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A comparison of cytoplasmic revertants to fertility from different CMS-S maize sources

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Summary. The mitochondrial genome organizations of a number of independent culture-derived fertile CMS-S revertants with the nuclear genotype W182BN were compared to spontaneous field revertants with the genotypes WF9, M825/Oh07 and 38-11. Regions of the genome around sequences homologous to the terminal repeats of the linear S1 and S2 episomes characteristic of CMS-S mitochondria were used as hybridization probes on Southern blots of *Bam*HI and *Sal*I digested mitochondrial DNA. The results obtained suggest that the nuclear, not the cytoplasmic, genotype of the parent plant affects the type of novel mitochondrial DNA organization found in the revertant. The DNA reorganization during reversion from CMS-S in tissue culture appears to be similar to that observed in spontaneous revertants obtained during the normal plant life-cycle. Unlike the situation for reversion from CMS-T, no common DNA sequence or reading frame appeared to be lost or disrupted in revertants.

Key words: S-type cytoplasmic male sterility – Reversion to fertility – Mitochondrial genome organization – Recombination – Maize

Introduction

Cytoplasmic male sterility (CMS) is characterized by a failure of affected plants to produce viable pollen. The trait is maternally inherited, and most evidence associates it with changes in the mitochondrial genome. Maize cytoplasms that can exhibit CMS are classified as either T (Texas), C (Charrua) or S (USDA) on the basis of the effects of different nuclear genes that can restore fertility (*Rf* genes) (Duvick 1965; Laughnan and Gabay-Laughnan 1983). Normal (N) cytoplasms allow fertile pollen development in all nuclear backgrounds. These four cytoplasmic groups can also be differentiated by the restriction endonuclease digestion patterns of their mitochondrial DNA (mtDNA) (Pring and Levings 1978).

The mitochondrial genomes of S-type cytoplasms of maize are easily distinguished by the presence of two linear DNA episomes S1 (6,397 bp) and S2 (5,453 bp) in high copy number relative to the main mitochondrial genome (Pring et al. 1977). These linear DNAs contain identical 208 bp terminal inverted repeats (S-TIRs) and several unassigned open reading frames (URFs) (Levings and Sederoff 1983; Paillard et al. 1985). These URFs encode at least some of the large (>100 kd) polypeptides characteristic of mitochondria from S cytoplasms (Forde and Leaver 1980; Manson et al. 1986; Zabala et al. 1987; Zabala and Walbot 1988). URF3 of S1 shows homology to viral DNA polymerases, particularly the *Bacillus* 'phage Φ29 (Kuzmin and Levchenko 1987). In addition, there are proteins covalently bound to the 5′ termini of these episomes (Kemble and Thompson 1982) that may be involved in priming their replication by the same mechanism as that suggested for other linear DNA genomes, e.g. adenoviruses and 'phage Φ29 (Sederoff and Levings 1985).

S1 and S2 are also integrated into the high molecular weight (HMW) mitochondrial genome in S mtDNA. Some of the mitochondrial genome in S cytoplasms is present as linear molecules with S-TIR sequences at their termini (Schardl et al. 1984). These linear molecules probably arose by recombination between S1 or S2 and regions of the main mitochondrial genome (termed σ-σ', σ-φ', φ-φ', and φ-σ' by Schardl et al. (1984)) containing

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sequences homologous to their S-TIRs (Fig. 1). These S-TIR-homologous sequences can be thought of as 'targets' for recombination. The σ sequence contains the gene for subunit 1 of the cytochrome oxidase complex (*cox*1) (Isaac et al. 1985). The σ' and φ' sequences contain a 2 kb repeat that includes 186 bp identical to the S-TIRs (Lonsdale et al. 1988). This repeat shows strong homology to the unique end of the R1 episome of some South American maize cytoplasms (Weissinger et al. 1982; Leaver et al. 1985). This R1 homologous sequence is also found at the border of a recombinationally active repeat in N mtDNA (Houchins et al. 1986). In this paper we shall refer to the 2 kb sequence common to both σ' and φ' as R.

The nuclear background can affect the proportion of the genome that is linearized; in plants with the nuclear genotype WF9 the mitochondrial genome consists mostly of linear molecules, whilst in most other nuclear genotypes the mitochondrial genome contains a lower proportion of linear ends (Leaver et al. 1985; data not shown).

Some CMS-S sources of maize spontaneously revert to fertility. Subsequently, many of these reversion events are cytoplasmically inherited. The nuclear genotype affects the frequency with which these cytoplasmic revertants arise (Laughnan et al. 1981; Laughnan and Gabay-Laughnan 1983). All cytoplasmic revertants studied to date have exhibited mtDNA rearrangements associated with S episomal sequences, and most cytoplasmic revertants have lost free S1 and S2 (Levings et al. 1980; Kemble and Mans 1983; Schardl et al. 1985). The exceptions are cytoplasmic revertants with the nuclear genotype WF9, which retain S episomes at the same relative levels as their progenitor cytoplasms (Escote et al. 1985; Ishige et al. 1985).

We report characteristic mtDNA changes associated with reversion in the nuclear background W182BN. This genotype does not revert in the field, but the progeny of plants regenerated from tissue cultures from CMS-S plants are frequently fertile (Earle et al. 1987). The mtDNA rearrangements in these plants are compared with the changes seen in the mitochondrial genome from spontaneous field-derived cytoplasmic revertants with the nuclear backgrounds M825/Oh07, WF9 and 38-11. In the light of these observations, the molecular mechanisms underlying reversion and the possibility of identifying the lesion responsible for the CMS-S phenotype are discussed.

