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Fertility restoration and mitochondrial nucleic acids distinguish at least five subgroups among cms-S cytoplasms of maize (*Zea mays* L.)*

P. H. Sisco[1, 2, **], V. E. Gracen[1], H. L. Everett[1], E. D. Earle[1], D. R. Pring[3], J. W. McNay[3] and C. S. Levings, III[2]

[1] Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY 14853, USA
[2] Department of Genetics, North Carolina State University, Raleigh, NC 27695, USA
[3] Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA

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Summary. Differences in fertility restoration and mitochondrial nucleic acids permitted division of 25 accessions of S-type male sterile cytoplasm (cms-S) of maize into five subgroups: B/D, CA, LBN, ME, and S(USDA). S cytoplasm itself (USDA cytoplasm) was surprisingly not representative of cms-S, since only two other accessions, TC and I, matched its mitochondrial DNA pattern. CA was the predominant subgroup, containing 18 of the 25 accessions. The B/D and ME subgroups were the most fertile and LBN the most sterile. The exceptional sterility of LBN cytoplasm makes it the most promising of the 25 cms-S accessions for the production of hybrid seed. The most efficient means of quantifying the fertility of the subgroups was analysis of pollen morphology in plants having cms-S cytoplasm and simultaneously being heterozygous for nuclear restorer-of-fertility (*Rf*) genes. This method took advantage of the gametophytic nature of cms-S restoration. The inbred NY821LERf was found to contain at least two restorer genes for cms-S. Fertility differences were correlated with mitochondrial nucleic acid variation in the LBN, ME, and S(USDA) subgroups.

Key words: Cytoplasmic male sterility – cms-S – Mitochondrial DNA – Double-stranded RNA – Gametophytic restoration of fertility

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** Present address: USDA-ARS, Box 7620, North Carolina State University, Raleigh, NC 27695-7620, USA

Introduction

Cytoplasmic male sterility (CMS) is a maternally-inherited trait found in many plant species that has proved useful in emasculating the female parent of a hybrid cross (Edwardson 1970; Pearson 1981; Hanson and Conde 1984). The male-sterile phenotype is thought to be caused by interactions between nuclear gene products and cytoplasmic factors (Clayton 1950). In maize, these cytoplasmic factors are probably mitochondrial gene products (Leaver and Gray 1982). Nuclear restorer-of-fertility (*Rf*) genes overcome the nuclear/cytoplasmic incompatibility, restoring male fertility to plants having the sterilizing cytoplasm. The three major groups of CMS in maize are cms-C, cms-T, and cms-S, which differ in expression of sterility and in response to various *Rf* genes (Laughnan and Gabay-Laughnan 1983).

Cms-S is the only one of these groups having gametophytic restoration of fertility (Buchert 1961). Gametophytic restoration is determined by the nuclear genotype of the pollen grain, the haploid gametophyte. In tassels of cms-S plants heterozygous for restorer genes, pollen grains carrying *Rf* alleles develop normally, while pollen grains carrying only *rf* alleles collapse shortly before anthesis (Buchert 1961; Lee et al. 1980). In the cms-T and cms-C systems, restoration of fertility depends solely on the nuclear genotype of the mother plant, the diploid sporophyte, so that in plants heterozygous for *Rf*, all pollen develops normally (Warmke and Lee 1977; Lee et al. 1979).

Besides gametophytic restoration, cms-S plants are characterized by having in their mitochondria abundant quantities of two plasmid-like DNAs called S-1 and S-2 (Pring et al. 1977). The S-1 and S-2 molecules have terminal inverted repeats (Levings and Sederoff 1983) and are covalently linked at their 5′ ends to a protein (Kemble and Thompson 1982). S-1 and S-2 have received much attention, because they may be involved with the cms-S sterility mechanism (Levings et al. 1980; Laughnan et al. 1984) and have potential as vectors for gene transfer in maize (Levings and Pring 1979). The im-

portance of S-1 and S-2 for this study was that they provide a test for cms-S independent of the sterility phenotype (Kemble and Bedbrook 1979)[1].

Previous studies (Beckett 1971; Julis et al. 1976; Gracen et al. 1979; Kálmán and Dévényi 1982) had reported variation for fertility restoration and disease suspectibility among cms-S accessions, but no differences had been found in organellar (chloroplast or mitochondrial) nucleic acids (Pring and Levings 1978; Forde et al. 1980; Kemble et al. 1980). Recently Carlson and Kemble (1984) have shown that a 1.94 kb supercoiled plasmid, found in the mitochondria of almost all maize (Kemble and Bedbrook 1980; Kemble et al. 1983) is absent in eleven S cytoplasm accessions but present in four: I, J, S, and TC cytoplasms. No differences in plant traits were found to be correlated with the presence or absence of the 1.94 kb plasmid, however.

