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Reorganization of mitochondrial genomes of cytoplasmic revertants in *cms-S* inbred line WF9 in maize

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Summary. Cytoplasmic reversion to fertility in *cms-S* maize has been previously correlated with changes in mitochondrial genome organization, specifically with loss of the autonomously replicating linear plasmid-like DNAs, S1 and S2, and with accompanying alterations in the high molecular weight mtDNA (main genome) that specifically involved S1 and S2 sequences. These studies, however, dealt with cytoplasmic revertants occurring in the *cms-VG* M825 inbred line and in the *cms-VG* M825/Oh07 F_1 hybrid. This paper deals principally with patterns of mitochondrial DNA reorganization accompanying cytoplasmic reversion to fertility in the WF9 inbred line nuclear background. Here the free S1 and S2 plasmid-like DNAs are retained in the revertants. Mitochondrial DNA analysis by Southern hybridization using cloned fragments of S1 and S2 shows altered organization around S-homologous regions in the main mitochondrial genome of revertants as compared with that of the male-sterile parental controls, but the pattern of main genome changes involving these regions differs from that of the cytoplasmic revertants that occurred in M825 and M825/Oh07 backgrounds. Similar experiments using a clone of the cytochrome oxidase I (*COX* I) gene of maize as a probe indicate that reorganization in this region is also involved in the changes in mtDNA that accompany cytoplasmic reversion to male fertility in *cms-S* WF9. The heterogeneity in patterns of reorganization of the main mtDNA genome that accompany cytoplasmic reversion in the same and different nuclear backgrounds are discussed in relation to cytoplasmic male sterility (CMS).

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Introduction

The mitochondrial DNA (mtDNA) of maize plants carrying S-type male-sterile cytoplasm (*cms-S*) is characterized by the presence of autonomously replicating linear plasmid-like DNAs S1 (6.4 kb) and S2 (5.4 kb) in addition to the high molecular weight main mitochondrial genome (Pring et al. 1977; Kim et al. 1982). S1 and S2 are structurally similar to transposable elements in bacteria and some eukaryotes (Shapiro 1983) in that terminal inverted repeat (TIR) regions are present. The repeat regions are identical in S1 and S2 and are 208 bp in length (Levings and Sederoff 1983; Paillard et al. 1985). S1 and S2 share an additional 1254 bp homology adjacent to the TIR at what is now defined (Levings and Sederoff 1983) as the right end of each molecule. Sequences homologous to those of S1 and S2 are found in the high molecular weight mtDNA (main genomes) of both N-type (male-fertile) and S-type (male-sterile) cytoplasms (Levings et al. 1980; Thompson et al. 1980; Lonsdale et al. 1981; Kemble and Mans 1983), but appear to be virtually absent from the mtDNA of *cms-C* and *cms-T* male-sterile strains. Interestingly, it has been shown (Schardl et al. 1984) that the free S1 and S2 plasmids bring about a high degree of linearization of the main mitochondrial genome by recombining with sequences in that genome that are homologous to their TIR regions. As a result, a high proportion of the genome of *cms-S* mitochondria is believed to consist of linearized molecules terminated by S1 and S2 sequences.

Spontaneous reversion to male fertility has not been reported for *cms-C* and *cms-T* strains but both cytoplasmic and nuclear spontaneous reversions occur not infrequently in *cms-S* strains where the nuclear genotype governs the overall frequency of reversions as well as the relative distribution between those based on nuclear vs. cytoplasmic change (Laughnan and Gabay 1975; Laughnan and Gabay-Laughnan 1978; Laughnan et al. 1981; Gabay-Laughnan and Laughnan 1983).

Studies on the mtDNA of the first seven spontaneous cytoplasmic revertant strains all of which originated from the inbred line M825 background, indicated that the reversion event was in each case associated with disappearance of the linear S1 and S2 plasmid-like molecules (Levings et al. 1980). Further studies involving restriction enzyme fragment analysis coupled with molecular hybridization procedures indicated that various rearrangements involving the S-regions of the main mitochondrial genome are also associated with cytoplasmic reversion in these strains (Levings et al. 1980; Kemble and Mans 1983). Additional studies (Lonsdale et al. 1984; Schardl et al. 1985) confirmed the association of reversion with loss of the S1 and S2 episomes and showed that each of the five revertants studied carried a rearrangement in the left (unique) end of the integrated S2 sequence. The evidence also indicated that the reversion event is associated with reestablishment of the circular genome.

In contrast with the revertants from *cms-S* M825, where reversion is associated with loss of the autonomously replicating S1 and S2 episomes, in the *cms-S* WF9 inbred line background, S1 and S2 are retained in the revertants (Escote et al. 1985; Ishige et al. 1985).

In the study reported here three M825 revertants and five WF9 revertants were analyzed to determine whether the integrity of these integrated episomes is retained after reversion. The restriction enzyme used here was Pst I and probes pZmS4 and pZmS40, both of S2 origin, were employed. The remainder of the study involves restriction fragment analysis of further differences in mtDNA between the five WF9 revertants and their male-sterile source strains.

