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Fertile revertants from S-type male-sterile maize grown in vitro

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Summary. Plants were regenerated from callus cultures of maize inbred W182BN with the S(USDA) type of cytoplasmic male sterility (cms). Some regenerates from 16 of 18 separate cultures had fertile tassels. Many other regenerates, whose fertility could not be scored accurately because of abnormal plant morphology, produced fertile progeny after pollination with N cytoplasm W182BN. Revertant plants and/or progeny were obtained from all 18 cultures, which included the CA, D, LBN, and S sources of *cms*S. More revertants were recovered from cultures maintained as callus for 12 months than from 3–4 month old cultures. Several types of evidence (absence of segregation for fertility after selfing or pollination of revertants with standard W182BN, pollen viability counts, failure of revertants to restore sterile *cms*S lines to fertility, mitochondrial DNA analyses) indicated that the reversion to fertility involved cytoplasmic rather than nuclear alterations. All revertants examined lacked the S1 and S2 plasmid-like DNAs characteristic of the mitochondrial genome of sterile *cms*S lines. Most callus cultures lost S1 and S2 after 13–20 months in vitro. No revertants were seen among thousands of W182BN *cms*S plants grown from seed in the field or among plants from tissue cultures of W182BN with the C or T types of cms. The cytoplasmic revertants recovered from culture may be useful for the molecular analysis of *cms*S.

Key words: Cytoplasmic male-sterility (cms) – Reversion to fertility – Mitochondrial DNA – Maize – Somaclonal variation

Introduction

Plants recovered from tissue cultures of many different species show heritable variation (Larkin and Scowcroft 1981). Such somaclonal variation often shows Mendelian inheritance (Evans and Sharp 1983) and appears to involve alterations in nuclear genes. Although various alterations in organellar DNA of cell cultures have been described (e.g. Chourey and Kemble 1982; Kemble and Shepard 1984; McNay et al. 1984; Wilson et al. 1985), recovery of novel maternally inherited plant phenotypes from tissue cultures is rare. Tobacco plants with maternally inherited resistance to streptomycin and lincomycin have been obtained from cell cultures (reviewed in Maliga 1984). Resistance in these mutants is chloroplast-encoded, although concomitant alterations in the mitochondria have not been ruled out. The most notable example of a spontaneous culture-induced change specifically associated with mitochondrial (mt) DNA is the reversion of maize with the T (Texas) type of cytoplasmic male-sterility (cms) to fertility. Such reversion has been seen both in plants recovered after in vitro selection for resistance to the toxin produced by the fungal pathogen *Bipolaris maydis* race T (Gengenbach et al. 1977; Brettell et al. 1979) and in unselected plants regenerated from *cms*T cultures (Brettell et al. 1980; Umbeck and Gengenbach 1983). The shift to male-fertility shows a maternal pattern of inheritance which is correlated with alterations in mtDNA (Gengenbach et al. 1981; Kemble et al. 1982).

This paper reports a new example of maternally inherited somaclonal variation in a trait encoded by mitochondrial DNA, i.e. reversion to fertility from the S (USDA) type of cms in maize. Cytoplasmic revertants to fertility have previously been obtained from field-grown maize populations (Laughnan and Gabay-Laughnan 1983) but not in the inbred used in this study nor at the frequencies described here.

Materials and methods

Plant material

Zea mays L. inbred W182BN, an early maturity line widely used for hybrid production in the northeast USA, with the S, T or C (Charrua) types of cms was used to establish tissue cultures. Four different sources of *cms*S (CA, D, LBN, and S) were tested (Gracen 1982; Sisco et al. 1984). Male-fertile N cytoplasm W182BN, which carries no nuclear genes for fertility restoration, was used to pollinate sterile plants and fertile plants that shed pollen before or after silks were receptive.