Materials and methods

CMS-S seed with the nuclear genotype W182BN was obtained from the progeny of plants regenerated from immature embryo cultures after varying periods of time (Table 1) (Earle et al. 1987). Seed with the genotypes M825/Oh07-VG, 38-11-S, WF9-RD, WF9-ML and revertants thereof were obtained from plants grown at the University of Illinois. Seed from WF9-N, WF9-S and the WF9 revertant 85:6838 were supplied by Dr. V. E. Gracen, Cornell University.

Growth of maize plants and isolation of mitochondria was as described previously (Leaver et al. 1983), except that purification of mitochondria was carried out by discontinuous sucrose gradient centrifugation (Boutry and Briquet 1982). This purification technique gives higher yields of mtDNA, but the level of DNA degradation is above that seen after continuous gradient purification. Mitochondrial DNA was isolated by solubilization of the mitochondria in 0.5% N-lauroyl sarcosine (Sigma), 100 m*M* EDTA, 100 m*M* Tris-Cl (pH 8.0), 0.1 mg/ml Proteinase K (Boehringer) for 1 h at 60 °C, followed by CsCl density gradient centrifugation in the presence of 75 µg/ml ethidium bromide (Fox 1979). The ethidium bromide was removed by extraction with butan-1-ol, and the CsCl was removed by two ethanol precipitations followed by washing with cold 70% ethanol. Approximately 2–5 µg of mtDNA from each maize line was digested to completion with the chosen restriction endonuclease, fractionated on a 0.8% agarose (Sigma type II) gel and transferred to nylon filters (Hybond-N, Amersham). These filters were pre-hybridized for 30–60 min in hybridization buffer (0.6 *M* sodium chloride, 60 m*M* sodium citrate (4 × SSC), 0.1% (w/v) SDS, 50 m*M* sodium phosphate (pH 5.5), 0.2% (w/v) BSA, 0.2% (w/v) Ficoll, 0.2% (w/v) polyvinyl pyrrolidone (mol. wt. 40,000) and 200 µg/ml denatured herring sperm DNA) at 65 °C. As discussed later, ^{32}P labelled DNA probes were hybridized to the nylon filters at 65 °C in hybridization buffer overnight. The filters were then washed in one or two changes of fresh hybridization buffer (lacking herring sperm DNA) at 65 °C, followed by washing in 2 × SSC or distilled water for 30 min at room temperature. The filters were then exposed to pre-flashed Curix RP1 X-ray film (Agfa-Gevaert) for 1–7 days at −80 °C with the aid of intensifying screens.

The probes used in these experiments were derived as shown in Fig. 2. Probes complementary to single stranded m13 clones were labelled by second strand synthesis with α^{32}P dCTP (Hu and Messing 1982) using a 17 base oligonucleotide sequencing primer and separated from the template by boiling for 5 min before use. The double-stranded pUC8 clones, kindly supplied by Prof. C. S. Levings III (Department of Genetics, North Carolina State University), were labelled with α^{32}P dCTP by nick translation (Rigby et al.1977).

Results

W182BN revertants

Mitochondrial DNA was extracted from seedlings grown from kernels obtained from the progeny of plants (nuclear genotype W182BN, cytoplasm S or the related cytoplasms CA and LBN) regenerated from callus cultures after various periods of time (Table 1) (Earle et al. 1987). Many of the progeny of these regenerated plants are fertile, and lack the S1 and S2 DNAs characteristic of their sterile parents (Earle et al. 1987). Specific probes for S-TIR-linked sequences (Fig. 2) were hybridized to *Bam*HI or *Sal*I digests of the mtDNA from these regenerated plants. *Bam*HI does not cut S1, allowing one to distinguish between free S1, S1 integrated at a chromosome terminus (throughout this paper an asterisk is used

Table 1. Origins of the W182BN regenerants used in these experiments

Line	Fertility status	S-type cytoplasm subgroup	Culture	Time in culture (months)	S1 integrates[a]
1B	sterile	CA	398	3	(+)
2A	fertile	CA	398	3	+
2B	fertile	CA	398	3	+
3A	fertile	CA	406	15	*
5A	fertile	LBN	436	13	+
6B	fertile	S	81	17	−
7B	fertile	S	81	16	−
8A	sterile	S	460	12	(+)
9A	fertile	S	460	12	+
10A	fertile	S	468	3	+
11A	fertile	S	468	12	−
12A	sterile	S	484	3	(+)
13A	fertile	S	484	3	+
17A	fertile	S	484	4	+
18A	sterile	S	484	4	(+)
19A	fertile	CA	398	14	*
20B	fertile	CA	398	3	+

[a] + = present; (+) = present but at low level; − = absent; * = present but rearranged

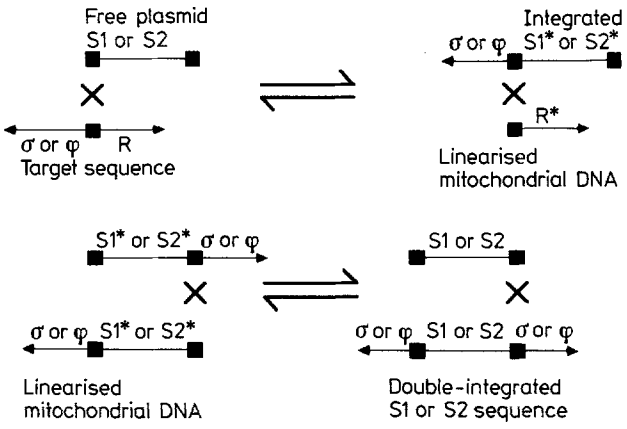

Fig. 1. Simplified diagrammatic representation of the S-TIR recombination system in CMS-S maize mitochondria (after Schardl et al. 1984). Free S1 and S2 DNAs can recombine with sequences homologous to their terminal inverted repeats (S-TIRs) (the σ-R and φ-R 'targets') present in the main mitochondrial genome to create linear chromosomes with S-TIRs at their termini. Linear chromosome termini are indicated with an *asterisk*. A pair of linear chromosomes can further recombine to form a double-integrated S1 or S2 sequence.