Materials and methods

Plant material

Cytoplasmic revertants R251, R409-7 and R423-12 used here occurred in inbred line M825 backgrounds. R251 occurred in a *cms-VG* M825/Oh07 F_1 plant; R409-7 and R423-12 occurred in strains whose nuclear genotypes were essentially M825 since they were the product of six backcrosses involving M825 as the recurrent male parent. The R409-7 and R423-12 source strains were *cms-ML* and *cms-S*, respectively: *VG, ML* and *RD,* mentioned below, are all members of the *cms-S* group.

R47-13, R47-15 and R47-16 are sibling cytoplasmic revertants that occurred in *cms-RD* WF9 line, and R1179-16 is an independently occurring revertant from the same source. R51-1 arose in inbred line *cms-ML* WF9. Male-sterile source strains for the revertants mentioned above were employed as controls.

Mitochondrial DNA isolation

The procedure used was basically that of Kemble et al. (1980). Four to five day old etiolated seedlings were ground in chilled mortar and pestle using freshly prepared ice-cold 0.01 M TES (pH 7.2), 0.5 M mannitol, 0.001 M EGTA, 0.2% BSA and 0.05% cysteine, at a volume three times the fresh weight of the seedling material. The ground tissue was filtered through four layers of cheesecloth and one layer of miracloth and then spun for 10 min at 4 °C, at 1000 × *g*. The supernatant was re-spun for 10 min at 12,000 × *g*, the pellet resuspended carefully, using a small brush, in 10 mls of the grinding medium, then spun again for 10 min at 1000×*g*. $MgCl_2$ and DNAase were added to the supernatant to a final concentration of 0.01 M and 10 µg/g fresh weight of tissue, respectively, and this solution was incubated in ice for 1 h. The solution was carefully layered over 10 mM TES (pH 7.2), 20 mM EDTA, and 0.6 M sucrose, and then spun at 12,000 × *g* at 4 °C for 20 min. The pellet was washed twice in the above-indicated sucrose solution, lysed in a solution consisting of 0.05 M Tris-HCl (pH 8.0), 0.01 M EDTA, 2% sarkosyl, and 0.012% autodigested pronase or proteinase K, and then incubated for 1 h at 37 °C in a waterbath shaker. Ammonium acetate was added to the lysate to a final concentration of 0.2 M. Further DNA purification involved a three-fold extraction with phenol-chloroform followed by ethanol precipitation overnight at −20 °C. The DNA precipitate was spun down at 9,000 rpm at 4 °C, washed twice with 70% ethanol, vacuum-dried, resuspended in sterile distilled water, and stored in −20 °C freezer.

To accomplish the isolation of high molecular weight mtDNA free of the low molecular weight plasmid-like elements, the pellet, following washes in the sucrose solution, was lysed in the indicated buffer without added pronase or proteinase. The lysate was mixed with CsCl (6 g CsCl for 6 mls of lysate) and ethidium bromide (EtBr), and then centrifuged at 60,000 rpm overnight. The fluorescent EtBr-DNA band was collected mixed with CsCl solution, and spun again in the ultracentrifuge. The DNA band was isolated, extracted several times with an equal volume of isoamyl alcohol to completely remove the EtBr, and then dialysed for 2 days in several changes of Tris-EDTA buffer (pH 8.0). This procedure, based on the fact that the S1 and S2 episomes possess terminally-bound proteins (Kemble and Thompson 1982), is effective in isolating the high molecular weight DNA which is not protein-bound. These preparations were analyzed for S1 and S2 sequences by running undigested total mtDNA in gels and probing it with S1 and S2 cloned fragments in Southern blots. An alternate procedure involved running total mtDNA in preparative gels and isolating the high molecular weight DNA band from agarose by the procedure of Benson (1984).

Restriction digestion, electrophoresis and Southern blotting

One-microgram DNA samples were digested overnight with restriction enzyme according to specified conditions. Digested mitochondrial DNA was loaded onto horizontal 1% agarose slab gels and electrophoresed at 60 V for 18 h in a medium consisting of 0.04 M Tris (pH 7.8), 0.02 M sodium acetate and 0.002 M EDTA. The gel was then stained in 0.5 µg/ml EtBr, rinsed in distilled water and photographed over a UV transilluminator with Type 55 Polaroid film using a red filter.

The gel was denatured in 1.5 M NaCl, 0.5 M NaOH, and then neutralized in 3 M sodium acetate (pH 5.5) prior to blotting to Biodyne A (Pall Biodyne Filter) according to the method of Southern (1975) using 20× SSC (3 M NaCl, 0.3 M Na-citrate, pH 7.0). Time allowed for transfer ranged from overnight to 48 h. After transfer the filter was baked in a 80 °C oven for 1 h.

