybridization of *Bam*HI-digested mtDNA from suspension-cultured cells with the genes encoding subunit II of cytochrome oxidase (*cox II*) and subunit 1 of NADH-dehydrogenase (*Nd1*). No alterations were observed in the mitochondrial genome of the callus and regenerants. The location of the genes for the α-subunit of F1-ATPase (*atpA*) and apocytochrome b (*cob*) in the mtDNA remained unchanged.

Our salient finding was of a plant with an altered mitochondrial genome as judged by *Eco*RI and *Bam*HI restriction analysis. This exceptional plant had retained the sterile phenotype like all of the other regenerants and the parent. The set of plasmid-like molecules of mtDNA remained the same as that in the control plant and in all of the regenerants, callus and suspension-cultured cells. The only type of plasmid-like molecule found in all of the DNAs was the 1.6-kbp minicircle, which is a feature of sterile cytoplasms. These structural changes in mtDNA were obviously a consequence of somaclonal variation during the in vitro cultivation of the sugar beet cells.

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Introduction

It is known that rearrangements in the plant mitochondrial genome can be induced by the in vitro culture of cells. Differences in mitochondrial genome organization between cultured and parent plants have been detected by restriction analysis in such plants as tobacco (Dale et al. 1981), pearl millet (Ozias-Akins et al. 1987), wheat (Hartmann et al. 1989; Rode et al. 1988), *Brassica campestris* (Shirzadegan et al. 1989) and rice (Chowdhury et al. 1990; Saleh et al. 1990). Structural changes in mitochondrial DNA (mtDNA) have been observed during callogenesis in rice (Oono 1987) and also during regeneration from the callus of plants such as maize (Gegenbach et al. 1981), wheat (Galiba et al. 1986) and sugar beet (Brears et al. 1989).

Structural changes of this kind are attributable to the nuclear genome and are known as somaclonal variation. Similar changes can also be taken advantage of in the construction of new mitochondrial genomes of higher plants and also in the production of structural mutations in the nuclear and mitochondrial genes.

Of interest is the development of new mutant types of mitochondrial genomes in sugar beet with male-sterile cytoplasm. Cytoplasmic male sterility (CMS) is a trait encoded by the mitochondrial genome, and it is economically important in hybrid seed production. To our knowledge, Owen's type of sterility was the only source of the CMS trait in sugar beet. As a result, there arose the hazard of a ubiquitous infection with the same pathogen in all the sugar beet plants having the

same modified mtDNA organization and their eventual elimination. In fact, this was observed for maize with the CMS-T cytoplasm (Leaver and Gray 1982).

The aim of the work presented here was to study the structural alterations in the mitochondrial genome of sterile sugar beet that occur during cell cultivation and regeneration from callus. We attempted to answer the question of whether or not plants with the mutant genotype retain their original phenotype. Another question was whether a new type of CMS can arise as a consequence of rearrangements in the mtDNA of sugar beet.

Materials and methods

Plant material

A diploid CMS sugar beet line no. 22003 (supplied by the Kralice na Hane Breeding Station, Czechoslovakia) was used. The primary culture was derived in 1985 from axillary buds isolated from a flowering plant with confirmed pollen sterility.The cultivation continued on LS medium (Linsmaier and Skoog 1965) with one-half concentration of mineral salts, 20 g/l sucrose and 5 µg kinetin or BAP supplement. The culture was kept at 23° ± 2 °C under a 16-h photoperiod. Shoot tips propagated this way were used as explants for the induction of embryogenic callus (Kubalakova 1990). The callus was cultivated on either standard MS (Murashige and Skoog 1962) or PGo (DeGreef and Jacobs 1979) medium without growth regulators. A suspension culture was induced from friable callus and cultivated on MS or PG medium. Plants, which were propagated in vitro, and regenerants from callus culture were planted in a sterile soil substrate. Rooted plants were transplanted first to a greenhouse and finally to an experimental field. About 25 plants propagated in vitro and about 80 plants regenerated from callus culture were examined annually under field conditions. Pollen grains were stained with acetocarmine, and anthers were sampled from buds just before opening. Sampling was carried out 3 times during the blossoming period. All of these plants had the sterile phenotype.