Table 2. Predicted hybridization of specific probes to S-TIR recombination products in mtDNA from CMS-S maize cut with the restriction endonucleases *Bam*HI and *Sal*I. No. of pluses (+) indicated the strength of the predicted hybridisation signal. * Indicates this fragment is derived from the terminus of a linear chromosome and is therefore diagnostic of linearization by S-TIR recombination. The notation used follows that of Schardl et al. (1984), whereby, for example, S2- and -S2 represent the right and left-hand termini of S2, respectively, with the unique end of S2 being the left-hand terminus

*Bam*HI	kb	IS1E5	M2C1	S2342	S1B5	*Sal*I	kb	S2341
φ-S1-φ	16.3			+	+	φ-S2-φ	19.3	+
σ-S1-φ, φ-S1-σ	14.4		+	+	+	φ-S2-σ, σ-S2-φ	14.2	+
σ-S1-σ	12.5		+	+	+	φ-S2*, S2-φ*	12.4	++
φ-S1*, S1-φ*	10.4			++	++	σ-S2-σ	10.3	+
σ-S1*, S1-σ*	9.4		++	++	++	σ-S2*, S2-σ*	7.9	++
S2-φ	7.9			++		S2	5.5	++++++
S2-σ	6.9		++	++				
S1	6.4			++++++	++++++			
φ-R	5.75	+++		+				
σ-R	4.75	+++	+++	+				
φ-S2	4.7			+				
S2-	3.9			++++++				
σ-S2	3.6		++	+				
R*	1.75	+++		+				
-S2	0.6			+				

to denote the terminal fragments of linear chromosomes, e.g., S1* and S2*) and S1 double-integrated into a mitochondrial chromosome at both ends (Fig. 1 and Table 2). *Sal*I does not cut S2, allowing for an analogous separation of S2 recombination products (Table 2). In addition, neither *Sal*I nor *Bam*HI differentiates between σ′ and φ′ as the R repeat includes sites for both of these enzymes.

These experiments showed many differences between the fertile and sterile regenerants (the sterile regenerants 1B, 8A, 12A and 18A were in all four cases identical to their sterile progenitors) (Figs. 3–5). 1S1E5, a probe for the R repeat (Fig. 2), reveals that all of the fertile regenerants lack a 1.75 kb *Bam*HI fragment characteristic of S mtDNA (Fig. 3). This fragment is the R linear chromosome end (denoted R*) generated by S-TIR recombination (Fig. 1, Table 2). In addition, the fertile regenerant 6B lacks the largest hybridizing 5.75 kb *Bam*HI fragment (Fig. 3, lane 16), which is the φ-R 'target' sequence (Fig. 1 and Table 2), indicating that not all fertile regenerant plants have the same mtDNA alterations. Probing

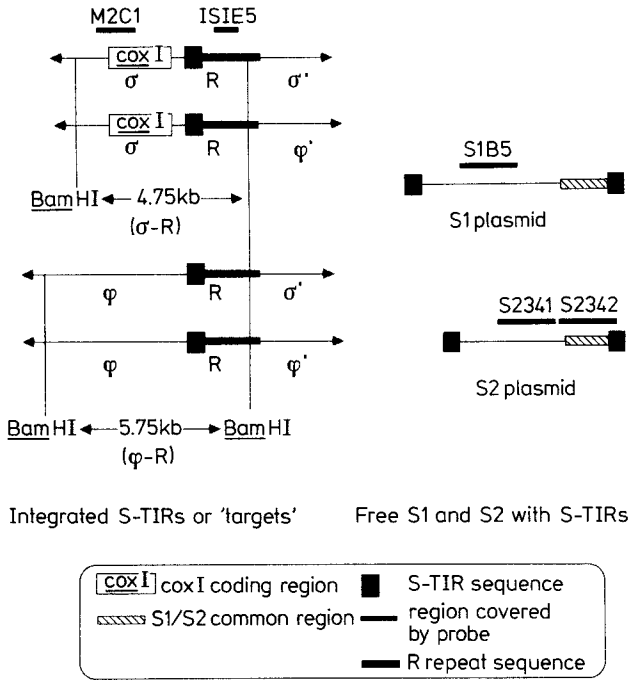

Fig. 2. The derivation of the hybridization probes used to investigate mtDNA from cytoplasmic revertants. M13 clones: M2C1 – *MspI* clone specific for the σ sequence, and derived from the 3' end of the *coxI* gene (Isaac et al. 1985); IS1E5 – *Sau*IIIA clone specific for the R repeat; S1B5 – *Hind*III clone specific to S1. pUC 8 clones: S2341 – *Pst*I clone specific to S2; S2342 – *Pst*I clone of S2 containing 1330 bp common to S1 and S2