Probes, Southern hybridization, and rehybridization

S1 and S2 cloned fragments in pBR322 were gifts from Dr. R.J. Kemble, and genomic clones of maize cytochrome c oxidase subunit I (*COX* I pBN 6601) and II (*COX* II pZmE1) and apo-

cytochrome b (*COB* pZmEH680) were kindly provided by Dr. C.J. Leaver. Isolation of plasmids was carried out by the alkaline lysis procedure detailed by Maniatis et al. (1982).

Cloned fragments were labeled by nick-translation using ^{32}P-dCTP (NEN) and BRL Nick-Translation Kit (Bethesda Research Laboratories). Labeled fragments were purified by passing through a Sephadex G-50 Medium column. The minimum probe activity involved in individual filter hybridizations was 1×10^6 cpm. Filters were prehybridized for at least one hour in the same solution and under the same conditions as were involved in radioactive hybridization. Hybridizations were carried out under aqueous conditions in 5x Denhardt's buffer (100X stock = 2% w/v Ficoll-400, 2% w/v PVP-360, and 2% BSA), 5x SSPE (20× stock = 3.6 M NaCl, 0.2 M sodium phosphate at pH 8.3, 0.02 M EDTA), 0.2% SDS and 500 µg/ml denatured calf thymus DNA, at 65 °C overnight.

After hybridization the filters were washed once in 1x SSC for 1 h at 65 °C, then three times in 0.1x SSC at 65 °C for a total of 3 h. The filter was then plastic-wrapped and autoradiographed in XAR-5 X-ray film at −80 °C.

For rehybridization of filters, the previous probe was removed by washing the filter twice in 0.4 M NaOH at 37 °C for a total of at least 3 h. The filter was then rinsed in 5× SSPE prior to prehybridization and rehybridization.

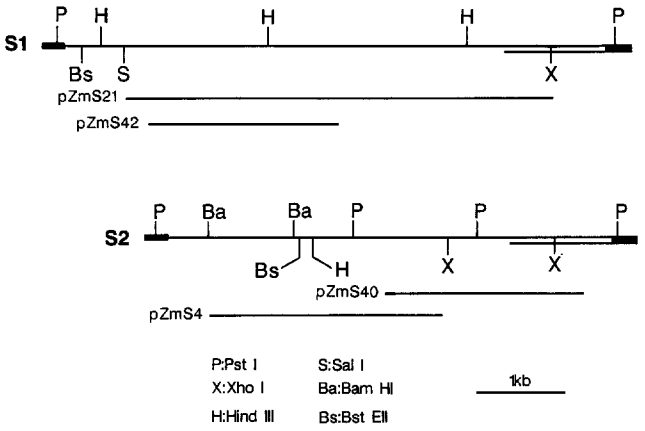

Fig. 1. Restriction enzyme map of S1 and S2 showing the origins of cloned S1 and S2 fragments used as probes in Southern hybridizations. (■ = terminal inverted repeats, TIRs; ═ = S1 and S2 region of homology)

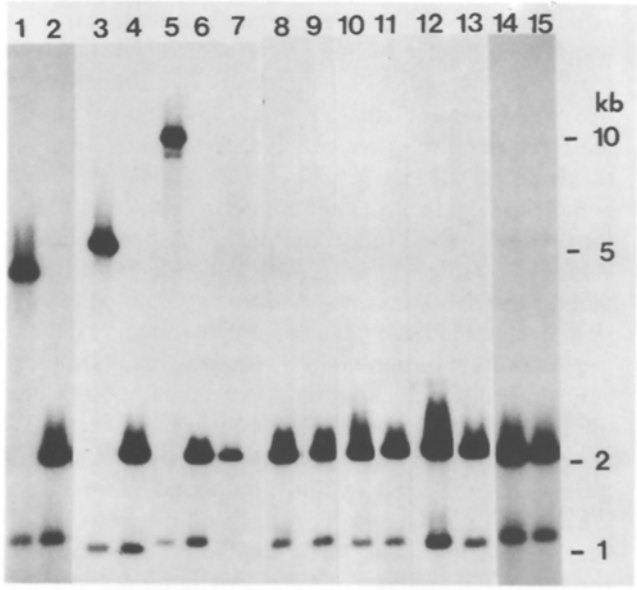

Fig. 2. Pst I digests of high molecular weight mitochondrial DNA of M825 and WF9 revertants and sterile controls probed with pZmS4. *Lane 1* revertant R251; *2* sterile control for R251; *3* revertant R409-7; *4* sterile control for R409-7; *5* revertant R423-12; *6* sterile control for R423-12; *7* M825 normal fertile cytoplasm; *8* revertant R47-13; *9* revertant R47-15; *10* revertant R47-16; *11* revertant R1179-16; *12* total mitochondrial DNA from RD-WF9 sterile; *13* RD-WF9 sterile; *14* revertant R51-1; *15* sterile ML-WF9

Results

Figure 1 shows the origin of the cloned S-episome subfragments relative to some of the restriction sites previously mapped on the S1 and S2 episomes (Kim et al. 1982; Levings and Sederoff 1983; Paillard et al. 1985). These cloned fragments were used as probes in the Southern hybridizations. pZmS4 is an S2-specific fragment while pZmS42 is S1-specific; pZmS21 carries most of the S1 sequences including part of the S1-S2 region of common homology; pZmS40 was cloned from S2 and carries most of the S1-S2 region of homology and has a short sequence overlap with pZmS4 (Thompson et al. 1980).

