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Molecular aspects of cytoplasmic male sterility in perennial ryegrass (*Lolium perenne* L.): mtDNA and RNA differences between plants with male-sterile and fertile cytoplasm and restriction mapping of their *atp6* and *coxI* homologous regions

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Summary. *Lolium perenne* L. male-sterile and fertile cytoplasms contain different mitochondrial genomes, as revealed by Southern hybridization with a number of heterologous mitochondrial probes. In addition, transcriptional patterns of *atp6* and *coxI* genes distinguish both cytoplasmic types. The majority of the *L. perenne* sequences from male-sterile and fertile cytoplasm showing homology with these two genes has been cloned and mapped by restriction digestion. A complex genomic organization, especially concerning *coxI* homologous sequences, was found in the male-sterile cytoplasm. Furthermore, during the course of these studies tissue-culture-induced mtDNA mutations in a number of *coxI*-containing sequences were detected in regenerated plants.

Key words: *Lolium perenne* – Mitochondrial genome – *atp6* – *coxI* – tissue culture

Introduction

Cytoplasmic male sterility (cms) in higher plants is a widespread phenomenon that is important – and in some crops indispensable – in the commercial production of F_1 hybrid seed. In perennial ryegrass the failure to produce functional pollen was first reported in 1931 (Jenkin). However, the introduction of F_1 hybrid varieties in perennial ryegrass has been hampered by the lack of suitable cms and maintainer types. The *L. perenne* cms type used in this study is stable and originates from a *L. perenne* × *L. multiflorum* hybrid (Wit 1974).

A great deal of evidence points to the mitochondria as carriers of the genetic determinants constituting the cytoplasmic component of cms (Hanson and Conde 1985). Apart from circumstantial evidence, as obtained by restriction fragment or Southern blot analyses of mitochondrial genomes from male-sterile and fertile cytoplasms, the most compelling evidence for mitochondrial involvement comes from somatic hybridizations. Thus, it was shown for *Petunia* that the male sterility of the hybrid progeny segregated independently from plastid type, which implies a role for the mitochondria (Clark et al. 1985). In addition, in *Petunia* the presence of a particular mitochondrial DNA (mtDNA) arrangement derived from the cms fusion partner coincided with the expression of male sterility (Boeshore et al. 1985). Further research identified an open reading frame of chimeric character, i.e., consisting of fragments not normally contiguous in the genome (Young and Hanson 1987). In maize cms-T another chimeric gene is implied as causing cms (Dewey et al. 1986). Mutation or deletion of this *T-urf-13* gene leads to restoration of male fertility (Wise et al. 1987; Rottmann et al. 1987).

In this study we will show differences between the mtDNA organization of male-sterile and fertile perennial ryegrass plants. Furthermore, evidence will be presented or *atp6* and *coxI* homologous transcripts differing between both cytoplasmic types. The majority of the (partial) copies of both of these genes from male-sterile as well as fertile cytoplasm has been characterized by restriction mapping. Finally, we found tissue-culture-induced mitochondrial mutations involving some of the mapped *coxI* copies.

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Materials and methods

Plant materials

In this study the following perennial ryegrass (*L. perenne* L.) genotypes were used: the cms line Lp9, its nearly isogenic maintainer Lp10, and the fertile varieties Lp2 and Lp6 (provided by Barenbrug Holland BV, Wolfheze).

Preparation of total DNA

Total DNA was isolated essentially according to Dellaporta et al. (1983) from green shoots.