We here present differences in mitochondrial nucleic acids correlated with variation in fertility restoration in at least three subgroups of cms-S.

Materials and methods

Definition of terms

CMS: cytoplasmic male sterility (noun) or cytoplasmically-male-sterile (adjective).

CMS accession: An independently-discovered source of CMS, designated by capital letters. For example, the EK accession is a CMS source discovered in the open-pollinated maize variety 'Early King' (Beckett 1971). Some authors use the terms "EK cytoplasm" or "cms-EK" for the EK accession (Beckett 1971; Gracen and Grogan 1974), while others refer to accessions as "strains" (Laughnan and Gabay-Laughnan 1983).

CMS group: A group of accessions, categorized by their response to restorer-of-fertility (*Rf*) genes. The three major CMS groups identified in maize have been named C, S, and T for the C ("Charrua"), S ("USDA") and T ("Texas") accessions (Beckett 1971; Gracen and Grogan 1974; Laughnan and Gabay-Laughnan 1983).

CMS subgroup: A subgroup of accessions within the three major CMS groups, identified by variations in fertility restoration and mitochondrial nucleic acids. The subgroups are named for accessions typical of the subgroup (e.g. the CA subgroup is named for the CA accession).

cms-C, cms-S, and cms-T: In this paper, as in Laughnan and Gabay-Laughnan (1983), these terms refer to the three major CMS groups, not to the accessions for which the groups were named. Some authors, however, use cms-C, cms-S, and cms-T to refer to particular accessions (e.g. Beckett 1971).

S(USDA): This term is used here to refer to the original S accession, for which the S group is named. "S" is derived from "USDA", because the original S accession was sent to D. F. Jones by Merle T. Jenkins of the United States Department of Agriculture (Duvick 1965).

[1] S-1 and S-2 molecules in very low molar amounts have been reported in cms-C (Koncz et al. 1981). Our unpublished results agree with this finding. S-1 and S-2 are usually not visible in ethidium-bromide-stained gels of mitochondrial DNA from cms-C, however, while they are prominent molecules in cms-S mitochondrial DNA (Fig. 5)

S-Rf genes: Nuclear genes that restore male fertility to cms-S plants. The "standard" S-*Rf* gene is *Rf3*, located in the long arm of Chromosome 2, but other S-*Rf* loci exist (Laughnan and Gabay 1978; Gabay-Laughnan and Laughnan 1979).

Plant material

The 25 cms-S accessions in the Cornell cytoplasm collection were investigated (Table 1). Cornell obtained these accessions in two inbred backgrounds, N6 and 38-11. Subsequently the cytoplasms were backcrossed eight to 15 times to 36 inbreds adapted to New York (Gracen and Grogan 1974; Sisco et al. 1982b).

Male fertility studies

Intragroup variation in fertility restoration was investigated in four ways:

Method 1. Tassels of inbreds

As various cytoplasms are backcrossed into a particular maize inbred, their nuclear genotype approaches uniformity. Thus, after an adequate number of backcrosses (six or more), the remaining variation between cytoplasms within a specific nuclear background is likely due to non-nuclear (e.g. mitochondrial or chloroplast) genes. Cornell's cytoplasm collection (Sisco et al. 1982b) and earlier work by Beckett (1971) permitted comparison of tassel fertility among cms-S accessions in over 40 inbreds. Initial inferences on fertility variation among cms-S accessions were taken from Beckett (1971) and Gracen and Grogan (1974). Additionally, the entire Cornell collection was grown out in the Cornell summer nurseries of 1980 and 1981 (half of the collection each year). Each inbred/cytoplasm combination was rated for tassel fertility, using the 1–5 scale developed by Duvick (1965) and Beckett (1971) that is noted in the legend of Table 1.

Method 2. Tassels of plants having cms-S sterile cytoplasm and segregating for various *Rf* genes for S-type male sterility

The data of Gracen and Grogan (1974) indicated that there were *Rf* genes specific for some accessions of cms-S. To determine whether these *Rf* factors for S accessions were single genes, a genetic analysis similar to that of Kheyr-Pour et al. (1981) was done. All crosses (F_1, F_2, and backcrosses) initially were made in "normal" (non-sterilizing) cytoplasm, so that both *Rf* and *rf* alleles were transmitted through pollen. The final cross was made onto cms-S sterile plants, and the tassels of the progeny were rated for male fertility. Tassels were rated "fertile" if anther exsertion appeared normal. This method took the greatest time (four generations) and the most space of the four methods. Method 2 is summarized in Table 2.