Culture procedures

In 1981, 1983, and 1984, tissue cultures were established from 1.0–2.0 mm immature embryos from field or greenhouse grown cms plants. After ears were surface-sterilized with 30% Chlorox + 0.5% PEX detergent, embryos were excised, placed scutellum up on nutrient medium solidified with 0.88% Bacto-agar or 0.22% Gel-Rite (Kelco Corp.), and cultured in the dark at 25 °C. The medium contained MS (Murashige and Skoog 1962) salts, 100 mg/l myo-inositol, 0.4 mg/l thiamine, 4% sucrose, 2 or 5 mg/l 2,4-D and in some experiments 20 mM proline. Organized areas that developed from the scutellum were subcultured at approximately 4 week intervals for 3 to 31 months before transfer to medium lacking 2,4-D for plant regeneration. In some cases tissue to be regenerated was first grown on hormone-free medium with MS or N6 (Chu et al. 1975) salts and 10% sucrose; after several weeks it was shifted to hormone-free MS medium with 4% sucrose in the light (16 h/day, 70–100 $\mu E \cdot m^{-2} \cdot s^{-1}$, mixed cool-white and Grolux bulbs). Small shoots/plantlets were transferred onto filter paper moistened with liquid MS medium (0 2,4-D, 2% sucrose) in Magenta GA-7 boxes or test tubes. Well-rooted plantlets with several leaves were potted in vermiculite watered with ¼ strength MS salts and kept in a growth chamber (12 h/day, 70–100 $\mu E \cdot m^{2} \cdot s^{1}$) for several weeks. They were then transferred to Cornell soil-less mix (Boodley and Sheldrake 1973) in 20 cm pots, kept in a walk-in growth chamber (12 h/day, 300 $\mu E \cdot m^{-2} \cdot s^{-1}$) for several weeks, and then grown to maturity under greenhouse conditions.

Ratings of male-fertility

Regenerates and their progeny were rated for male-fertility according to the 1–5 scale of Beckett (1971), in which 1 is a completely sterile tassel with no anthers exserted, 3 is a tassel with some fertile anthers exserted, and 5 is a fully fertile tassel. Regenerated plants are listed as "fertile" if they exserted at least some anthers which shed fertile pollen, "sterile" if they had a tassel with sterile anthers, and "none" if they had a terminal ear instead of a tassel. Most regenerates listed as fertile had ratings of 3 on the 1–5 scale, while fertile progeny of regenerates were usually rated as 5. Fertility of pollen from plants with exserted anthers was determined by staining in acetocarmine and counting stained pollen in a population of several hundred grains at 100× magnification.

Progeny tests

If possible, regenerated plants were selfed; usually they were pollinated with seed-grown W182BN-N. Pollen was applied to silks in tassels or terminal ears as well as to any normal ear shoots. The seeds obtained were grown either in the greenhouse or in the field in Aurora, NY during the summers of 1983–1986. These plants were rated for fertility and crossed in various combinations. Some crosses and fertility ratings were also done in winter nurseries in Homestead, FL during 1984–1986. R1 populations were usually small because of the limited number of seeds available but R2 and later populations consisted of 12–24 plants.

Analysis of mitochondrial DNA

Assays for S1 and S2 mitochondrial episomes (Pring et al. 1977) were done using the rapid assay procedure of Kemble and Bedbrook (1979) as modified by Sisco et al. (1984). Dark-grown coleoptiles (3–4 days old), young green leaves of regenerated or seed-grown plants, and callus cultures were ground with mortar and pestle either in high salt buffer (0.8 M Tris, 0.4 M NaH_2PO_4, 40 mM Na_2EDTA, pH 8.3) or in a low salt buffer (0.4 M mannitol, 1 mM EGTA, 0.05% cysteine, 1 mM dithiothreitol, 10 mM TES, 0.1% bovine serum albumin). The low salt buffer gave better results. Leaf and callus preparations were usually extracted with phenol. Samples containing unrestricted mtDNA from 500–1,000 mg of coleoptiles or leaves or 1–2 g of callus were loaded onto 0.8% agarose gels (Sigma Low EEO agarose) and electrophoresed for approximately 18 h at 35 V. Gels were photographed with Polaroid Type 55 film.

Terminology

Plants regenerated from culture (R0 plants) are designated by the *cms*S source (CA, S, etc.) of the original material, the number of the culture (S-1981, S-460, S-468, etc.) and a plant number (S-1981-19, S-1981-22, S-480-58, etc.). In some cases a regenerated plant produced several tillers, each of which was rated for fertility. Such tillers are designated by decimals after the plant number (S-1981-31.1, S-1981-31.2, etc.). Progeny of R0 plants are termed R1; progeny of R1 plants are R2, etc.

Results

Morphology of plants regenerated from W182BN cmsS cultures

In 1981, a tissue culture was established from W182BN *cms*S (source S) on medium containing 5 mg/l 2,4-D and subcultured on the same medium. Plants were regenerated by transfer of tissue to medium lacking 2,4-D after 15 to 24 months of subculture. Twenty-nine regenerated plants (counting tillers) were grown to maturity (Table 1). The plants were stunted, ranging in height from 6 to 140 cm with a mean of 38 cm; W182BN plants grown from seed in the greenhouse are approximately 200 cm high.