Preparation of mitochondrial DNA

Mitochondrial DNA was prepared from actively growing suspension cultures of *L. perenne*, which were initiated and grown as described (Creemers-Molenaar et al. 1989). The procedure was carried out at 4 °C up to mitochondrial lysis. The cells were harvested by filtration over 88-μm nylon mesh and homogenized for 3 min by mortar and pestle in 2 ml of homogenization buffer [0.5 *M* mannitol, 50 m*M* TRIS-HCl (pH 7.5), 10 m*M* KH_2PO_4, 1 m*M* EGTA, 5 m*M* 2-mercaptoethanol, 0.25% PVP-25, 0.2% BSA] per gram of cells (fresh weight) in the presence of sea sand. The suspension was filtered over nylon and squeezed to obtain the remaining fluid. The residue was then reextracted twice. The pooled filtrates were centrifuged for 10 min at 1,500 × *g* and $MgCl_2$ and DNase were added to the supernatant to give final concentrations of 10 m*M* and 50 $\mu g \cdot ml^{-1}$, respectively. After incubation for 60 min at 4 °C 20 m*M* EDTA was added and the suspension was centrifuged once more for 10 min at 1,500 × *g*. The resulting supernatant was layered on top of a 7.5-ml sucrose cushion (0.6 *M* sucrose, 10 m*M* EDTA) and spun for 25 min at 12,000 × *g* in a Sorvall SS-34 rotor. The mitochondrial pellet was resuspended in lysis buffer [50 m*M* TRIS-HCl (pH 8.0), 10 m*M* EDTA, 2% sarcosyl, and 200 μg/ml proteinase K] and incubated for 30 min at 37 °C. The DNA was purified by phenol/chloroform extraction and precipitated with ethanol. The pellet was dried under vacuum and taken up in 10 m*M* TRIS-HCl (pH 8.0), 1 m*M* EDTA (TE).

Preparation of total RNA

Total RNA was isolated as described by De Vries et al. (1982) from green shoots.

Heterologous probes

The mitochondrial genes used as probes were those coding for ATPase subunits 6 (Dewey et al. 1985a), 9 (Dewey et al. 1985b), and A (Schuster and Breenicke 1986) and for cytochrome *c* oxidase subunits 1 (Isaac et al. 1985), 2 (Fox and Leaver 1981), and 3 (Hiesel et al. 1987).

Subcloning

Subclones from selected lambda phages (see below) were made by cloning gel-eluted (Tautz and Renz 1983) restriction fragments into plasmid pGEM 9-Zf(-) (Promega).

Restriction endonuclease digestions

Restriction enzymes from various manufacturers were used as recommended except for the digestion of total DNA, where twice the recommended amount of enzyme was used during a 2-h incubation period. In addition, the restriction reaction mix was supplemented with spermidine to a concentration of 2.0 m*M*.

Southern blotting and hybridization

Following separation of restriction fragments on 0.8% agarose gels in 40 m*M* Tris-acetate (pH 7.8), 1 m*M* EDTA, and 0.5 $\mu g \cdot ml^{-1}$ EtBr, the DNA in the gel was depurinated and denatured by successively soaking the gel for 5 min in 0.25 *M* HCl and twice for 15 min in 0.5 *M* NaOH, 1.5 *M* NaCl. Finally, the gel was equilibrated twice for 10 min in the blotting solution (1 *M* NH_4Ac) prior to capillary transfer of the DNA onto nitrocellulose or nylon membranes (Hybond-N from Amersham or GeneScreen from NEN) (Kafatos et al. 1979; Smith and Summers 1980). After transfer, for a minimum of 3 h or overnight, the filter was either baked for 2 h at 80 °C in vacuo or air-dried and UV-crosslinked with an optimized dose using a 30-W Philips germicide lamp.

Plasmid DNA to be used as probe was isolated as described by Birnboim and Doly (1979) and modified by Maniatis et al. (1982). This DNA or gel-eluted restriction fragments (Tautz and Renz 1983) were used for random primed labeling with digoxigenin-11-dUTP. The hybridizations were performed and the DNA/DNA hybrids were visualized by colorimetric (Boehringer Mannheim) or chemiluminescent detection (Bronstein et al. 1989). Colorimetric detection was carried out as suggested by the manufacturer. Chemiluminescent detection was carried out following the same protocol, up to addition of alkaline phosphatase substrates; the filter was then incubated in 100 m*M* TRIS-HCl (pH 8.0), 100 m*M* NaCl, and 50 m*M* $MgCl_2$ supplemented with AMPPD (Tropix) at a concentration of 0.26 m*M* instead of NBT/BCIP. After 20 min the substrate solution was removed from the plastic bag containing the filter, leaving only the adherent solution. Luminography was performed by flattening the resealed bag into an autoradiography cassette and overlaying it with Kodak-X-Omat S film for 45 min.