Organization of integrated S2 sequences

With Pst I digestion (Fig. 1), an intact integrated S2 sequence should give internal fragments of 1.6, 1.4 and 2.2 kilobases (kb). To determine whether integrated S2 sequences are intact with respect to Pst I restriction sites, mitochondrial DNA of the various strains under study was digested with Pst I after free episomal sequences were removed as described earlier. Southern blots of these digests were hybridized to pZmS4 which should detect the 1.4 and 2.2 kb fragments (Fig. 1). Figure 2, lanes 1, 3 and 5 correspond to the three cytoplasmic revertants that occurred in the M825 background. As shown, all the revertants have the internal 1.4 kb fragment but the 2.2 kb fragment is replaced by a higher molecular weight fragment, the size of which varies in different revertants. On the other hand, the *cms-S* male-sterile M825 parental sources (lanes 2, 4 and 6) generate the expected 2.2 kb internal fragment. This pattern of loss characterizes all six revertants studied by Lonsdale et al. (1984), and Schardl et al. (1985). We have also found this association between cytoplasmic reversion and loss of the 2.2 kb fragment of integrated S2 in these six revertants, and in an additional

19 cytoplasmic revertants that occurred in *cms-S* type strains carrying the M825 nuclear background. For five of the revertants, sequence analysis (Schardl et al. 1985) revealed that in each case there was a deletion of part of the TIR sequence at the left (unique) end of integrated S2, and that the deletion included the Pst I site. The evidence indicates that these deletions arise through special recombinational events and it is reasonable to infer that all revertants occurring in M825 background share this feature. Pst I digests of main mtDNA from the M825 revertants and their sterile progenitors, when probed with pZmS40 (Fig. 1) showed the expected 1.6 kb fragment (data not shown), indicating that the Pst I site in the TIR at the right end of S2, and the associated sequences, are unaltered in the integrated S2. This agrees with the findings for the five revertants analyzed by Schardl et al. (1985).

When the high molecular weight mitochondrial DNAs from WF9 cytoplasmic revertants and their sterile progenitors were subjected to the same Pst I analysis as described above, both expected Pst I-generated fragments were detected using probe pZmS4. As shown in Fig. 2, the 1.4 and 2.2 kb fragments are present in all five WF9 revertants (lanes 8 to 11, and lane 14) and in their sterile controls (lanes 13 and 15) and, in sharp contrast with the M825 revertants, no S2-homologous higher molecular weight fragments are detected. Lane 12 is a Pst I digest of total mitochondrial DNA from the RD-WF9 male-sterile source probed with pZmS4; it shows that the internal fragments, 1.4 kb and 2.2 kb, generated from integrated S2 sequences are the same as those generated from autonomous S2 episomes that are present in the total mitochondrial DNA preparation. When the Pst I digests of the mtDNA from the WF9 cytoplasmic revertants and their sterile controls are probed with pZmS40 the expected 1.4 and 1.6 internal S2 fragments are identifiable (data not shown). We have seen (Escote et al. 1985; Ishige et al. 1985) that the WF9 cytoplasmic revertants, unlike those of M825 origin, do not lose the free S1 and S2 plasmids. The above observations involving Pst I digests indicate that these types of revertants differ in regard to the integrated S2 sequences; all the M825 revertants studied to date exhibit a rearrangement of the left region of integrated S2, whereas in the WF9 revertants the integrated S2 sequences are intact.

On the other hand, the integrated S1 sequences of both WF9 and M825 cytoplasmic revertants are intact as shown by the detection of the 6.2 kb S1 fragment (Fig. 1) in Pst I digests hybridized to clone pZmS42 and/or clone pZmS40 which carries most of the S1 and S2 region of homology (data not shown).

In the studies described in the following paragraphs total mtDNA isolated from WF9 cytoplasmic revertants and from their sterile controls was digested with several restriction enzymes, blotted and hybridized to radioactively labeled S1 and S2 cloned fragments shown in Fig. 1. In such digests the restriction fragments generated from the free S-episomes are easily distinguished from other S-homologous sequences because they are relatively more abundant as a consequence of the ability of S-episomes to self-replicate faster than the main genome. These free episomal fragments show brighter fluorescence in ethidium bromide stained gels (not shown) and much wider bands in autoradiograms of hybridized Southern blots.