The sugar beet plants of the sterile msSOAN-31 and fertile SOAN-31 and SOAN-742 lines were kindly provided by Dr. S. Maletsky (collection of the Population Genetics Laboratory of the Institute of Cytology and Genetics).

Isolation of mtDNA

Mitochondria from callus and suspension-cultured cells were isolated in principle as previously described for sugar beet seedlings (Dudareva et al.1988a, b); the lysis of the mitochondria and further preparation of mtDNA were carried out according to Rogers and Bendich (1985). MtDNA was isolated from sugar beet roots as described elsewhere (Rogers and Bendich 1985).

Electrophoresis and Southern blotting

Samples of mtDNA were digested with *Bam*HI, *Eco*RI and *Sal*I restriction endonucleases under standard conditions and then fractioned in horizontal 0.8% agarose gels in TAE buffer (Maniatis et al. 1982). Gel electrophoresis of the undigested mtDNA was carried out in 1.5% agarose gels. The gels were stained with 0.5 mg/l ethydium bromide, photographed and transferred to nitrocellulose filters according to Southern (Maniatis et al. 1982). The products of *Hin*dIII and *Eco*RI restriction of phage lambda DNA were used as molecular weight standards.

Hybridization analysis of mtDNA

Southern blots were probed with the cloned mitochondrial genes. Genomic clones of maize subunit II of cytochrome c oxidase (*cox II*), alpha subunit of F1-ATPase (*atpA*) and apocytochrome b (*cob*) were gifts from Dr. C. J. Leaver, Edinburgh University, Scotland. The clone of subunit 1 of the *Oenothera* NADH-dehydrogenase gene (*Nd1*) was provided by Dr. A. Brennicke.

Minicircle c from sugar beet mtDNA was kindly provided by Dr. C. Thomas, John Innes Institute, Norwich, UK.

Integrated minicircle c and mitochondrial gene sequences were recovered from the recombinant plasmids by electroelution and then labelled by random priming (Maniatis et al. 1982). The hybridization and filter washings were done under standard conditions (Maniatis et al. 1982). The filters were autoradiographed on X-ray film at −70 °C.

Results

Restriction analysis of callus and suspension-cultured cells mtDNAs

MtDNA was isolated from a plant of a sterile line, callus and in vitro-cultured cells derived from the sterile sugar beet plant lines. *Bam*HI restriction analy-