Northern blotting and hybridization

Total RNA (10 μg/lane) was denatured with glyoxal, omitting DMSO, and fractionated on 1.5% agarose gels in 10 m*M* NaH_2PO_4, pH 7.0 (Thomas 1980). The RNA was blotted onto GeneScreen in an overnight transfer, using 50 m*M* NaH_2PO_4 (pH 7.0), 5 m*M* EDTA as blotting buffer. Subsequently the filter was air-dried, UV-irradiated (see above) and finally deglyoxylated by boiling it for 5 min in 20 m*M* TRIS-HCl (pH 8.0), 1 m*M* EDTA.

^{32}P-labeled probes were prepared by nick-translation and hybridized overnight to filters in 1 *M* NaCl, 1% SDS, 10% dextran sulfate, and 100 μg/ml calf thymus DNA at a probe concentration of 10 ng/ml. Washing of the filters was carried out according to the GeneScreen protocol. Autoradiography was done for various exposure times using Fuji-RX film without intensifying screens.

Construction and screening of lambda libraries from mtDNA

mtDNA from Lp9 and Lp6 was partially digested with *Sau*3A, dephosphorylated by calf intestinal phosphatase treatment, and ligated into *Bam*HI-*Eco*RI double-digested lambdaGEM-11 vector (Promega). The ligation mixture was packaged in vitro and amplified on a lawn of *E. coli* NM539.

The resulting libraries were screened by plating them at low density (between 500 and 1,000 pfu per 9-cm diameter petri dish) on TB medium (1% Tryptone, 0.5% NaCl), with agarose (0.6%) replacing agar in the top layer, and incubated overnight. Plaque lifts were prepared (Benton and Davis 1977) using Hybond-N, and the DNA was crosslinked to the air-dried membrane using UV-irradiation with an optimized dose (see above). Prior to hybridization, filters were pretreated as described by Kincaid and Nightingale (1988). Hybridization with gel-eluted and

digoxigenin-11-dUTP-labeled restriction fragments was performed as described above for Southern hybridizations.

Positives were selected and the phages were used to prepare plate lysates on TB medium, with standard agarose and Ultrapure DNA-grade agarose (Bio-Rad) in bottom and top layers, respectively. Phage DNA was purified (Maniatis et al. 1982) and the insertions were characterized by restriction digestion followed by Southern hybridization.

Results

Mitochondrial DNA differences between male-sterile and fertile cytoplasms

The mitochondrial DNA was prepared from a relatively young suspension culture (4 months) of the male-sterile Lp9 material and from an older suspension (9 months) initiated from fertile Lp2 material. The isolation of mtDNA from green tissues was found to be unsuitable, since only highly variable yields of impure mtDNA could be obtained.

Souther blot analyses of *Eco*RI-digested *L. perenne* mitDNAs with heterologous probes allow clear discrimination between the two cytoplasmic types (Fig. 1). Nevertheless, all probes – except *atpA* – hybridize to at least one *Eco*RI fragment shared by both types. Each probe – again, except *atpA* – detects more than one fragment in both cytoplasmic types, indicating the presence of *Eco*RI sites within the homologous regions or within multiple (partial) copies of the hybridizing regions. The most pronounced example of a complex pattern is provided by the *coxI* probe hybridizing to *Eco*RI fragments from male sterile cytoplasm (Fig. 1 d).

While mtDNA variation as judged by Southern blot analyses of total DNA was virtually absent from a whole range of fertile cytoplasms including Lp10, similar analyses did reveal two male-sterile cytoplasms containing mtDNA differing from Lp9 (results not shown).

Mitochondrial transcripts in plants with male-sterile and fertile cytoplasm

Total RNA from vegetative green material was used to monitor mitochondrial transcripts in male-sterile and fertile plants by Northern blot hybridization with *atp6*, *coxI*, *coxII*, and *coxIII* probes.

All probes hybridized to multiple transcripts, but only the *atp6* and *coxI* probes revealed differences between both cytoplasmic types (Fig. 2). The major *atp6* transcript in fertile cytoplasm was 1.45 kb in size, while male-sterile cytoplasm contained one slightly longer, abundant transcript of 1.55 kb. Some minor, identical transcripts also appeared in both cytoplasmic types.