Figure 3 is a display of Xho I digests of total mtDNA from WF9 cytoplasmic revertants and their sterile control sources probed with pZmS4. The widest band, about 3 kb in size, is a free episome fragment corresponding to the left-end Xho I fragment of S2 detectable by pZmS4 (Fig. 1). The other bands correspond to regions of the main mitochondrial genome contiguous with the left end of integrated S2. As shown in Fig. 3, the RD-WF9 revertants R47-13, R47-15 and R47-16 (lanes 1, 2, 3, respectively) show identical pZmS4-homologous bands. Since these three are sibling revertants, and possibly derive from the same revertant sector on a parental ear, this is not unexpected (Escote et al. 1985). They differ from the RD-WF9 sterile parent (Fig. 3, lane 4) in that there is a new band of about 10 kb found in the revertants but not in the sterile, a major band of approximately 4.5 kb is more abundant in the revertants than in the sterile, and one band of about 8.5 kb is found only in the sterile parent. R1179-16, also of RD-WF9 origin (Fig. 3, lane 6), on the other hand differs from these three revertants. It has the 6.3 kb and 3.9 kb bands also present in the three sibling revertants and in the sterile control but it does not show the 10 kb band, and the 4.5 kb band is greatly reduced as in the sterile parent. In fact, R1179-16 differs from its sterile control only in having lost the 8.5 kb band, just as the other revertants did. Thus, while the RD-WF9 revertants showed differences in regard to integrated S2 sequence organization, they have in common the absence of the 8.5 kb Xho I-generated band that distinguishes them from the RD-WF9 sterile parent.

Agarose gel displays of Xho I digests of mtDNA from the WF9 revertants and their sterile controls, blotted and probed with pZmS21, the nonunique S1 subfragment, are shown in Fig. 4. pZmS21 hybridizes to Xho I fragments carrying the integrated left end of S1, as well as to the left end of the free S1 episome which is about 5.4 kb; because it carries part of the S1-S2 homologous region, pZmS21 also detects the 1.4 kb internal Xho I S2 fragment (Fig. 1). The three RD-WF9 sibling revertants (Fig. 4, lanes 1 to 3) show identical hybridization patterns with pZmS21. They differ from the sterile parent (lane 4) in that a fragment of about 10.5 kb is present only in the sterile parent and fragments of 12 and 8 kb are found only in the sibling re-

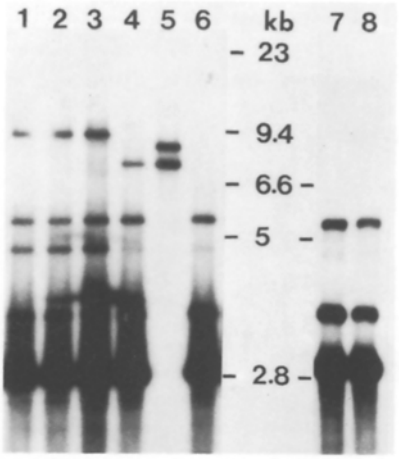

Fig. 3. Xho I digests of total mitochondrial DNA of WF9 revertants and sterile controls probed with pZmS4. *Lane 1* revertant R47-13; *2* revertant R47-15; *3* revertant R47-16; *4* sterile RD-WF9; *5* normal fertile cytoplasm WF9 line; *6* revertant R1179-16; *7* revertant R51-1; *8* sterile ML-WF9

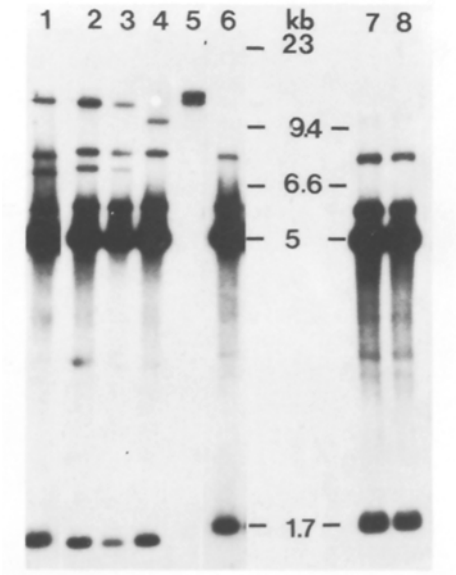

Fig. 4. Xho I digests of total mitochondrial DNA of WF9 revertants and sterile controls probed with pZmS21. *Lane 1* revertant R47-13; *2* revertant R47-15; *3* revertant R47-16; *4* sterile RD-WF9; *5* normal fertile WF9; *6* revertant R1179-16; *7* revertant R51-1; *8* sterile ML-WF9

vertants. Thus, with reversion, two new pZmS21-homologous bands appear and this is accompanied by loss of the parental 10.5 kb band. On the other hand, RD-WF9 revertant R1179-16 (Fig. 4, lane 6) has the 9 kb and 6.3 kb fragments in common with the three other revertants and the male-sterile parental source but lacks the two new bands present in the sibling revertants, as well as the parental-specific 10.5 kb band. In-