Nineteen plants had only terminal silks and no tassels; five had sterile tassels (Table 1). R0 plants 19, 50, 51 (both tillers), and 53 had partially fertile tassels (rated 3), with 50–81% stainable pollen. The recovery of fertile plants from a *cms*S culture was particularly striking because even plants regenerated from cultures of male-fertile W182BN frequently had sterile tassels or none at all (Earle and Gracen 1985). Fertile tassels on regenerates from N cytoplasm cultures were often rated 3, so the subnormal fertility of the S revertants is probably a consequence of the culture procedures and plant growth conditions used.

Table 1. Male-fertility of plants and progeny from a W182BN *cms*S (source S) maize culture established in 1981 (S-1981)

R0 no.	Age[a]	R0 tassel[b]		R0 pollen[c]	R1 seeds[d]	R1	R1 pollen[c]	R2[f]
19	15	Fertile	(3)	–	3 (3)	No Germ.	–	–
22	17	Sterile	(1)	–	3 (2)	Fertile	89%	Fertile
26	17	None		–	11[e] (6)	Fertile	–	Fertile
27	17	None		–	2[e] (1)	Fertile	85	Fertile
31.1	16	Sterile	(1)	–	4 (2)	Fertile	–	Fertile
31.2	16	Sterile	(1)	–	2 (1)	Fertile	90	Fertile
32.2	15	None		–	3 (3)	Fertile	85	Fertile
43	20	None		–	2[e] (1)	Fertile	88	Fertile
50	23	Fertile	(3)	51%	3[e] (2)	Fertile	93	–
51.1	24	Fertile	(3)	68	0	–	–	–
51.2	24	Fertile	(3)	81	0	–	–	–
53.2	23	Fertile	(3)	50	0	–	–	–

+2 sterile R0 plants and 15 R0 plants without tassels from which no seeds or R1 progeny were obtained

– Not examined

[a] No. of months S-1981 cultures were grown on medium containing 5 mg/l 2,4-D before transfer to medium without 2,4-D for plant regeneration

[b] Figure in parentheses is the fertility rating on Beckett's 1–5 scale (1971)

[c] Percentage of pollen grains staining with acetocarmine

[d] All R1 seeds were from R0 plants pollinated with seed-grown W182BN-N plants, not from selfed R0 plants. The first figure is the number of R1 seeds obtained; figure in parentheses is number of R1 seeds used in progeny tests

[e] R1 seeds were obtained from ear shoots rather than from pollination of terminal silks

[f] All R2 plants were from seed of selfed R1 plants

In 1983 and 1984, additional cultures were established from embryos of W182BN *cms*S (sources CA, D, LBN and S). Callus was initiated on medium containing 2 or 5 mg/l 2,4-D (±20 mM proline) and subcultured on medium with 2,4-D for 3 to 31 months before transfer to regeneration medium. One aim in setting up these cultures was to confirm the results of the 1981 work, thereby ruling out the possibility of a labelling error. It was also of interest to determine how early reversions to fertility occurred, whether they occurred in sources of *cms*S other than S, and whether cultures grown on 2 rather than 5 mg/l of 2,4-D also yielded revertants.

Results from these studies, summarized in Table 2, are consistent with those from 1981 (Table 1). Although most regenerates had terminal ears or sterile tassels, many fertile R0 plants were again observed. Fifteen of the 17 new cultures produced some fertile R0 plants. Tillers from a single regenerate sometimes differed in fertility. Viability of pollen from fertile tassels was variable but was at least 50% in 35/43 cases and over 75% in 17/43 (data not shown). The 2,4-D level on which cultures were maintained did not appear to influence the fertility of regenerated material (data not shown).

Inheritance of male fertility in cmsS revertants

Although examination of the R0 plants showed that reversion to fertility occurred in many independent cultures from all 4 sources of *cms*S, it underestimated the extent of in vitro reversion. Many more revertants were identified in the R1 generation.

A few seeds were obtained from 9 of the 1981 regenerates, including 2 of the fertile ones (Table 1). All 1981 R1 progeny tested were fully fertile (rated 5), including those from R0 plants with sterile or absent tassels (Table 1). Fertile R1s were recovered from seeds produced by pollination of ear shoots (R0 plants No. 26, 27, 43, and 50) as well as from seeds obtained by pollination of terminal silks, so the revertant R0 plants were apparently not chimeral. The progeny obtained after pollination of R0 plants with W182BN-N showed no segregation for fertility, and all had at least 85% plump darkly stained pollen grains. These results suggest that the reversion to fertility was maternally transmitted. *Cms*S plants heterozygous for Rf_3, the nuclear gene that restores fertility to *cms*S (Laughnan and Gabay-Laughnan 1983), normally show gametophytic restoration; that is, only pollen grains carrying the Rf_3 allele (50%) are viable. All selfed or sibbed progeny of plants S-1981-22, 26, 27, 31, 32.2, and 43 were fully fertile in the R2 (Table 1), R3, and subsequent generations, as would be expected with maternal inheritance.