Differences between mitochondrial transcripts with *coxI* homology from male-sterile and fertile cytoplasms seem to be of a quantitative nature. However, it is not clear, for example, whether or not the apparently single 2.70-kb transcript in male-sterile cytoplasm actually consists of the two minor transcripts of almost equal size in fertile cytoplasm. Similarly, the very abundant 2.25-kb transcript in fertile cytoplasm seems to be present in male-sterile cytoplasm as well, but at a low concentration. In fact, the 2.25-kb transcript in fertile cytoplasm may be a complex of transcripts of slightly variable size.

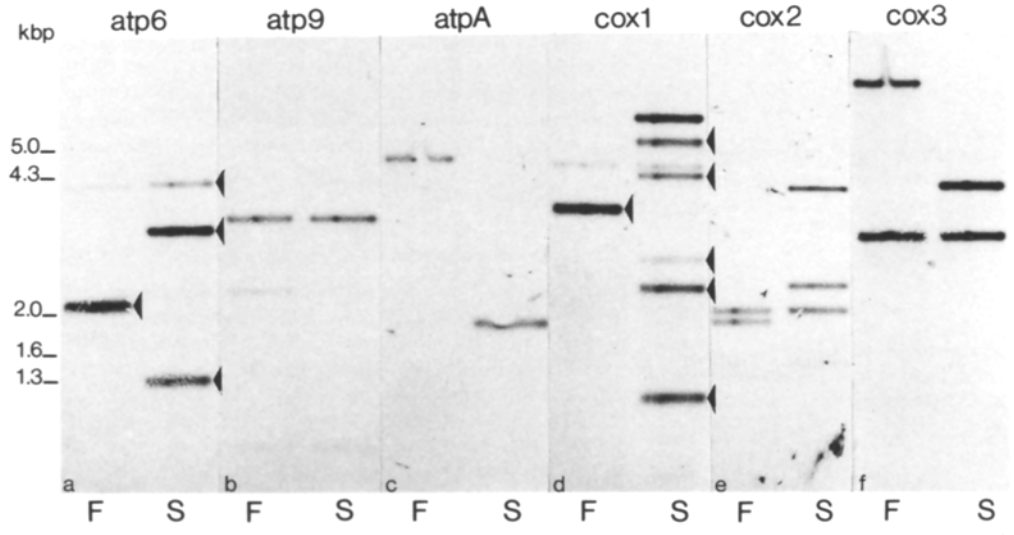

Fig. 1 a–f. Southern blot hybridization of *Eco*RI-digested mtDNA of *L. perenne* Lp9 (S) and Lp2 (F) with digoxigenin-11-dUTP-labeled probes *atp6* (**a**), *atp9* (**b**), *atpA* (**c**), *coxI* (**d**), *coxII* (**e**), and *coxIII* (*f*). *Arrowheads* point to the fragments, which were (partially) cloned. DNA-DNA hybrids were detected as described in "Materials and methods" with NBT/BCIP as alkaline phosphatase substrates. Molecular weight is indicated in kilobase pairs (kbp)

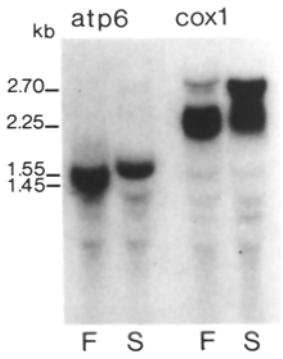

Fig. 2. Northern blot hybridization of total RNA from Lp9 (S) and Lp10 (F) using ^{32}P-labeled *atp6* and *coxI* as probes. The size of the transcripts is indicated in kilobases (kb)