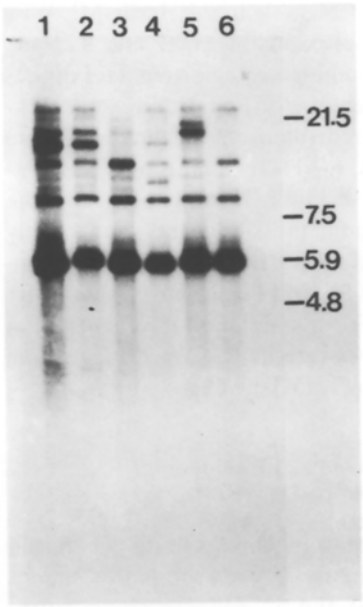

Fig. 5. Sal I digests of total mitochondrial DNA of WF9 revertants and sterile controls probed with pZmS4. *Lane 1* revertant R47-15; *2* revertant R47-16; *3* revertant R1179-16; *4* sterile RD-WF9; *5* revertant R51-1; *6* sterile ML-WF9

terestingly, with Xho I digestion, the pattern of changes observed in integrated S1 sequences (probe pZmS21) is similar to that for integrated S2 sequences (probe pZmS4). Although the sibling revertants differed from the R1179-16 revertant by two new bands homologous to pZmS21 that are present only in the former, the RD-WF9 revertants have in common the loss of the 10.5 kb parental band. The significance of this parental-specific band warrants further study.

While Xho I digestion reveals differences between the RD-WF9 cytoplasmic revertants and their sterile progenitor, it did not do so between the ML-WF9 cytoplasmic revertant R51-1 and its parental ML-WF9 sterile progenitor (Figs. 3 and 4, lanes 7 and 8 in both figures). The same was true when Bst EII and Hin dIII restriction enzymes were used (data not shown). Note that the RD-WF9 and ML-WF9 sterile parental lines also differ in hybridization patterns produced with both the S1 and S2 probes following Xho I digestion of their mtDNAs (Figs. 3 and 4, lanes 4 and 8 in both figures). Differences between the RD and ML sterile sources are also revealed when Bst EII and Hin dIII digests are hybridized with the S1 and S2 probes (data not shown).

So far, only Sal I restriction enzyme has differentiated between R51-1 and its sterile parent ML-WF9. Sal I does not cut S2, thus the free S2 episome is shown as an intact 5.4 kb band in Sal I digests of total mtDNA probed with pZmS4 (Fig. 5, lanes 1 through 6). Two high molecular weight fragments (approximately 15 kb and 14 kb) showing homology to pZmS4 in R51-1

(Fig. 5, lane 5) are not detectable in the sterile ML-WF9 line (lane 6). Three other bands of 23, 12 and 9 kb are common to both. S1-homologous regions in Sal I digests of the mitochondrial genome of R51-1 and sterile ML-WF9 are similarly altered (data not shown). Sal I also reveals differences in RD-WF9 revertants and their parental strain as shown in Fig. 5, lanes 1 to 4. As is the case with Xho I digestion, the sibling revertants, only two of which are shown (lanes 1 and 2), differ from revertant R1179-16 (lane 3), and all the revertants differ from the sterile RD-WF9 source (lane 4). Note also that Sal I differentiates between sterile RD-WF9 (Fig. 5, lane 4) and sterile ML-WF9 (Fig. 5, lane 6).

Organization of cytochrome c oxidase subunit I sequences

To determine whether rearrangements in mitochondrial DNA during reversion to fertility are limited to regions showing homology to S1 and S2, genomic clones carrying the coding sequences for corn cytochrome c oxidase subunit I (*COX* I) (Isaac et al. 1985), apocytochrome b (*COB*) (Dawson et al. 1984) and cytochrome c oxidase subunit II (*COX* II) (Fox and Leaver 1981) were used as probes in Southern hybridizations of digested total mtDNA. Figure 6 shows the hybridization of cloned *COX* I to the same Sal I blot shown in Fig. 5. While the three sibling revertants (only 2 are shown here) are different from R1179-16 in regard to organization of the S regions, they all show the same organization of the *COX* I region in their main mitochondrial genomes with Sal I enzyme (Fig. 6, lanes 1 to 3). The same was observed with enzymes Hin dIII, Xho I and Bam HI (data not shown). The sterile parental RD-WF9 shows the same four *COX* I-hybridizing bands as do all the revertants, but in addition it has three minor bands of 15, 12 and 10 kb, and two major bands of 8.5 and 4 kb (Fig. 6, lane 4) which are absent in the RD-WF9 revertants. Revertant R51-1 differs from its sterile parent by one band of about 10 kb found only in the sterile parent (Fig. 6, lane 6) and a 15 kb band present in the revertant only (Fig. 6, lane 5).

Bst EII digests probed with the *COX* I cloned fragment show four bands of 11, 12, 14 and 15 kb in all the RD-WF9 revertants analyzed (Fig. 7, lanes 1 to 3). Sterile RD-WF9 (lane 4) shows seven major *COX* I-hybridizing bands in addition to the four found in the revertants. R51-1 (lane 5) shows the same pattern of hybridization with the *COX* I probe as the RD-WF9 revertants, and the sterile ML-WF9 parental (lane 6) has two bands in addition to the four *COX* I bands it has in common with the revertants.