Seed recovery from regenerates was somewhat better in 1983 and 1984 than in 1981 because W182BN-N pollinators were planted in the greenhouse every week; therefore, more extensive progeny tests were done. These again revealed maternal inheritance of reversion. Almost all fertile R0s tested produced only fertile R1 progeny (Table 2). This was true regardless of whether

Table 2. Male-fertility of plants and progeny from W182BN maize cultures with different source of *cms*S

*cms*S source	Culture no.	R0 plants			R0→R1 progeny[a]					R1→R2 progeny[b]	
		Fertile	Sterile	Total	F→F	F→S	S→F	S→S	S→S+F	F→F	S→S
S	1981	5	24	29	1/1	0/1	7/7	0/7	0/7	6/6	–
	460	2	18	20	2/2	0/2	5/11	6/11	0/11	6/6	5/5
	468	4	33	37	2/2	0/2	13/21	6/21	2[c]/21	14/14	7/7
	472	1	2	3	–	–	1/1	0/1	0/1	1/1	–
	484	4	13	17	2/2	0/2	6/11	4/11	1[d]/11	5/5	3/3
CA	12	3	13	16	–	–	0/4	4/4	0/4	–	–
	395	1	3	4	–	–	1/1	0/1	0/1	–	–
	398	5	17	22	4/4	0/4	3/7	4/7	0/7	5/5	3/3
	399	2	8	10	1/1	0/1	3/4	1/4	0/4	–	1/1
	405	0	11	11	–	–	3/4	1/4	0/4	2/2	1/1
	406	2	18	20	0/1	1[e]/1	6/6	0/6	0/6	5/5	–
LBN	11	2	6	8	1/1	0/1	2/2	0/2	0/2	2/2	–
	422	1	5	6	1/1	0/1	0/2	2/2	0/2	1/1	1/1
	423	1	8	9	–	–	1/1	0/1	0/1	–	–
	427	0	15	15	–	–	3/6	3/6	0/6	2/2	3/3
	433	3	5	8	1/1	0/1	–	–	–	1/1	–
	436	2	6	8	–	–	2/2	0/2	0/2	2/2	–
D	5	13	13	26	8/9	1[e]/9	1/3	2/3	0/3	–	–
Total	18	51	218	269	23/25	2/25	57/93	33/93	3/93	52/52	24/24

[a] No. of fertile (F) or sterile (S) R0 plants giving all fertile, all sterile or a mixture of sterile and fertile (S+F) R1 progeny/R0 plants tested

[b] No. of fertile (F) R1 lines giving all fertile R2 progeny and the number of sterile (S) R1 progeny giving all sterile R2 progeny/lines tested. No F R1→S R2 or S R1→F R2 lines were observed

[c] 16 S plants and 1 F plant; 19 S plants and 1 F plant

[d] 2 S plants and 1 F plant

[e] From R0 selfed by pollination of ear shoot

the R0 silks pollinated were in terminal ears or in normal ear shoots or whether R0s were selfed or sibbed with seed-grown W182BN. Two R0 plants with fertile tassels (CA-406-22.2 and D-5-2.3) produced only sterile progeny. In both cases, the seeds tested were obtained from self pollination of ear shoots so it is possible that these two fertile R0 plants were chimeral and the cytoplasm of the ovules pollinated was not revertant.

The R1 progeny of many sterile R0s from the 1983 and 1984 cultures were also fertile, but some sterile R1 plants were also seen (Table 2). This recovery of some sterile R1 progeny contrasts with the results from the S-1981 culture (Table 1) and is probably due to the inclusion of R0 plants recovered from callus cultures only a few months old (see below). In a few cases (3/93), progeny of sterile R0s segregated for fertility, with a single fertile plant seen (Table 2). Mixed seed during planting might account for these rare fertile plants in an otherwise sterile row.

Pollen viability of the fertile R1 plants from the 1983 and 1984 cultures was always > 85% and often as high as 95% (data not shown). Ratings of the R2 plants tested followed the fertility of the R1s, with no segregation of selfed fertile R1s and no new shifts to fertility from the sterile R1s (Table 2).