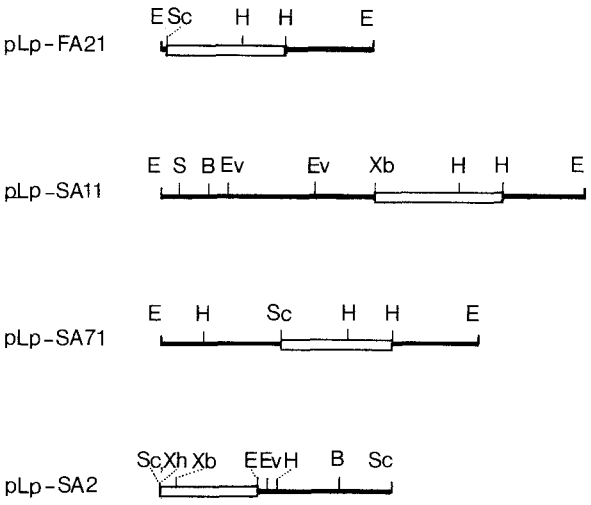

Fig. 3. Restriction maps of subclones from Lp6 (pLp-FA21) und Lp9 (pLp-SA11, pLp-SA71, and pLp-SA3) with *atp6* homology. The *open boxes* indicate the fragments hybridizing to the *atp6* probe. B = *Bam*HI, E = *Eco*RI, Ev = *Eco*RV, H = *Hin*dIII, Sc = *Sac*I, S = *Sal*I, Xb = *Xba*I, Xh = *Xho*I

Characterization of atp6 and coxI homologous sequences from male-sterile and fertile perennial ryegrass

Isolation of recombinant phages. The complex arrangement of mitochondrial sequences containing *atp6* and *coxI* homology and their cytoplasm-specific transcriptional patterns prompted a more detailed study into the genomic arrangement of these genes.

Lambda phage libraries containing mtDNA from both cytoplasmic types were screened with maize *atp6* and *coxI* probes, to identify the phages carrying the hybridizing *Eco*RI fragments visible in Fig. 1 a and 1 d. The DNA from the hybridizing phages was characterized by agarose gel electrophoresis and Southern blot hybridization with the corresponding probe.

The *atp6* homologous phages containing mtDNA from fertile cytoplasm were almost identical, indicating that the *atp6* gene in this cytoplasm is located in one genomic environment. Similarly, the uniformity of the *coxI* homologous phages containing mtDNA of fertile origin suggests that the major *coxI* copy in fertile cytoplasm also resides in a single genomic location in the mtDNA.

The phases containing the *atp6* and *coxI* homologous sequences from male-sterile material represented many of the hybridizing fragments seen in Fig. 1 a(S) and 1 d(S) (indicated by arrowheads). Restriction digestion of the phage DNAs demonstrated that the majority of the hybridizing fragments from male-sterile cytoplasm are probably located in different genomic environments; only the phages with the *coxI* homologous 5.3- and 2.3-kbp *Eco*RI fragments from male-sterile cytoplasm (Fig. 1 d) shared restriction fragments.

Characterization of phage subclones with atp6 homology. The phage DNAs, with the *atp6* homologous *Eco*RI fragments indicated by arrowheads in Fig. 1 a, did not contain additional *Eco*RI fragments hybridizing to this probe (data not shown). The single *atp6* homologous *Eco*RI fragment of each phage clone was subcloned and mapped by restriction digestion (Fig. 3).

The main feature of these subclones is the presence of a small *Hin*dIII fragment containing part of the region with *atp6* homology. The 2.2-kbp *Eco*RI fragment from fertile cytoplasm and the 3.4-kbp *Eco*RI fragment from male-sterile cytoplasm – subclones pLp-FA21 and pLp-SA71, respectively – appear to be highly similar to the right of their common *Sac*I site.

The 1.3-kbp *Eco*RI fragment in male-sterile cytoplasm has not been obtained intact; only a partial *Sac*I subclone (pLp-SA2) was found. Hybridization of pLp-SA2 to a Southern blot with *Eco*RI-digested total DNA showed that it probably does not contain *atp6* sequences, since it did not hybridize to any of the other fragments detected by the maize *atp6* probe (data not shown).

Characterization of phage subclones with coxI homology. The subclones were derived from the phage DNAs carrying *coxI* homologous *Eco*RI fragments corresponding to those detected in Fig. 1 d. Four out of five subclones were obtained from phage DNAs in which the full complement of homologous sequences were present on a single *Eco*RI fragment. Only the subclone containing the 1.2-kbp *Eco*RI fragment (pLp-SC23) from male-sterile cytoplasm had to be cloned as a *Sac*I fragment to prevent the *coxI* homologous region from being disrupted by *Eco*RI digestion (Fig. 4).