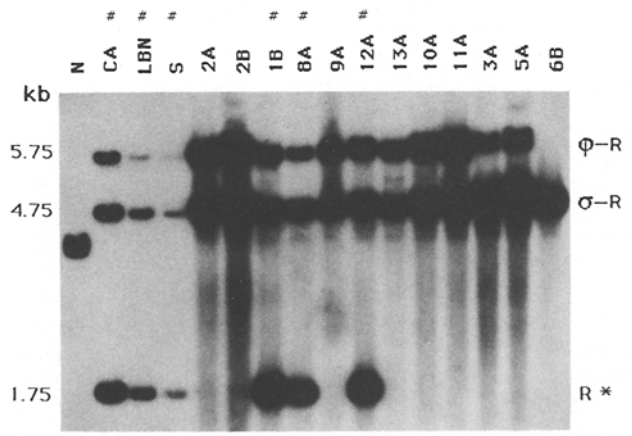

Fig. 3. Hybridization of IS1E5 (specific for the R repeat) to a Southern blot of *Bam*HI-digested mtDNA from W182BN regenerant lines compared with mtDNA from fertile (N) and sterile progenitor (CA, LBN, S) lines; * denotes fragment derived from the termini of linear chromosomes; # denotes cytoplasms that confer male sterility; 1B, 8A and 12A are male sterile regenerant lines and retain the 1,75 kb R* linear chromosome terminus. All other regenerant lines are fertile (revertant) and lack this fragment. All lines except 6B retain the φ-R and σ-R targets characteristic of CMS-S, 6B lacks φ-R. No consistent differences were observed between fertile regenerants from CA (2A, 2B, 3A), LBN (5A) or S (6B, 9A, 10A, 11A, 13A). Differences in hybridization signal intensity are due to inconsistencies in the amount of mtDNA loaded per lane

the fertile regenerant mtDNA with M2C1 (σ) (Fig. 4) confirms the presence of the σ-R target sequence and also confirms the lack of linear chromosome end fragments (there is no 9.4 kb fragment indicating the σ-S1*, S1-σ* linear ends). The mtDNA from fertile regenerant lines also lacks the 3.6 kb σ-S2 and 6.9 kb S2-σ fragments. Furthermore, probing these DNAs with the S2 specific sequence S2341 (Fig. 2) shows that all of these fertile regenerants apparently lack these S2 sequences, either free or integrated (data not shown).

The most interesting finding from the M2C1 probing is the presence in some of the fertile regenerants of two σ-containing *Bam*HI fragments of over 12 kb (Fig. 4). The size of these fragments suggests that they could include double-integrated complete copies of S1. Probing the same blot with S2342 (Fig. 2) after removing the M2C1 probe with 0.4 M NaOH detects three fragments of over 12 kb (Fig. 5a), the smaller two coincident with the pair that hybridize to M2C1. The same pattern is obtained using a probe specific for S1 (S1B5, Fig. 2) on similar revertant mtDNAs (Fig. 5b). This is as predicted if these fragments are complete S1 double-integrates (the three fragments being φ-S1-φ, φ-S1-σ/σ-S1-φ and σ-S1-σ, as in Table 2). The fertile regenerants 3A and 19A contain a single S1-hybridizing fragment of 12.5 kb (Fig. 5b), which, in the case of 3A, does not hybridize to M2C1 (Fig. 4). It seems likely therefore that in these regenerants there has been a rearrangement in an integrated S1 sequence, which may have involved the loss of the attached σ sequence. This rearrangement probably included the loss of one S-TIR, or else the original arrangement would be able to reform by recombination with the σ-R/φ-R targets still present in these revertants.

Neither the sterile progenitor lines nor the sterile regenerant lines contain detectable levels of these S1 integrate fragments and not all of the fertile regenerant lines exhibit the S1 integrate fragments to the same extent (Figs. 4 and 5). The presence of these fragments appears to depend on the age of the callus when plants were regenerated from it (Table 1). Fertile revertant lines established from plants regenerated from cultures less than 5-months-old contain high levels of S1 integrates, whilst those regenerated from cultures over 1-year-old have apparently lost these integrates, implying that these structures are unstable or selected against in culture. The exceptions are the novel recombinant arrangement found in 3A and 19A that is present in plants regenerated after 14–15 months in culture, and the S1 integrates present in 9A, despite the fact that this line was established from a 12-month-old culture (Fig. 5b). However, this culture (460, Table 1) seems anomalous as it was the only culture capable of producing sterile regenerants after 12 months (e.g., 8A), and thus may be considered 'slow'.

One important observation about the independent reversion events seen in these regenerant plants is their