Thus the reorganization of mtDNA sequences accompanying reversion to fertility does not exclusively involve S1 and S2 homologous sequences, but those of the *COX* I probe and possibly other mitochondrial genes as well. So far, however, no differences between WF9 revertants and their sterile parents have been detected for sequences homologous to the *COB* gene and the *COX* II gene following digestion with the six restriction enzymes indicated in Fig. 1 (data not shown).

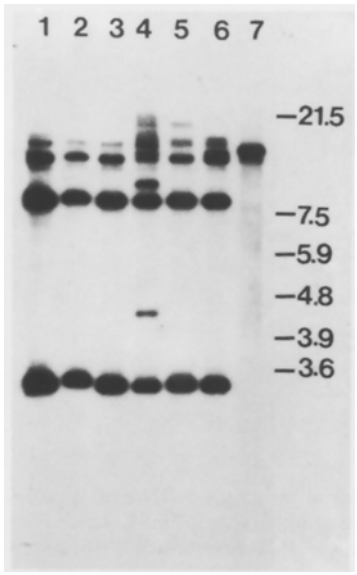

Fig. 6. Sal I digests of total mitochondrial DNA probed with *COX* I. *Lane 1* revertant R47-15; *2* revertant R47-16; *3* revertant R1179-16; *4* sterile RD-WF9; *5* revertant R51-1; *6* sterile ML-WF9; *7* normal fertile cytoplasm WF9

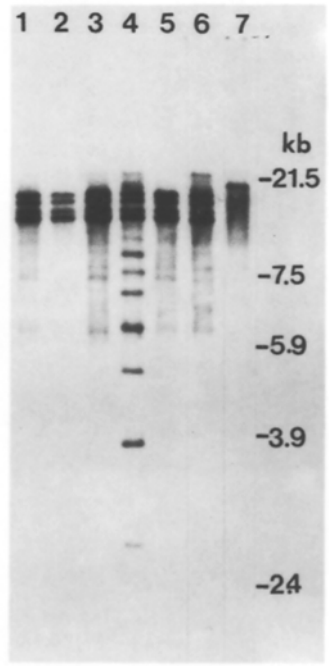

Fig. 7. Bst EII digests of total mitochondrial DNA probed with *COX* I. *Lane 1* revertant R47-15; *2* revertant R47-16; *3* revertant R1179-16; *4* sterile RD-WF9; *5* revertant R51-1; *6* sterile ML-WF9; *7* normal fertile WF9 line

Discussion

In all the cytoplasmic revertant strains studied thus far reversion to fertility is associated with rearrangements in the main mitochondrial genome. The patterns of rearrangement vary among the different revertants but some are clearly characteristic of a given nuclear background. In the M825 nuclear background the mitochondria lose the autonomously replicating forms of S1 and S2 upon reversion. Furthermore, the revertants in this background resemble the parental sterile sources in regard to intact S1 sequences in the main mitochondrial genome. The integrated form of S2, however, is characterized by loss of the Pst I site of the TIR at the left end of S2 in all 25 M825 revertants examined to date. This change leads to the replacement of the expected 2.2 kb fragment from Pst I digestion by a high molecular weight fragment apparently carrying most of the 2.2 kb fragment plus contiguous sequences of the main mitochondrial genome. The size of this fragment varies in different revertants indicating some considerable variation in the molecular characteristics of these rearrangements. The fact that the loss of this Pst I site by recombination (Schardl et al. 1985) occurs in all the M825 revertants suggests that the event is closely associated, perhaps causally, with reversion to fertility in the M825 background.

In the WF9 nuclear background, the mitochondrial genomic rearrangements which accompany cytoplasmic reversion do not involve loss of the linear S1 and S2 episomes, indicating that their presence per se in the mitochondria is not associated with the sterility-fertility phenotype. Moreover, the characteristic loss of a Pst I site of the integrated S2 region in M825 cytoplasmic revertants is not observed in the WF9 revertants. Nevertheless, regions flanking both S1 and S2 are reorganized upon reversion in the WF9 nuclear background, though the rearrangements, summarized below, were not identical in the several revertants.

All four revertants in RD-WF9 background lost one S1- and one S2-homologous main mitochondrial DNA Xho I fragment that is found only in the sterile RD-WF9 parent. Perhaps these parental-specific fragments are products of a unique S1 and S2 organization in the main mitochondrial genome of male-sterile source RD-WF9 that prevents the normal expression of male fertility. If so, loss of such organization might well be associated with reversion to the male-fertile condition. The ML-WF9 revertant, R51-1, differs from the RD-WF9 revertants in regard to integrated S-region organization but this is not surprising since the ML-WF9 sterile and RD-WF9 sterile source lines themselves differ in mtDNA organization.