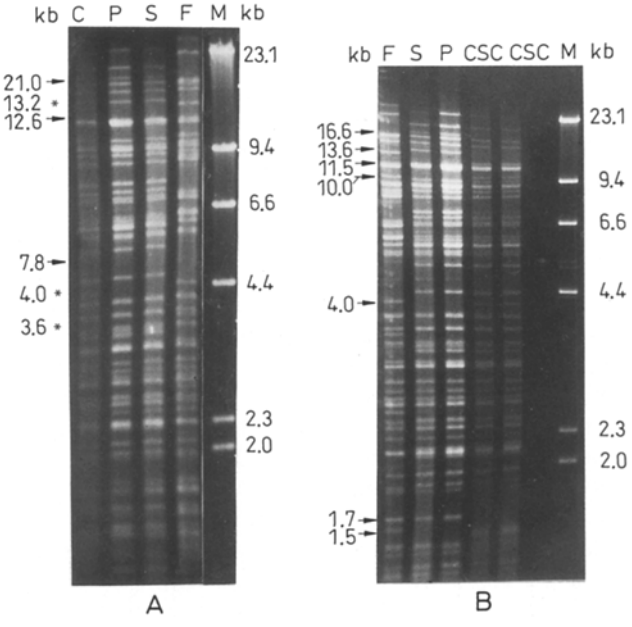

Fig. 1A, B. Agarose gel electrophoresis of *Bam*HI-digested mtDNAs from sterile line no. 22003 (*P*), callus (*C*) derived from a plant of this line and cell suspension culture (*CSC*). Sterile line msSOAN-31 (*S*) and fertile SOAN-31 line (*F*) were used as sterile and fertile standards. In **A** the arrows indicate the position of callus-specific fragments; in **B**, the differences between the suspension culture and the parental plant mtDNAs. *Hin*dIII restriction fragments of lambda phage DNA (*M*) were used as molecular weight markers (kb)

sis of mtDNA was used to identify culture-associated mtDNA rearrangements. The mtDNA of the control plant, the callus and cell suspension culture differed in a set of *Bam*HI fragments (Fig. 1). Callus mtDNA contained several *Bam*HI fragments that were missing from the mtDNA of the control plant; one of these, however, a 4.0-kb fragment, was present in the mtDNA from cultured cells. Three of these additional fragments, 13.2 kb, 4.0 kb and 3.6 kb, were observed among the mtDNA restriction products of the fertile line SOAN-31 used as a standard (asteriks on Fig. 1A). Marked alterations were detected in the mtDNA of the cell suspension culture. The mtDNA of cultured cells differed in restriction pattern from those of the control plant, callus and fertile line SOAN-31: the two *Bam*HI fragments, 16.6 kb and 13.6 kb, which were characteristic of the mtDNA pattern from the parental sterile line were missing or present in small copy number in the restriction pattern of the cultured cells, and the 1.7-kb *Bam*HI fragment of the parental and fertile mtDNA did not appear in the mtDNA of cultured cells. The additional 10.0-kb and 4.0-kb *Bam*HI mtDNA fragments of the cell suspension culture were also present in the mtDNA of the fertile plants, the last (4.0 kb) being characteristic of callus mtDNA. It should be noted that the restriction pattern of the mtDNA cultured cells contained 11.5- and 1.5-kb frangments that were specific to it. Obviously, a portion of the rearrangements which occurred in callus mtDNA was retained by the mtDNA of suspension-cultured cells; concomitantly alterations occurred making the mtDNA of the cultured cells structurally similar to that of the fertile plant. Unique rearrangements were also observed.

Hybridization analysis of mtDNAs

Southern blots of *Bam*HI-digested mtDNA from the control plant, callus and suspension-cultured cells were probed with *cox II*. Previous hybridization experiments with *cox II* have shown that it is feasible to distinguish fertile from sterile sugar beet lines and that it is also possible to identify sterile cytoplasms of different origin (Dudareva et al. 1988a). Therefore, we used the *cox II* gene as the informative probe in the analysis of alterations in the sugar beet mitochondrial genome. The hybridization patterns of the control plant and the callus were identical (Fig. 2a). The mtDNA of the cultured cells contained an additional 3.0-kb *Bam*HI fragment that hybridized weakly with the probe; this fragment was absent from the mtDNA of the fertile and sterile lines (Fig. 2b).

We failed to find any differences in the location of *Nd1* between the callus and the control plant mitochondrial genomes (data not shown). Using the *Nd1* gene probe, we found that the mtDNA of cultured cells