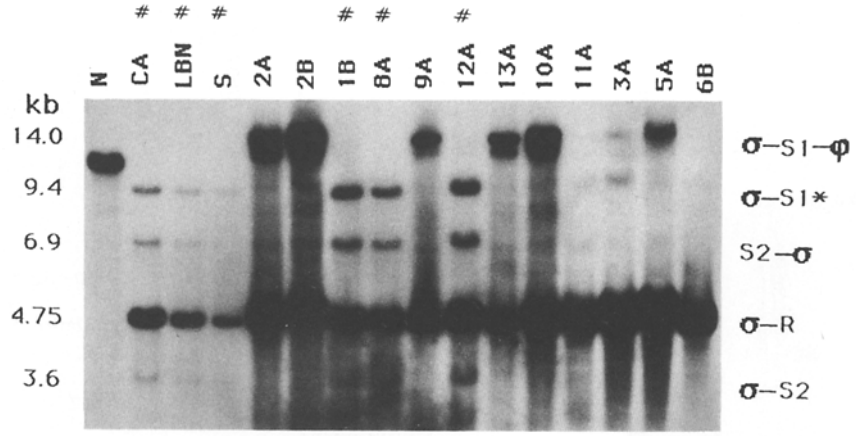

Fig. 4. Hybridization of M2C1 (specific for the σ sequence) to a Southern blot of *Bam*HI-digested mtDNA from W182BN regenerant lines compared with mtDNA from fertile (N) and sterile progenitor (CA, LBN, S) lines; * denotes fragments derived from the termini of linear chromosomes; # denotes cytoplasms that confer male sterility. The sterile regenerant lines 1B, 8A and 12A show a pattern indistinguishable from that of the progenitor CMS-S lines (CA, LBN, S). All fertile regenerants retained the σ-R target but lost the products of S episome recombination seen in the sterile lines. However, many of the fertile regenerants show two hybridizing fragments in the 12–15 kb region. These can be interpreted as the σ-S1-φ and σ-S1-σ double integration products (Table 2), which are not visible in the sterile lines. No consistent differences are observed between fertile regenerants from CA (2A, 2B, 3A), LBN (5A) or S (6B, 9A, 10A, 11A, 13A). Differences in hybridization signal intensity are due to inconsistencies in the amount of mtDNA loaded per lane

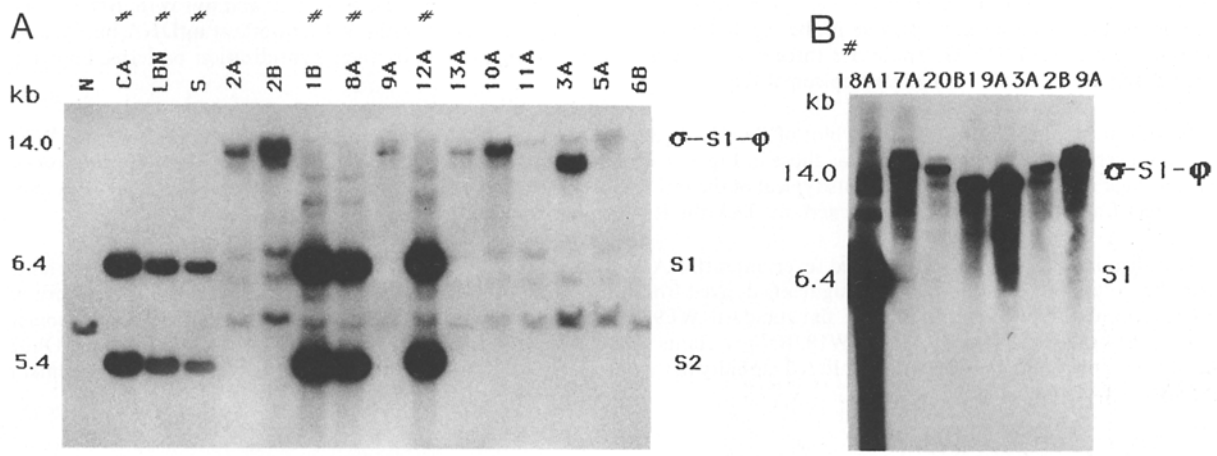

Fig. 5a and b. Hybridization of **a** S2342 and **b** S1B5 to Southern blots of *Bam*HI-digested mtDNA from W182BN regenerant lines compared with mtDNA from fertile (N) and sterile progenitor (CA, LBN, S) lines; # denotes cytoplasms that confer male sterility. **a** the sterile regenerants 1B, 8A and 12A show a pattern indistinguishable from that of the progenitor CMS-S lines (the faint signals appearing in the sterile regenerants are only apparently absent from the progenitor lines because of the lower DNA loadings in these lanes). The two smaller of the three large (c. 14 kb) fragments visible in the DNA from 2A, 2B, 9A, 13A, and 10A are coincident with those in the previous probing (Fig. 4). 3A exhibits an anomalous smaller hybridizing fragment. The numerous faint bands hybridizing in most lanes are due to the small amount of S-TIR homology present in this probe. **b** The three high molecular weight fragments detectable with the S1/S2 probe S2342 hybridize equally well to the S1B5 S1-specific probe, helping to confirm that they represent the φ-S1-φ, σ-S1-φ and σ-S1-σ double integration products. The probe also hybridizes to the anomalous smaller fragments of the lines 19A and 3A. 18A is a sterile regenerant line obtained from the same culture at the same time as the fertile regenerant line 17A. 19A was derived from the same culture as 20B, though it was derived from a plant regenerated nearly 1 year later

similarity with each other. No differences were seen amongst revertants that could be correlated with the cytoplasmic genotypes S, CA or LBN, even though the mitochondrial genomes in these cytoplasms can be differentiated on the basis of restriction endonuclease patterns (although they are not distinguished by the probes used in this study), and these cytoplasms are phenotypically distinguishable in the presence of some restorer genes (Sisco et al. 1985). The minor differences observed between fertile regenerants were apparently independent of cytoplasmic origin. However, the mtDNA alterations in these revertant plants seemed to differ in certain impor-

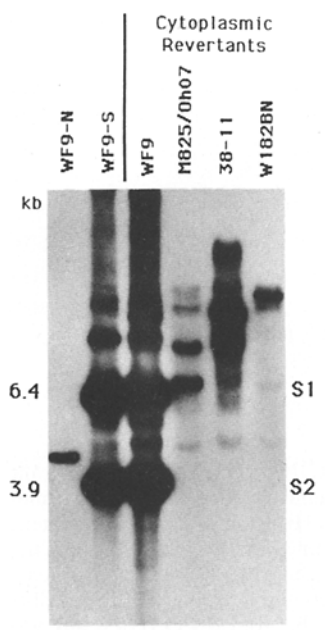

Fig. 6.