We have seen that in WF9 revertants the presence of the S1 and S2 episomes is not directly related to the expression of male sterility or male fertility, and that the same is true in the case of the R1 and R2 episomes in the RU cytoplasm of Latin American races of maize (Weissinger et al. 1982). Nevertheless, the possibility that S1 and S2 sequences in the main mitochondrial genome are involved in rearrangements that lead to fertility or sterility cannot be ruled out. So far, in every one of the 30 cases studied, cytoplasmic reversion in S-type cytoplasm has been associated with rearrangement of the S1 and/or S2 regions of the main mitochondrial genome. The evidence for linearization of the main mitochondrial genome (Schardl et al. 1984) suggests that there is an equilibrium between the autonomous and integrated states of S1 and S2 in *cms-S* strains. Perhaps, in these strains, cytoplasmic reversion is the result of an occasional event that produces a new alignment of integrated S elements that interrupts this equilibrium and leads to the change from male-sterile to male-fertile condition.

The WF9 revertants and their sterile sources were tested for homology with clones *COX* I, *COX* II and *COB*. It should be noted that the *COX* I gene is present in multiple copies in the mitochondrial genome of *cms-S* maize and that the *COX* I gene clone used here carries flanking sequences (Isaac et al. 1985). While *COB* and *COX* II organization appears the same in the WF9 cytoplasmic revertants and their sterile sources (data not shown), *COX* I organization differs. RD-WF9 revertants which vary in terms of S sequences, exhibit the same organization of *COX* I-homologous sequences. They differ from the parental RD-WF9 in that *COX* I-homologous fragments present in the sterile are lost in the revertants. However, there is no *COX* I band in the revertants that is not present in the sterile source. The ML-WF9 revertant also differed from its parental sterile line. A *COX* I-homologous fragment present in the sterile ML-WF9 is missing in the revertant, and vice-versa. Thus, the rearrangements in the mitochondrial genome accompanying *cms-S* reversion to male fertility are not exclusively localized to S1 and S2 regions. Moreover, cytoplasmic revertants in the M825 *cms-S* background are also associated with rearrangements of *COX* I sequences (data not shown). It is interesting to note that the *COX* I clone pBN6601 (from *cms-S*) used in these studies has a region starting at about −175 base pairs upstream from the initiation codon which is homologous to 187 of the 208 base pairs of the TIR of S1 and S2 (Isaac et al. 1985). Whether this has something to do with the susceptibility of S1 and S2 sequences and the *COX* I region to rearrangements remains to be determined. TIR regions were previously reported as hotspots for recombination in the mitochondrial genome (Schardl et al. 1984).

Reorganization of S-homologous regions and of *COX* I regions is clearly associated with cytoplasmic

reversion in *cms-S* M825 and WF9 inbred lines, but the great diversity of these rearrangements among the revertants studied, even among those occurring in the same nuclear background, leaves us with no obvious clues regarding the basis for cytoplasmic reversion in *cms-S* strains.

It has been suggested (Schardl et al. 1985) that *cms-S* male sterility may be due to the high proportion of linear S1 and S2 episomes in the male-sterile plant which leads to an imbalance in the copy numbers of mitochondrial transcripts. It is argued that the effect of this imbalance is most acute during early states of pollen development and results in pollen abortion.

Since it has now been shown that cytoplasmic reversion is also associated with rearrangements of *COX* I clone sequences, and that copy numbers of these sequences are most often quite different in the revertants and their sterile progenitors, S-type male sterility may be explained as the result of imbalance in *COX* I transcripts, as argued in the case of S1 and S2 sequences in the previous paragraph. It should be noted, however, that the *COX* I clone used here carries flanking sequences and it can not be inferred that the hybridizing fragments necessarily carry *COX* I gene sequences.

Whatever may be the cause of cytoplasmic reversion in *cms-S*, the event must be regarded as under control of the nuclear genotype since it has been shown that different inbred lines carrying the same *cms-S* cytoplasm exhibit striking differences in frequency of reversion as well as in the proportion of reversions that are cytoplasmic vs. nuclear in character (Laughnan et al. 1981; Carlson et al. 1982; Gabay-Laughnan and Laughnan 1983). For example, among thousands of *cms-S* male-sterile plants examined in inbred line N6, we have never observed a cytoplasmic reversion event, while in inbred line *cms-VG* M825, 10% of the plants exhibit some level of cytoplasmic reversion to male fertility. Beyond this it has been shown that there is nuclear gene control over the relative replication rates of the mtDNA S1 and S2 autonomous elements (Laughnan et al. 1981; Carlson et al. 1982); in most inbred line backgrounds examined the S1 and S2 episomes occur in equimolar amounts, but in the M825 nuclear background there is a relative suppression of S2, and in the inbred line 38-11 a relative suppression of S1. It is clear that a nuclear gene product(s) governs the event that leads to the rearrangement of sequences in the main mitochondrial genome that in turn is manifested as a change from male-sterile to male-fertile phenotype.

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