Crosses in which revertants were used to pollinate sterile W182BN plants with the CA, LBN, or PR sources of *cms*S provided additional evidence for maternal inheritance of reversion. Pollen from 22 revertants (from 12 different cultures and the CA, LBN, and S sources of *cms*S) failed to restore sterile *cms*S lines to fertility (data not shown). Progeny from *cms*S lines pollinated with revertants LBN-11-24 and LBN-427-36 segregated 1 and 5 for fertility, initially suggesting that these revertants might be heterozygous for a nuclear fertility restoration (*Rf*) gene. In further tests, pollen from the plants rated 5 did not restore sterile lines to fertility, so that the fertility seen after the initial crosses probably had an environmental rather than genetic basis. The high R1 pollen fertility (97 and 92% respectively), lack of segregation in the R1 and R2 generation, and loss of the S1 and S2 plasmids (see below) in LBN-11-24 and LBN-427-36 also indicated that they are cytoplasmic revertants like the others.

Effect of time in culture on reversion to fertility

The 1983 and 1984 studies showed that reversion to fertility can occur well before the 15 or more months of subculture on medium containing 2,4-D used with the

Table 3. Effect of time in culture on reversion of W182BN *cms*S maize to fertility

*cms*S source	Culture no.	Culture age[a] (months)	Revertants[b]	R1 progeny[c]	
				Fertile	Sterile
S	1981	15–24	12	8	0
	460	3–4	0	0	3
		12	7	7	3
	468	3	2	1	8
		12	13	13	0
		21	2	1	–
	472	3	1	1	0
		14	1	–	–
	484	3–4	8	7	4
		22	2	1	0
CA	12	4	3	0	4
	395	15–21	2	1	0
	398	3	2	2	4
		12–15	6	5	0
	399	3	0	0	1
		13	5	4	0
	405	3	0	0	2
		12–13	3	3	0
	406	12–15	8	6	1
LBN	11	11–17	4	3	0
	422	3–4	2	2	2
	423	8	0	–	–
		14–27	2	1	0
	427	3–8	0	0	3
		12	3	3	0
	433	12	1	1	0
		31	2	–	–
	436	14	4	2	0
D	5	8	14	9	2
Total		3–4	6/10 cultures[d]	5/10[e]	9/10[e]
		11 or more	15/15 cultures[d]	14/14[e]	2/14[e]

[a] No. of months cultures were grown on medium containing 2 or 5 mg/l 2,4-D before transfer to medium lacking 2,4-D for plant regeneration
[b] No. of R0 plants with fertile tassels and/or fertile progeny
[c] No. of R0 plants that produced fertile or sterile R1 progeny
[d] No. of cultures with revertants/cultures
[e] No. of cultures with fertile (or sterile) R1 progeny/cultures from which R1 seeds were tested

1981 materials (Table 3). Revertants were recovered from several cultures maintained on 2,4-D for only 3–4 months (S-468; S-472; S-484; CA-12; CA-398; LBN-422); however, reversion increased with time in culture. Only 6/10 3–4 month old cultures produced some

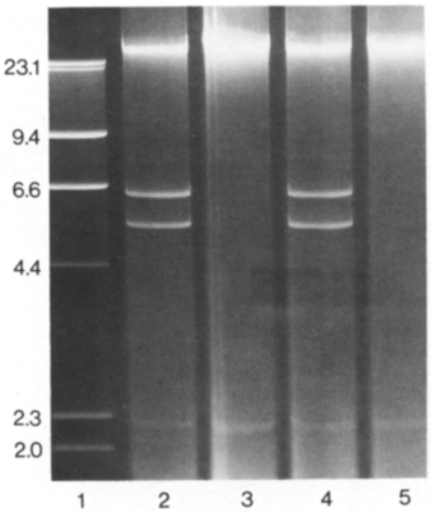

Fig. 1. Agarose gel electrophoresis of DNA from coleoptiles of W182BN *cms*S maize lines, showing absence of S1 and S2 plasmids in fertile revertants. DNA from approximately 1.5 g of tissue was loaded in *lanes 2–5*. *Lane 1* Molecular weight markers (Hind III digest of λ DNA) with sizes in kb. *2* 6.2 and 5.2 kb plasmids present in control W182BN *cms*S. *3* No plasmids in fertile progeny from regenerate S-1981-32.2. *4* Plasmids present in sterile progeny from CA-405-4.2 (regenerated after 3 months of callus culture). *5* No plasmids in fertile progeny from CA-405-16 (regenerated after 13 months of callus culture)

revertant plants/progeny but all cultures had done so by 12–15 months. Moreover, 9/10 3–4 month old cultures produced some sterile progeny but only cultures S-460 and CA-406 still produced any steriles after 12 months. Revertant and non-revertant lines were recovered simultaneously from cultures S-460, S-468, S-484, CA-398, CA-406, LBN-422, and D-5. The callus cultures thus appear to be chimeral, at least for a time.