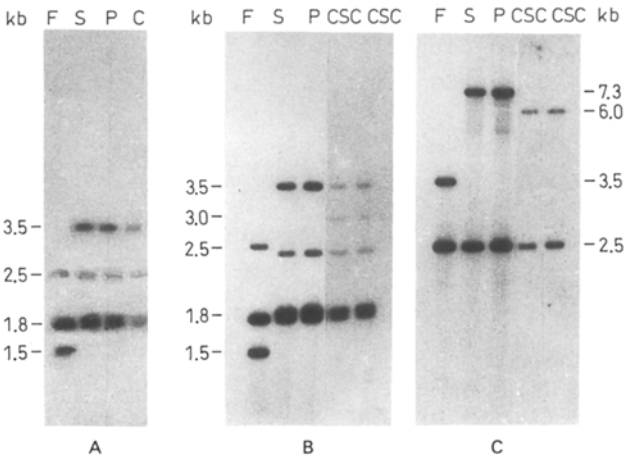

Fig. 2A–C. Hybridization of *Bam*HI-digested mtDNAs from callus (*C*), cell suspension culture (*CSC*), parental sterile line (*P*), sterile msSOAN-31 (*S*) and fertile SOAN-31 (*F*) lines with *cox II* (**A**, **B**) and *Nd1* (**C**) probes

differed in a set of hybridizing fragments from the mtDNA of sterile line msSOAN-31 and callus (data not shown) as well as from the mtDNA of fertile line SOAN-31. MtDNA of the cultured cells contained a 2.5-kb fragment similar to those found in the mtDNAs of fertile and sterile sugar beet lines. However, mtDNA of the cultured cells contained a specific 6.0-kb fragment that hybridized with the *Nd1* probe; there was no 7.3-kb fragment in the parental sterile cytoplasm (Fig. 2c).

Blot hybridization of the mtDNA of the control plant, callus and cultured cells with the probes *atpA cob* did not disclose any differences (data not shown). Hence, while hybridization analysis with the *cox II* and *Nd1* probes revealed significant rearrangements in suspension-cultured mtDNA, we did not succeed in identifying rearrangements in callus mtDNA when using the labelled genes.

Analysis of alterations in plasmid-like molecules

It has been demonstrated that the mitochondrial genome of sugar beet as well as containing high-molecular-weight DNA also contains low-molecular-weight, plasmid-like circular molecules: minicircles a (mc a, 1.6 kbp), b (mc b, 1.5 kbp), c (mc c,1.4 kbp) and d (mc d, 1.3 kbp) (Powling 1981; Hansen and Marcker 1984; Thomas 1986). The genomes of the fertile sugar beet lines differ from those of the sterile line in the number and type of these molecules. Minicircle c and one or two other types of minicircles – a, b or d – are present in the set of plasmid-like molecules of mtDNA from fertile lines, whereas mtDNA from sterile lines of *Beta vulgaris* L. contains only a single type of minicircle in most cases (Powling 1981; Powling and Ellis 1983;

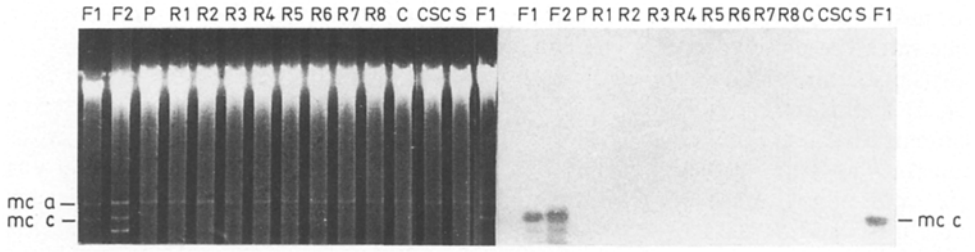

Fig. 3. *Left* Electrophoresis on 1.5% agarose gel of undigested mtDNAs from a parental sterile sugar beet line (*P*), callus (*C*), cell suspension culture (*CSC*) and eight plants, regenerated from callus (R1–R8). Fertile lines SOAN-742 (*F1*) and SOAN-31 (*F2*) and sterile line msSOAN-31 (*S*) were used as fertile and sterile standards. *Right* Autoradiographs of the above-mentioned DNAs hybridized with the labelled minicircle c probe

Dudareva et al. 1988a, b; Weihe et al. 1991). Moreover, it has been shown that the reversion to sterility is accompanied by the loss of minicircle c from the fertile sugar beet mitochondrial genome, and that the sterile "reverted" plants contain only minicircle a (Dudareva et al. 1990).