Method 3. Tassels of F_1 plants from crosses between cms-S sterile inbreds and inbreds partially male fertile in S cytoplasm

Another way of differentiating the fertility restoration capacity of the 25 cms-S accessions is to make crosses between cms-S sterile plants and inbreds carrying "weak" restorer genes (inbreds rated "3" according to the fertility scale of Table 1). The use of such F_1 hybrids brings out differences not evident in the inbreds themselves, yet requires only two generations and simple observation of tassel fertility. In 1974, 18 of the cms-S accessions in the inbred W182BN, which is sterile in cms-S, were crossed to the inbred CO192, rated "3" in most S accessions (Table 1). The F_1 progeny, one row of 24 plants per cross, were grown out in 1975 and 1981 and rated for tassel fertility on the 1–5 scale. In 1981, a special study was begun to test the restoration capacity of the B and D acces-

Table 1. Tassel fertility[a] of the cms-S collection at Cornell University

Inbreds-	A239	A495	A619	A632	A636	AyX65	AyX138	AyX145	AyX157	AyX187y-1	AyX187y-2	Ay499	Ay191-71	Ay303E	Ay490-2A	B8	C153	CO113	CO150	CO192	CO220	CrS4HLA	MS64-7	MS89A	MS1334	NYD410	NY63-71-1	NY821	NY821LERf	Oh43	Oh51A	Pa884P	SD10	Va20	W64A	W182BN
S-Group cytoplasms																																				
B/D subgroup:																																				
B	–	5	5	–	–	–	–	–	–	–	–	3	–	1	3	–	5	5	–	1	3	–	4	–	–	–	–	–	4	5	–	5	–	–	5	–	1
D	1	5	5	5	–	5	–	3	5	5	5	–	–	–	3	5	5	–	–	3	–	4	–	1	4	1	2	3	5	5	4	–	5	–	1	1
CA Subgroup:																																				
CA	1	–	3	3	3	3	–	3	3	3	3	3	1	5	1	3	3	–	–	3	–	1	5	1	3	1	–	1	5	5	3	1	1	5	1	1
EK	1	4	3	3	5	–	1	3	–	3	3	3	1	–	3	3	3	3	1	3	5	1	5	1	3	–	2	1	5	5	3	1	1	4	1	1
F	–	3	3	–	3	–	–	–	–	–	3	–	–	–	–	3	3	–	–	3	–	1	–	–	–	–	–	–	5	–	2	–	–	4	–	1
G	1	3	3	3	3	3	–	–	3	3	3	3	1	5	3	3	3	–	1	3	–	2	–	1	3	1	1	1	5	5	3	1	–	4	1	1
H	–	3	3	–	–	–	1	3	3	3	3	3	1	5	1	3	–	3	1	3	5	1	–	1	3	1	1	1	5	–	2	1	–	4	1	1
IA	–	3	3	3	3	–	–	–	3	3	3	3	–	–	3	3	3	–	1	3	–	1	–	–	3	1	2	1	5	5	3	1	2	4	1	1
J	1	3	3	3	3	–	–	3	–	3	3	3	1	4	1	3	4	–	–	3	–	1	–	1	3	–	–	1	4	–	3	–	–	4	1	1
K	1	–	3	3	3	3	–	–	3	3	3	3	–	5	3	–	3	–	1	3	–	1	–	–	3	1	2	1	5	5	3	–	1	4	1	1
L[b]	–	–	–	–	–	–	–	3	–	–	–	–	–	5	–	–	–	3	1	3	5	–	5	–	–	–	–	–	–	–	–	–	–	4	–	–
M	–	–	3	3	3	3	1	–	–	–	3	–	–	–	1	3	4	3	–	3	5	1	5	2	–	–	–	1	5	5	3	1	–	4	1	1
ME(N6)[c]	1	–	3	3	3	3	–	3	3	3	3	3	–	–	–	3	3	–	–	–	–	1	–	1	3	1	–	1	5	5	–	–	2	4	1	–
ML	1	3	3	3	3	–	–	3	3	3	3	3	–	4	1	3	3	–	1	–	–	–	–	1	3	1	2	1	5	5	3	–	1	5	–	1
MY	1	3	3	3	3	3	–	1	–	3	3	3	1	–	3	3	3	–	1	3	–	2	–	2	3	1	1	1	5	5	3	–	1	4	1	–
PS	1	3	–	3	3	–	–	3	3	3	3	3	1	–	1	3	3	3	1	3	–	2	5	1	3	1	1	1	5	5	3	–	1	4	1	1
R	–	3	3	–	–	–	–	3	–	3	3	–	1	–	–	3	4	3	–	3	–	2	–	–	–	–	–	1	5	–	3	1	–	4	–	1
SD	1	–	3	1	3	–	1	–	3	3	3	3	–	5	1	3	3	3	–	3	5	–	5	1	–	1	–	1	5	5	3	–	1	5	1	1
TA	1	3	3	3	3	–	–	3	–	3	3	–	1	–	–	3	3	–	1	3	–	1	5	1	–	–	–	1	5	–	2	–	–	5	1	1
VG	–	3	3	–	–	–	–	–	–	–	3	–	–	3	–	3	3	–	1	3	–	1	–	–	–	–	–	1	5	–	3	–	–	5	–	–
W	–	3	3	–	–	–	–	1	–	–	3	–	3	–	–	3	3	–	1	3	–	1	5	–	–	–	–	1	5	–	3	–	–	5	–	1
LBN Subgroup:																																				
LBN[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
ME Subgroup:																																				
ME(38-11)[c]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–	–	–	–	–	–	–	–	–	3
S(USDA) Subgroup:																																				
I	–	3	3	3	–	–	–	3	3	3	3	3	1	3	3	3	3	3	1	3	–	2	5	1	3	1	1	1	5	5	3	–	1	5	1	1
S(USDA)	1	–	3	3	–	–	–	–	–	3	3	–	–	–	–	3	3	–	–	3	–	1	–	1	–	–	–	1	5	–	3	–	–	4	1	1
TC	1	3	3	3	3	–	–	3	3	3	3	3	–	–	–	3	4	–	1	3	–	1	–	1	3	1	1	1	5	–	3	–	1	3	1	1