Loss of S1-S2 plasmids in cmsS revertants and callus cultures

Additional evidence that reversion to fertility involves a cytoplasmic alteration was obtained by examination of mtDNA from some of the revertants. Presence or absence of the S1 and S2 plasmids in coleoptiles of the progeny of regenerates correlated perfectly with fertility of the plants; all sterile lines contained them and all fertile lines tested lacked them (Fig. 1, Table 4). Mitochondrial studies of the 1981 revertants were done only on progeny (R3 coleoptiles), but some R0 plants from the later cultures were also examined. All R0 plants tested, except S-460-121, lacked S1 and S2 (Table 4). Progeny of S-460-121 were sterile while all others tested were fertile. Analysis of leaf samples of W182BN R0 plants was therefore an effective way of predicting the fertility of their progeny. It is likely that field trials of

Table 4. Loss of S1 and S2 plasmids in fertile revertants from W182BN *cms*S maize tissue cultures

*Cms*S source	Culture no.	R0 no.	R0 plants		Progeny	
			Tassel[a]	Plasmids[b]	Fertility	Plasmids[c]
S	1981	27	0		F	–
		31.2	0		F	–
		32.2	S		F	–
		43	0		F	–
	460	58	0		S	+
		81	S		S	+
		106.1	S	–	F	
		106.2	S	–		
		108	0	–	F	
		109	F	–	F	
		111	F	–		
		121	0	+	S	
		122	0	–	F	
	468	70.2	F		F	–
		77	S		S	+
		84	S		S	+
		98.2	0	–	F	
		99.2	0	–	F	
		105.1	0	–	F	
		105.2	0	–	F	
		115.2	0	–	F	
		118	S	–	F	
		119	0	–		
		125	0	–	F	
	472	126	F	–		
	484	65.1	S		F	–
		65.2	S		F	–
		67.2	S		S	+
		80	S		F	–
CA	398	2.3	0		S	+
		3	0		S	+
		20.1	F	–	F (20.2)[d]	
		26	0	–		
		28.1	S	–		
		28.2	0	–		
		31	0	–		
		32.3	?	–	F (32.1)[d]	
	399	19.1	0	–		
		19.2	F			
		19.3	F			
		19.4	S	–		
	405	4.1	S		S	+
		16	0	–	F	
	406	22.1	F	–		
		24	S	–		
		33	0	–		
		34	0	–	F	
		38	0	–		
		40	0	–	F	
LBN	11	24	0		F	–
		63.1	F			–
	422	7	F		F	–
		13	0		S	+
	423	71	0	–		
		72	0	–		
		73	0	–		
		74	0	–		
	427	9	0		S	+
		35.1	0		F	–
		36	0		F	–
	433	42.1	0	–		
		52	F		F	–
	436	41	0	–		
		43.1	F	–		
		43.2	F	–		
		46	S		F	–
		47	0	–		
D	5	2.1	F		F	–
		5.1	F		F	–
		6	F		F	–

[a] F: fertile tassel; S: sterile tassel; 0: no tassel, only terminal silks
[b] Assayed using leaf tissue
[c] Assayed using coleoptiles from seedlings of progeny, usually from the R1 or R2 generation but in some cases from R3 or R4
[d] Fertility rating is for progeny from a different tiller of the R0 plant assayed

the plasmid-free lines not yet tested will reveal that they too are fertile. The high percentage of fertile, plasmid-free lines is probably due to the fact that the mtDNA analyses of R0 plants were done only on material recovered after about 12 months of culture, not on the earliest regenerates.

Twenty-four callus cultures were examined for the presence of S plasmids after various periods of culture (Table 5). The CA and D callus cultures set up in 1984 were positive for the plasmids after 5.5 months. By 13 months the plasmids were absent from 9/12 1983 cultures. After 19–20 months, all 1983 cultures examined were plasmid-free. The S-1981 culture, examined after 40 months, also lacked the plasmids. Four cultures (CA-398, LBN-427, LBN-436, and D-6) lost the plasmids between successive assays separated by only 1–2 months. Comparison of Tables 3 and 5 shows that fertile plants/progeny were recovered from callus cultures that still showed S1 and S2 (e.g. S-460, CA-398, CA-406). In those cases, either the callus was chimeral for presence of plasmids or the loss of plasmids occurred during the process of plant regeneration. Once a callus was plasmid-free, all regenerated plants/progeny were fertile.