Assuming that the size of the *L. perenne coxI* open reading frame will resemble its 1.6-kbp maize equivalent, only the 3.4-kbp clone from fertile cytoplasm (pLp-FC22) and one 2.3-kbp clone from male-sterile cytoplasm (pLp-SC71) would be able to comprise a full-

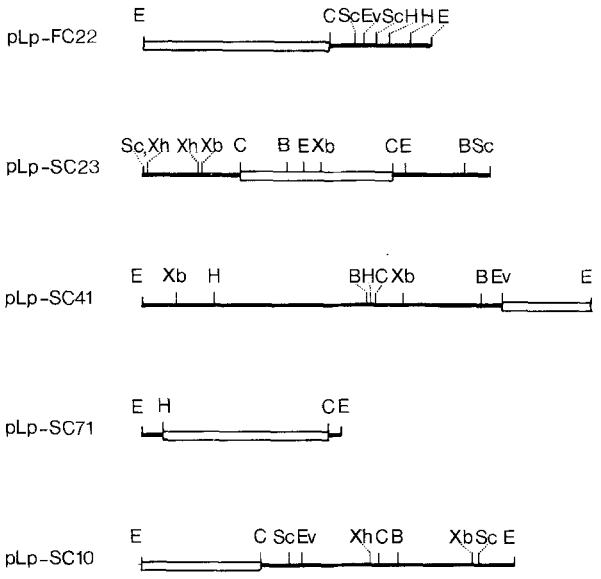

Fig. 4. Restriction maps of subclones from Lp6 (pLp-FC22) and Lp9 (pLp-SC10, pLp-SC23, pLp-SC41, and pLp-SC71) with *coxI* homology. The *open boxes* indicate the fragments hybridizing to the *coxI* probe. B = *Bam*HI, C = *Cla*I, E = *Eco*RI, Ev = *Eco*RV, H = *Hin*dIII, Sc = *Sac*I, S = *Sal*I, Xb = *Xba*I, Xh = *Xho*I