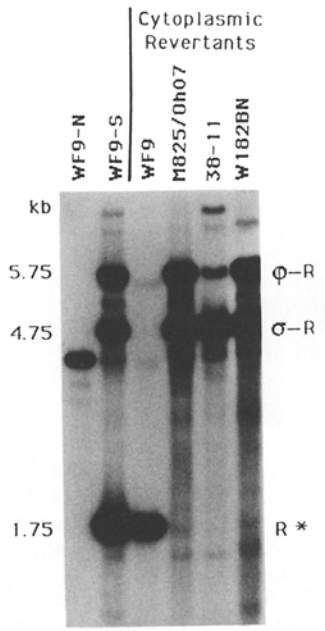

Fig. 7.

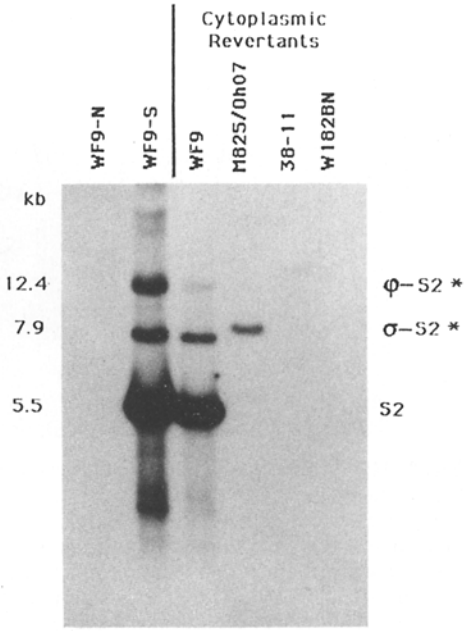

Fig. 8.

Fig. 6. Hybridization of S2342 to a Southern blot of *Bam*HI-digested revertant mtDNA compared to fertile (N) and sterile (S) WF9 mtDNA. The revertants shown are: (i) WF9-S 85:6838; (ii) M825/Oh07-VG 801-8; (iii) 38-11-S 81-115-2; and (iv) W182BN-S fertile regenerant line 10A. This general S episome probe highlights the free S1 and S2 present in the WF9 revertant mtDNA but lacking from the other revertant mtDNAs. The other three revertants are easily distinguishable by their hybridization patterns, implying different rearrangements in their integrated episomal sequences

Fig. 7. Hybridization of IS1E5 to a Souther blot of *Bam*HI-digested revertant mtDNA compared to fertile (N) and sterile (S) WF9 mtDNA. The revertants shown are the same as those in Fig. 6. * Denotes fragments derived from the termini of linear chromosomes. All revertants lack at least one of the fragments typical of the mtDNA from the sterile WF-S line. The WF9 revertant lacks both (σ-R and φ-R) target fragments; the other three revertants lack the R* chromosome terminus

Fig. 8. Hybridization of S2341 to *Sal*I-digested revertant mtDNA compared to fertile (N) and sterile (S) WF9 mtDNA. The revertants shown are the same as in Fig. 6. * Denotes fragments derived from the termini of linear chromosomes. The hybridization pattern of the WF9 revertant closely resembles that of the standard WF9-S, with both fragments characteristic of linearized chromosomes detectable, as well as free S2. The 38-11 and W182BN revertants lack any significant homology to the probe at all. The M825/Oh07 revertant shows one prominent band with an altered mobility to that of the typical S pattern; this is the partially deleted rearrangement described by Schardl et al. (1985)

tant respects from those reported previously for spontaneous CMS-S cytoplasmic revertants with other nuclear genotypes, and hence we have compared a set of typical revertants with different nuclear backgrounds using the same probes as for the W18BN fertile regenerants.

Comparison of W182BN revertants to revertants from other sources

Extensive studies of the mtDNA from CMS-S cytoplasmic revertants have been published only for spontaneous field-derived revertants with the nuclear genotypes M825/Oh07 and [WF9/M825]/M825 (Levings et al. 1980; Kemble and Mans 1983; Schardl et al. 1985). In these revertants, recombination between integrated S2 sequences and homologous sequences elsewhere in the genome led to the deletion of the left-hand S-TIR of the integrated S2 sequences. In addition, the revertant mtDNA lacked any fragments diagnostic of linear chromosomes, and lacked free S1 and S2 (Schardl et al. 1985). In contrast, mtDNA from revertants with the nuclear genotype WF9 retained free S1 and S2 (Escote et al. 1985; Ishige et al. 1985), and the integrated S1 and S2 sequences remained intact (Escote-Carlson et al. 1988).