Table 5. Presence of S1 and S2 plasmids in W182BN *cms*S maize callus cultures

Cms-S source	Culture no.	Year[a]	Age of culture (months)						
			5.5	11	12	13	15–17	19–24	36–40
S	1981	1981							–
	460	1983			+	+			
	468	1983		–	–	–			
	472	1983						–	
	484	1983		–		–			–
CA	7[b]	1984	+					–	
	12	1984	+						
	17[b]	1984	+						
	395	1983				–		–	
	398	1983			+	+	+	–	
	399	1983				–		–	
	405	1983				–			
	406	1983			+	+			
LBN	11	1983					–	–	
	423	1983			–	–		–	
	427	1983		+	+	–			–
	433	1983			–	–			
	436	1983			+	–			
D	1[b]	1984	+						
	2[b]	1984	+						
	3[b]	1984	+						
	4[b]	1984	+						
	5	1984	+					–	
	6	1984	+					+,+,–[c]	

[a] Year cultures were initiated
[b] Not used for plant regeneration
[c] Assays at 22 and 23 months were positive; at 24 months, plasmids were no longer apparent

Plants from cultures of W182BN with cmsC and cmsT

A total of 111 plants were regenerated from 2 cultures of W182BN *cms*C cultures ranging in age from 13 to 50 months. None of these plants had a fertile tassel, nor were any R1 progeny (obtained by pollination with W182BN-N) from 27 of these R0s fertile. Later generations from some of these R1s were also sterile. Similarly, none of 45 plants regenerated from 13 different cultures of W182BN *cms*T were fertile; 23 had sterile tassels and the rest had no tassels. R1 progeny from 12 of these R0s also remained sterile. Most of the progeny tests of *cms*T lines used regenerates from 3–5 month old callus but progeny from a 25 month old culture were also sterile.

Discussion

Laboratory and field studies conducted over the past 5 years indicate that fertile revertants can readily be recovered from tissue cultures of several sources of *cms*S with the W182BN nuclear background. The *cms*S sources represented 4 of the 5 different *cms*S subgroups identified by Sisco et al. (1985). All 18 cultures examined gave revertants. Of the 8 cultures from which plants were regenerated both at 3–4 months and after a year or more, only 4 gave revertants at 3–4 months while all did at the later time. Seven of these 8 younger cultures also produced some sterile progeny while only one of the older cultures still produced some steriles. After loss of the S1-S2 plasmids, which usually occured between 1 and 2 years in vitro, cultures gave only fertile progeny. In effect, therefore, there was a reversion frequency of 100%, provided cultures were maintained long enough.

This high frequency of reversion can be related to previous studies in several ways. One is to compare reversion at the whole plant level with that in vitro. Certain maize strains carrying *cms*S revert rather frequently to male fertility in the field (Laughnan and Gabay-Laughnan 1983). The nuclear genotype strongly influences both the frequency of such reversions and whether they are nuclear or cytoplasmic in origin. For example, reversions of *cms*S plants in inbred M825 occur at a high frequency and are predominantly cytoplasmic while in inbred WB 4 reversions occur at a lower frequency and are typically nuclear (Laughnan et al. 1981). Backcrossing M825 into a number of lines carrying *cms*S resulted in an increase in

the spontaneous reversion rate from 1% to an average of 8% and an increase in the percentage of cytoplasmic revertants from 17% to over 95% (Laughnan and Gabay-Laughnan 1983) from 28 different inbred by *cms*S cytoplasm combinations and a total of 15,702 plants examined, 80 revertants were found (Gabay-Laughnan and Laughnan 1983). These occurred in 18 of the 28 combinations and represented 0.51% of the total examined.

In their field studies, Gabay-Laughnan and Laughnan (1983) found cytoplasmic revertants from *cms*S to fertility in the nuclear background Wf9. We have also observed frequent field reversion of a series of *cms*S sources in the Wf9 inbred background, but not in the W182BN background. We have observed many thousands of W182BN plants in 22 different sources of *cms*S over a ten year period without detecting any fertile revertants. Our results suggest that reversion of *cms*S to fertility does not occur at the whole plant level in W182BN. The high frequency of in vitro reversions from W182BN thus contrasts markedly with the whole plant results with the same inbred, which suggests that different factors may control in vitro and in vivo reversion. In vitro reversion from W182BN *cms*S also seems to occur much more frequently than whole plant reversion in inbred M825.