The low-molecular-weight fraction of mtDNA can be separated by 1.5% agarose gel electrophoresis. Figure 3 presents an electrophoregram of mtDNAs from a parental sterile sugar beet line (P), regenerated plants (R1–R8), callus (C), and a cell suspension culture (CSC). For comparison we analysed mtDNAs from fertile lines SOAN-31 (F1), SOAN-742 (F2) and sterile line msSOAN-31 (S). The mitochondrial genomes of the parental line, callus and cell suspension culture contained plasmid-like molecules of the same type – minicircle a. In contrast, mtDNAs of fertile lines had one or two additional types.

Blot hybridization of minicircle c with mtDNA from the sterile line, the derived callus and suspension cell cultures confirmed the absence of minicircle c from their mitochondrial genomes. This is in contrast to mtDNA from fertile cytoplasms, which contain a sequence homologous to that of minicircle c. Thus, electrophoretic analysis did not make it possible to find differences in the content of plasmid-like molecules in the mtDNA of sugar beet cells during in vitro culturing.

Analysis of mtDNA of callus-regenerated plants

The next step was to analyse the effect of regeneration on the mitochondrial genome structure. Among eight sugar beet plants, regenerated from callus, we recovered one plant with an altered mitochondrial genome. The mtDNA of this plant contained larger amounts of two *Bam*HI fragments of 4.7 and 2.1 kb, and had three additional fragements of 17.0, 5.3 and 3.6 kb that were either missing or present in smaller copy number in the mtDNA of suspension-cultured cells and callus [except for the last fragment of 3.6 kb

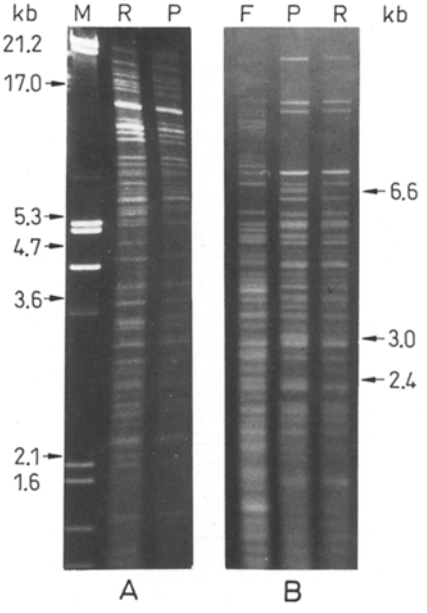

Fig. 4A, B. Electrophoresis of *Bam*HI- (**A**) and *Eco*RI (**B**)-digested mtDNAs from the sterile sugar beet plant regenerated from callus (*R*), parental sterile line (*P*) and fertile line SOAN-31 (*F*). *Hin*dIII and *Eco*RI restriction fragments of lambda phage DNA were used as molecular weight markers (kb)

that was also characteristic of callus mtDNA (Fig. 4A)]. MtDNA from this regenerant differed from that of the other regenerants and the control plant by the absence of a single 6.6-kb *Eco*RI fragment and a lower content of 3.0-kb and 2.4-kb fragments (Fig. 4B). Restriction analysis with the *Sal*I enzyme failed to detect any differences among regenerants (data not shown). Hybridization analysis of the *Bam*HI-, *Eco*RI- and *Sal*I-digested DNAs with the *cox* II, *cob*, *Nd1* and *atpA* genes, used as probes, did not reveal significant differences in the organization of their mitochondrial genomes as compared with the control (data not shown). Nor was any variation observed in the content of plasmid-like molecules of mtDNA from any of the

regenerants. Electrophoretic analysis of mtDNAs from all of the regenerants demonstrated the presence of a single type of minicircle a, similar to that found in the sterile cytoplasm. This conclusion is supported by the result of hybridization of the regenerant mtDNA with the cloned minicircle c sequence (Fig. 3).