Figure 6 shows mtDNA from field-derived revertants with the nuclear genotypes WF9, M825/Oh07, and 38-11 compared with mtDNA from the fertile W182BN regenerant 10A digested with *Bam*HI and probed with S2342. The hybridization pattern of each of the revertant mtDNAs is easily distinguishable, and as three out of four of the revertants had the same original cytoplasm, it is likely that the differences are due to nuclear gene ef-

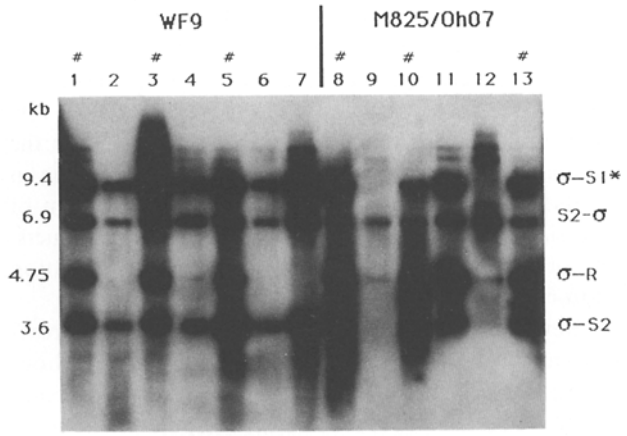

Fig. 9. Hybridization of M2C1 to a Southern blot of *Bam*HI-digested mtDNA from WF9 and M825/Oh07 revertants and corresponding sterile progenitor lines. * Denotes fragments derived from the termini of linear chromosomes. # Denotes cytoplasms that confer male sterility. *1* WF9-ML: *2* CR from 1 (81H-51-1); *3* WF9-RD; *4* CR from 3 (81-47-13); *5* WF9-RD; *6* CR from 5 (81-47-16); *7* CR from 5 (82-1179-16); *8* M825/Oh07-VG; *9* CR from 8 (793); *10* M825/Oh07-VG; *11* NR from 10 (Rf-m); *12* CR from 13 (799); *13* M825/Oh07-VG. CR = cytoplasmic revertant; NR = nuclear revertant. All four of the cytoplasmic revertants from WF9 parents (*2*, *4*, *6*, and *7*), whether derived from RD or ML cytoplasm, show similar patterns. The only change from the parental pattern is the loss of the 4.75 kb σ-R target fragment. The 9.4 kb σ-S1*/S1-σ* linear chromosome end is retained. The M825/Oh07 cytoplasmic revertants have lost this 9.4 kb fragment. The 3.6 kb S2 containing fragment is also lacking; this is the end of S2 deleted in these revertants (Schardl et al. 1985). The σ-R fragment is reduced in quantity in these M825/Oh07 revertants, but is still detectable at this level of exposure (note that the M825/Oh07 revertant depicted in Fig. 6 retained normal levels of this fragment). The nuclear revertant (*11*) shows the same mtDNA hybridization pattern as sterile M825/Oh07. The autoradiograph has been overexposed to reveal a pair of faintly hybridizing fragments (at > 9.4 kb) that represent the φ-S1-σ and σ-S1-σ double-integration products

fects. The most strikingly different revertant pattern is that of the WF9 revertant, which has retained S1 and S2 in high copy number, as previously reported (Escote et al. 1985; Ishige et al. 1985), and which, with this general S episome probe, is difficult to distinguish from its sterile parent.

To gain information on which mtDNA alterations are affected by nuclear background, other hybridization probes specific to R (IS1E5) and S2 (S2341) were employed. Figure 7 shows mtDNA from the same revertants cut with *Bam*HI and probed with IS1E5. As expected, the M825 and W182BN revertants lack the 1.75 kb *Bam*HI R* fragment, as does the 38-11 revertant. This would be expected if the mtDNA organization reverted to a circular form during reversion to fertility. In contrast, the WF9 revertant mtDNA does contain this fragment. Therefore, WF9 revertants, in addition to retaining free episomes (Fig. 6), also retain the linear chromosomes characteristic of the CMS-S parent. However, the IS1E5 probe does unambiguously separate the WF9 revertant from its sterile parent (and also from the other cytoplasmic revertants) by highlighting the conspicuous lack of the S-TIR target fragments σ-R and φ-R in this revertant. Similarly, differences between the WF9 revertant and the others emerge after probing *Sal*I digested mtDNA from the same revertants with S2341 (Fig. 8). The WF9 revertant closely resembles the typical S pattern, including the 7.9 kb σ-S2* linear end and the 12.4 kb φ-S2* linear end. Of the other mtDNAs, the W182BN revertant lacks any detectable homology to this probe, the 38-11 revertant shows only faint hybridization (at positions not corresponding to any predictable recombination products), and the M825/Oh07 revertant shows a single prominent band, with a mobility different from that of any of the bands in the standard S track; this is the rearranged integrated form of S2 previously reported (Schardl et al. 1985).

To provide further evidence that independent reversion events within any one nuclear background are similar, we compared several revertants from each of the backgrounds WF9 and M825/Oh07 (Fig. 9). Hybridization of M2C1 to these mtDNAs confirms earlier observations. All WF9 revertants retain linear chromosomes (e.g., the 9.4 kb σ-S1/S1-σ* fragment) but lose S-TIR targets (the 4.75 kb σ-R fragment), whilst the M825/Oh07 revertants lose fragments characteristic of linear chromosomes.