Reversions to fertility have not been detected at the plant level in any *cms*C or *cms*T maize lines. *Cms*C also appears very stable in vitro; we failed to detect any revertants after regeneration of W182BN *cms*C material, even after 50 months of culture. However in vitro reversions from *cms*T to fertility in the nuclear backgrounds A188, W22, and Wf9 have been reported by several laboratories (Gengenbach et al. 1977; Brettell et al. 1979, 1980; Umbeck and Gengenbach 1983). Our failure to observe reversion from cultured W182BN *cms*T material may be due either to the nuclear background used or to the limited number of cultures and progeny examined. A role of nuclear background is suggested by the fact that we observed fertile tassels on 8 of 9 plants regenerated from 2 Wf9 *cms*T cultures maintained as callus for 7–9 months (Earle, unpublished data). Length of time in culture also appears to be important since some fertile revertants from W182BN *cms*T have recently been recovered from 14-month old callus cultures (Kuehnle, unpublished); most of the *cms*T plants examined in the present work were from cultures only 3–5 months old.

The culture-derived W182BN revertants examined to date fit a cytoplasmic rather than nuclear pattern of inheritance. Absence of segregation in R1 and later generations, pollen fertility counts, failure of revertants to restore sterile *cms*S lines to fertility, and loss of the S1 and S2 mtDNA plasmids are all consistent with this conclusion. Whether the predominantly maternal inheritance of reversion in W182BN is a consequence of in vitro culture or of the nuclear genotype used remains to be seen.

It is not clear what aspects of the culture environment induce the observed alterations in fertility and mtDNA. To demonstrate high frequency of reversion, it was important to have long-term cultures that retained the ability to regenerate plants and to make special efforts to recover seeds even from morphologically abnormal plants. The fact that almost all long-term W182BN callus cultures lost the S1 and S2 plasmids suggests that the relevant changes occur at the callus rather than the plant level. Furthermore, most revertant R0 plants do not appear to be chimeral, as evidenced by recovery of fertile progeny even after pollination of ear shoots distant from the fertile tassel. *Cms*S callus cultures are probably chimeral for some months since fertile and sterile plants could be obtained from the same culture. In W182BN loss of plasmids in leaves of small regenerates correlated perfectly with the fertility of their progeny. This correlation may permit study of factors (e.g. media, culture conditions, etc.) affecting reversion in W182BN via assay of leaves from small regenerated plants, without the necessity of growing plants to maturity or doing progeny tests.

The absence of S1 and S2 in all W182BN revertants examined is consistent with the first reports on field-derived *cms*S cytoplasmic revertants. In those lines, which had the M825 nuclear background, loss of the plasmids was correlated with rearrangements of the main mitochondrial genome (Levings et al. 1980; Kemble and Mans 1983; Schardl et al. 1985). Recent studies by Escote et al. (1985) indicate that cytoplasmic reversion to fertility does not require loss of the plasmids. Field revertants with a Wf9 nuclear background retain the plasmids but do show other mtDNA changes. Chourey and Kemble (1982) found that free S1 and S2 plasmids were absent in 2 year old callus cultures of *cms*S Wf9 and that their sequences were integrated into the high molecular weight mtDNA. This change was correlated with a shift from organized to friable callus morphology, a difference we did not observe in W182BN cultures that lost the plasmids. Because no plants were recovered from Chourey and Kemble's Wf9 cultures, no conclusions could be drawn about the relevance to cms of the observed mtDNA changes. We have established cultures of Wf9 with *cms*S to see whether revertants can be recovered in vitro from this inbred and whether they lose or retain the S plasmids.

Although a number of differences between mtDNA of fertile maize and the S, C and T types of cms have been identified (Pring and Levings 1978), it is difficult to determine which are causally related to male sterility. Revertants to fertility are particularly valuable for such studies since the revertant and parental lines can be directly compared. The cytoplasmic revertants identified by Laughnan and Gabay-Laughnan (1983) have been used in this way (Levings et al. 1980; Schardl et al. 1984, 1985). The large array of new revertants described here provides additional material, in a different nuclear background and in several *cms*S sub-

groups, for elucidation of the molecular basis of the S type of cms. Comparisons of different revertants with sterile material from the same cultures and with fertile and sterile versions of the standard inbred W182BN are under way (Leaver et al., unpublished data). Besides contributing to understanding of cms, molecular and field studies of culture-derived mitochondrial variants may identify other novel maternally inherited phenotypes with potential for maize improvement programs.

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