On the basis of the field experiments, all of the regenerants were phenotypically sterile, like the control plant. Thus, from an examination of mtDNAs from eight callus-regenerated sugar beet plants we recovered a single plant having an altered mitochondrial genome structure. It should be emphasized that this regenerant retained the sterile phenotype.

Discussion

The present results demonstrate that in vitro cultivation of higher plant cells induces rearrangements in their mtDNA.

The plasticity of the sugar beet mitochondrial genome may be accounted for by its structure. The mtDNA of sterile sugar beet consists of approximately 486 kbp and contains one inverted and four direct repeats which are involved in intra- and intermolecular recombination, thereby producing changes in the set of mtDNA molecules (Lonsdale et al. 1988). Mutations of the deletion and insertion types can also involve repeats.

This is the first report on alterations in the mitochondrial genome of sugar beet at the callus stage as revealed by electrophoretic analysis. These rearrangements, however, did not affect the location of the *atpA*, *cob*, *cox* II and *Nd1* genes in callus mtDNA. Some of the callus mtDNA rearrangements were inherited by the cells of the suspension culture; however, new rearrangements, including reversions, arose, and these increased the similarity between the mtDNA of cultured cells and that of the fertile plants.

Our results agree well with earlier reports on the structure of higher plant mitochondrial genomes. Rearrangements in mtDNA as a result of the in vitro culturing of cells occur in the mtDNA of tobacco (Dale et al. 1981), maize (Chourey and Kemble 1982), wheat (Hartmann et al. 1989; Rode et al. 1988), *Brassica campestris* (Shirzadegan et al. 1989).

In some higher plants, changes in the content of plasmid-like mtDNA molecules have been observed. Cases in point are: (1) cultured cells of *Brassica campestris*, loss of 1.3-kbp plasmid-like DNA molecules; (2) callus cells of sterile maize, disappearance or presumable transposition of S-1 and S-2 plasmid-like molecules (Earle et al. 1987). However, in the present experiments the mtDNAs from callus, suspension-cultured cells, the regenerants and the control plant all, without exception, showed exclusively the "a" minicircle, a feature of *Beta vulgaris* L. sterile lines.

This was taken to mean that only high-molecular-weight DNA has undergone rearrangements during cell culturing and regeneration. Environmental stress may act as an inducer of rearrangements in the mitochondrial genome during in vitro cell culturing. This hypothesis is plausible when one recalls that under stressful conditions, superoxide and hydroxyl radicals may be generated; these can elicit double-stranded breaks in DNA, producing rearrangements in the genome.

Our salient finding was the single regenerant with a modified mitochondrial genome structure that we found among eight regeneranted plants. The *Bam*HI and *Eco*RI patterns of the mtDNA from this exceptional regenerant differed from the pattern obtained for all of the other regenerated plants. It is noteworthy that rearrangements in the mitochondrial genome of this exception arose de novo and that they were not transmitted from the callus. Thus, changes in the mtDNA occurring under selection during callogenesis were eliminated: those cells survived whose mtDNA remained structurally close to that of the parent. Interestingly, the exceptional regenerant had the same sterile phenotype as all of the other regenerants. The recovery of a phenotypically sterile regenerant with an altered mitochondrial genome offers a new possibility for having another source of cytoplasmic male sterility besides Owen's.

The screening of 30 sterile callus-regenerated plants allowed Brears et al. (1989) to identify a sterile plant with a rearranged mitochondrial genome. The restriction pattern shown by this plant was similar to that obtained from plants with fertile cytoplasm, although not identical. This regenerant also retained the sterile phenotype. Alterations in mtDNA have been shown for the regenerants of other higher plants such as rice (Oono 1987), wheat (Galiba et al. 1986) and maize (Gegenbach et al. 1981; Earle et al. 1987).

Consequently, the method of in vitro culture provides a real possibility for constructing new mitochondrial sugar beet genomes.

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