Discussion

In summary, all the CMS-S revertants to fertility studied lack some S-TIR-linked fragments found in their sterile progenitors (Table 3). In some cases the loss of certain fragments is linked with the appearance of new fragments presumably generated by recombinational rearrangements. Beyond these general similarities, the revertants from each source differ (Table 3). WF9 revertants, which retain the free episomes of their parents but lack the σ-R and φ-R target sequences, can be considered to be in a class of their own. The W182BN, M825 and 38-11 revertants resemble each other in having lost free episomes and linear chromosome ends but (mostly) retaining the targets. These revertants differ, however, in the fate of the integrated episomal sequences. Revertants with the genotype W182BN appear to have lost S2 sequences entirely, the 38-11 revertant has reduced levels, whilst M825 revertants retain S2 sequences abundantly in a rearranged form. In addition, the 38-11 revertant contains rearranged integrated S1 sequences in high copy number, whilst the M825 revertants contain only the rare intact double-integrated S1 copies. During the culture

Table 3. Comparison of cytoplasmic revertants from different CMS-S sources

Features	CMS-S	Nuclear background of revertants to fertility[a]			
		WF9	M825/ Oh07	38-11	W182BN
Free S1, S2	+	+	−	−	−
σ-R target	+	−	+	+	+
φ-R target	+	−	+	+	+
S1* linear ends	+	+	−	−	−
S2* linear ends	+	+	−	−	−
R* linear ends	+	+	−	−	−
Integrated intact S1	(+)	(+)	(+)	−	+
Integrated intact S2	(+)	(+)	−	−	−
Integrated rearranged S1	−	−	−	+	−
Integrated rearranged S2	−	−	+	−	−

[a] + = present; − = absent; (+) = present at low levels

period, the W182BN calli appear to lose the terminal S1 integrates with a concomitant increase in the amount of S1 sequence found in the double-integrated form. Over a further period of several months these double-integrated forms are lost, except in the case of two cultures (3A and 19A) where the integrated forms are retained, apparently with a deletion. The regenerants from these cultures thus resemble the 38-11 revertant.

These results suggest a link between the type of molecular events during reversion and the nuclear genotype. This is reinforced by the observation that the nuclear genotype greatly affects the rate of reversion of field grown plants, e.g. M825 plants can revert at a rate of up to 10% per generation (Laughnan et al. 1981), whilst plants with the W182BN genotype have never been known to revert spontaneously. Other effects of the nuclear genotype on the mitochondrial genome are known; genotypes M825 and 38-11 influence the ratio of free S1 to S2 (Laughnan et al. 1981) and their integration products (data not shown), and this may be reflected in which integrated sequences are predominant in the revertants. For example, sterile 38-11 plants contain more free and integrated S2 than S1; the revertants retain integrated (but rearranged) S1 sequences but have reduced levels of integrated S2 sequences. In general, for M825 plants the situation is reversed. The most suggestive link, however, is the fact that the mitochondrial genome in sterile WF9 plants, though normal with respect to the free episomes, exhibits unusual stoichiometries of the S-TIR recombination sequences, specifically lower than normal proportions of the target sequences (Leaver et al. 1985). Circumstantially this seems likely to have a bearing on the anomalous molecular events involved with reversion in plants with this genotype. Whatever the mechanism of reversion, it is likely to involve nuclear gene products, one or more of which may be involved in replication and/or recombination of S episomal sequences.

Reversion appears to occur after deletion or rearrangement of S-TIR-linked sequences in the mitochondrial genome. In the one study that examined the recombination points of these rearrangements, the extent of the homology between recombining sequences is small (Schardl et al. 1985), implying that such recombinations would be rare, and would require selection and/or amplification to become fixed in the genome. Such fixation of rare recombinant products may be a normal feature of maize mitochondrial genome evolution (Small et al. 1987), but what is remarkable during reversion is that several apparently random unlikely alterations occur within a single plant generation (or within 3 months of cell culture) and the fact that many similar or identical alterations occur in a number of independent plants. Which particular set of rearrangements occurs appears to be determined by the nuclear genotype of the plant.

The lesion responsible for pollen infertility in CMS-S maize is as yet unidentified. Cytoplasmic revertants seem an ideal system for studying the molecular events underlying the CMS phenotype, as they provide a number of independent comparisons to be made to their sterile progenitors. In addition, the mtDNA from all known revertants resembles that of their sterile progenitors much more closely than that from fertile plants with N cytoplasm.

Revertants from cultures with the CMS-T cytoplasm have proved invaluable in identifying the apparent underlying cause of T-type male sterility. In these revertants a 6.7 kb *Xho*I fragment present in the progenitor plants has been altered (Umbeck and Gengenbach 1983; Wise et al. 1987a). This fragment has been shown to encode a 13 kd polypeptide (Dewey et al. 1986, 1987; Wise et al. 1987b) present in mitochondria from sterile CMS-T plants but not in mitochondria from fertile revertant plants (Dixon et al. 1982; Wise et al. 1987b). Reversion from CMS-T is associated with the deletion or alteration of the gene encoding this polypeptide (Rottmann et al. 1987; Wise et al. 1987a). Thus, this 13 kd polypeptide is strongly implicated as a cause of sterility in plants with T cytoplasm, as its presence correlates with the sterility trait. Unfortunately, no such correlation exists for S-type mitochondria. No fragment has been identified that is lacking from all the revertants (Table 3), and although S-type mitochondria do synthesize variant polypeptides

encoded by the S episomes (Forde and Leaver 1980), these are also present in mitochondria from fertile WF9 revertants (Zabala et al. 1987; Zabala and Walbot 1988; data not shown). In conclusion, it can be said that reversion to fertility in both CMS-S and CMS-T revertants appears to occur by the fixation of recombinant molecules that have portions of the progenitor genomes missing, but the particular alteration responsible for the restoration to fertility in the case of CMS-S remains a mystery.

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