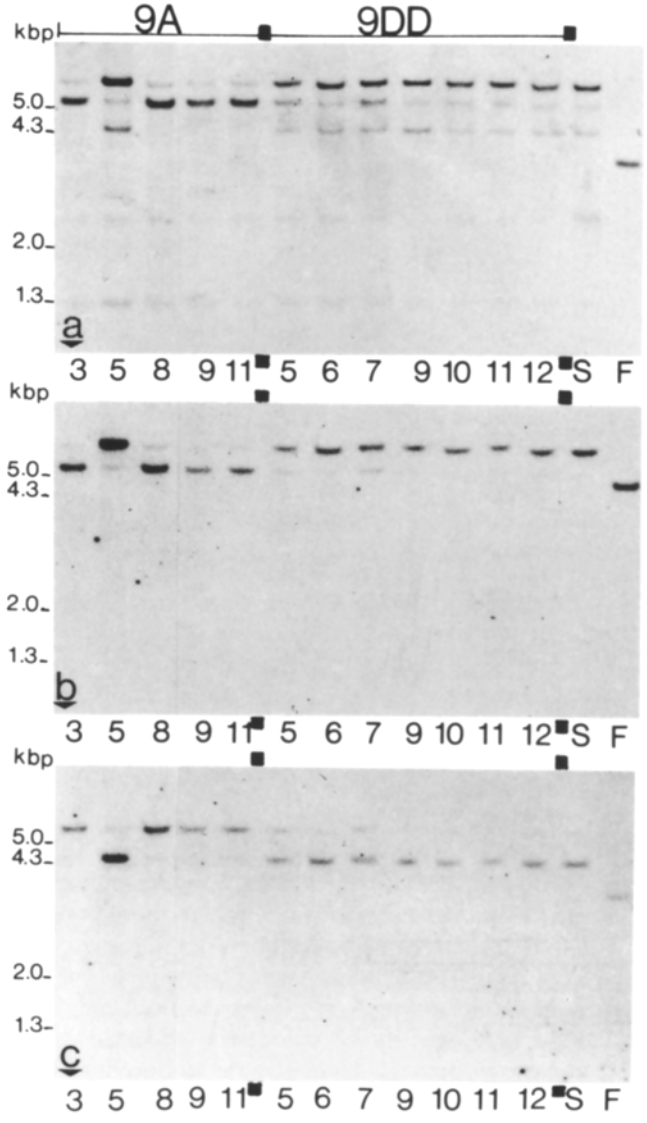

Fig. 5a–c. Southern blot hybridization of *Eco*RI-digested total DNA from plants of lines Lp9 (S), Lp10 (F) and regenerated plants from Lp9 suspension cultures 9A and 9DD. The regenerants from suspension culture 9A were obtained by plating it 9 (nos. 3 and 5), 80 (no. 8), 82 (no. 9), and 92 (no. 11) weeks after its initiation, and those from suspension culture 9DD when it was 5 (nos. 5, 6 and 7), 7 (no. 9), 11 (nos. 10 and 11), and 13 weeks (no. 12) old. The probes used were maize *coxI* (**a**), pLp-SC41 sequences to the left of the *Cla*I site (**b**), and the pLp-SC10 *Cla*I fragment (**c**), which were labeled with digoxigenin-11-dUTP. DNA-DNA hybrids were detected as described in "Materials and methods" using NBT/BCIP (**a**) or AMPPD (**b** and **c**) as alkaline phosphatase substrates. Molecular weight is indicated in kilobase pairs (kbp)

length *coxI* open reading frame. All of the other clones either include *coxI* homologous regions that are too short or else contain changes with respect to the "normal" copy present in fertile cytoplasm.

Clone pLp-SC10 bearing the 4.4-kbp *Eco*RI fragment from male-sterile cytoplasm (Fig. 1 d) could accommodate an almost complete copy of the *coxI* gene. It resembles clone pLp-FC22 from fertile cytoplasm up to a point beyond the actual *coxI* homology just to the right of its *Eco*RV site, beyond which both fragments deviate.

The *coxI* homologous region of clone pLp-SC23, which contains the 1.2-kbp *Eco*RI fragment from male-sterile cytoplasm, differs markedly from its fertile counterpart in clone pLp-FC22, for it contains an *Eco*RI site, a *Bam*HI site, and a *Xba*I site. The *coxI* homologous sequences to the left of the 1.2-kbp *Eco*RI fragment in clone pLp-SC23 are part of the 2.6-kbp *Eco*RI fragment in male-sterile cytoplasm, as was shown by Southern blot hybridization using these sequences as probes (data not shown). In the original phage, clone pLp-SC23 was probably located near the end of the insertion, since the *Sac*I and *Xho*I sites near its end appear to be derived from the vector.

The 5.3-kbp *Eco*RI fragment from male-sterile cytoplasm in clone pLp-SC41 seems to present a clear example of a partial *coxI* gene. First of all, the size of the *coxI* homologous region is much too small to include a full-length copy. Secondly, this homology does not continue into the adjoining fragment because – as already mentioned above – the 5.3-kbp fragment is the only fragment with *coxI* homology present in the phage DNA. Additional information confirming the absence of contiguous mtDNA fragments with *coxI* homology comes from studying the mtDNA compositions of plants regenerated from suspension cultures or protoplasts (Fig. 5). Southern blot hybridizations with the maize *coxI* as probe

demonstrate the specific amplification of a single 5.3-kbp *Eco*RI fragment in four out of five regenerants from suspension 9A (Fig. 5). The hybridization results in Fig. 5b, using the pLp-SC41 sequences to the left of its *Cla*I site as probe, serve to confirm the identity of the amplified 5.3-kbp fragment and pLp-SC41. In addition, they also reveal homology between the 5.3-kbp *Eco*RI fragment and the 7.4-kbp *Eco*RI fragment, apart from the obvious *coxI* homology (Fig. 5b).

The amplification of the 5.3-kbp fragment (Fig. 5) is accompanied by decreased concentrations of the 4.4- and 7.4-kbp fragments. This decrease is already evident in regenerant no. 3 from a suspension culture as young as 9 weeks. On the other hand, the second regenerant from this stage (no. 5) is almost identical to the untreated control, although it has somewhat higher concentrations of both the 4.4- and 7.4-kbp fragments. Thus, it would seem as if the fate of these three – 4.4-, 5.3-, and 7.4-kbp – fragments is somehow connected.

Figure 5c shows that the *Cla*I fragment of pLp-SC10 hybridizes not only to the 4.4-kbp but also to the 5.3-kbp fragment. As this *Cla*I fragment does not contain *coxI* sequences, its homology to the 5.3-kbp fragment (pLp-SC41) must lie to the left of the pLp-SC41 *Eco*RV site. Furthermore, Fig. 5b shows a lack of hybridization between the sequences to the left of the pLp-SC41 *Cla*I site and the 4.4-kbp *Eco*RI fragment (cloned in pLp-SC10). This implies that the hybridizing sequences on the 5.3-kbp *Eco*RI fragment are located between its *Cla*I site and its region with *coxI* homology. Probes *atp6* and *coxII* did not reveal any rearrangements in the regenerated plants (results not shown).

Discussion

The mitochondrial genome analyses of *L. perenne* cms and fertile lines by Southern blot hybridization are in agreement with the general observation that cms is associated with mtDNA differences, although some of the hybridizing fragments may result from tissue-culture-induced changes. A detailed picture of the mitochondrial genomes of *L. perenne* cms and fertile lines was obtained by analyzing mtDNA clones with *atp6* and *coxI* sequences, which were selected for closer examination because of their cytoplasm-specific transcripts and complex Southern hybridization patterns.

The most abundant *atp6* copy from fertile cytoplasm appears to be located in a single genomic environment, considering the homogeneity of the recombinant phages carrying it. Similarly, the uniformity of the phages with *coxI* homologous sequences from fertile cytoplasm also suggests only one location in the mitochondrial genome for the major *coxI* copy in this cytoplasmic type. The additional, minor hybridization signals with *atp6* and *coxI* probes [Figs. 1a(F) and 1d(F), respectively] could be due to the presence of low concentrations of complete *atp6* and *coxI* copies in different genomic environments. Alternatively, their faint signals may indicate the presence of partial copies of the *atp6* and *coxI* genes.

Unfortunately, the most prominent *Eco*RI fragment with *coxI* homology from male-sterile cytoplasm was not identified. The reason for this anomaly may be due to the mtDNA source used to construct the phage library, this being a suspension culture which had already been subcultured for more than a year. Therefore, the absence of a clone containing the 7.4-kbp fragment could perhaps be attributed to tissue-culture-induced mitochondrial genome alterations (McNay et al. 1984; Hartmann et al. 1987). In fact, regenerant no. 3 derived from a relatively young suspension culture – a progenitor culture of the one used for mtDNA preparation – already shows a strong decrease in the concentration of the 7.4-kbp fragment (Fig. 5). Further subculturing may have created even more mtDNA modifications in the suspension culture, which were retained in the plants regenerated from it (Gengenbach et al. 1981; Hartmann et al. 1989). Of course, the absence of this particular clone can also be explained by its relatively large size, which would make it more difficult to find a recombinant phage entirely encompassing it.

The importance of in-vitro-induced alterations is stressed by the observations of yet more fragments with *coxI* homology decreasing or increasing in concentration, i.e., the 4.4- and 5.3-kbp *Eco*RI fragments, respectively. The concurrent changes in the concentrations of the 4.4- and 7.4-kbp fragments suggest a physical association in the mitochondrial genome needing further research. The inverse ratio of 5.3- and 7.4-kbp *Eco*RI fragments in regenerated plants, as compared to control plants, and the homology between them – apart from the *coxI* sequences – may simply indicate a shift from one (sub)genomic arrangement to another. The overall effect of these rearrangements leads to regenerants in which the predominant fragment with *coxI* homology – contained on a 5.3-kbp *Eco*RI fragment – appears to be an incomplete copy. Tissue-culture-induced mitochondrial genome changes do not happen frequently since the maize *atp6* and *coxII* probes do not detect any changes, nor does every suspension culture yield aberrant regenerants.

Pseudocopies and chimeric genes, i.e., genes containing regions homologous to other mitochondrial genes, are not uncommon and have been found in maize, *Oenothera, Petunia*, soybean, and rice (Dewey et al. 1985a, 1986; Schuster and Brennicke 1986; Young and Hanson 1987; Grabau et al. 1988; Kadowaki et al. 1990). It is only when the partial or aberrant *coxI* copies encountered in this study are transcribed into open-reading-frame-containing transcripts that one could start considering them to be involved in the expression of cms

(Rouwendal et al. 1987). Thus, the existence of different transcripts with *coxI* homology in male-sterile and fertile cytoplasms can be taken as an indication for such an involvement, although it could be claimed that somewhat similar observations pertaining to the *atp6* gene could equally well be indicative of this gene's role in cms.

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