[a] Fertility scale: 1 no anthers exserted; 2 sterile anthers exserted; 3 partially fertile; 4 nearly fertile; 5 fully fertile. (–) the combination does not exist in the collection

[b] LBN cytoplasm, which has two prominent double-stranded RNAs, may be the same as L cytoplasm, from which it is derived. Tests to determine this are underway

[c] The ME cytoplasm that Cornell received in an N6 background was different in fertility restoration and mitochondral DNA from the ME received in a 38-11 background

sions by the inbred CrS4HLA. The data of Table 1 suggest that CrS4HLA has *Rf* genes for B and D, but not for other cms-S accessions. Crosses were made between Ay191-71, NYD410, and W64A, inbreds which are male-sterile in B and D, with the inbred CrS4HLA used as male. The F_1 progeny of these crosses were grown out ear-to-row in the Florida winter nursery of November, 1981, and the plants scored for tassel fertility. As a control for standard cms-S, the same crosses were also made in the CA accession.

Method 4. Pollen phenotype of F_1 plants from crosses between cms-S sterile inbreds and inbreds carrying S-*Rf* genes

Because fertility restoration of cms-S is gametophytic, only pollen carrying *Rf* allele(s) develops normally. Cms-S plants heterozygous for a single *Rf* gene usually have 50% functional, normal-appearing pollen (Buchert 1961). The other 50% of the pollen, carrying the *rf* allele, will collapse shortly before anthesis (Lee et al. 1980). The percentage normal-appearing pollen in F_1 progeny of crosses between cms-S steriles and restorer inbreds thus provides a rapid and quantitative method for comparing the fertility restoration among cms-S and among *Rf*-carrying inbreds.

Crosses were made using as females the 25 cms-S accessions in five inbreds male-sterile in S cytoplasm (Ay191-71, CrS4HLA, NY821, W64A and W182BN) and as males two inbreds that are fully male-fertile in S cytoplasm (MS64-7 and NY821LERf). It was not known at the time the crosses were made whether MS64-7 or NY821LERf carried *Rf3*, the standard S-*Rf* gene, or other S-*Rf* genes. The origin and exceptional restoration capacity of NY821LERf has since been reported (Sisco et al. 1982a). F_1 progeny of these crosses were grown out ear-to-row at Aurora, New York, in the 1981

Table 2. Summary of the Method 2 analysis of fertility restoration

Generation	Purpose	Example of genotype of crosses
1	Make F1 hybrids between inbreds containing *Rf* and *rf* alleles for cms-S. Use normal cytoplasm for female parent, so that both *Rf* and *rf* alleles will be transmitted through the pollen of the next generation	W182BN (*rfrf*) × NY821LERf (*RfRf*)
2	Make F2 and backcross generations. Use female having normal cytoplasm, for the same reason as in generation one	(W182BN × NY821LERf) ⊗ (W182BN × NY821LERf) × W182BN (W182BN × NY821LERf) × NY821LERf
3	Make plant-to-plant crosses between W182BN in S-type cytoplasm and the F1, F2, and backcross generations. Since the males in the crosses are in normal cytoplasm, both *Rf* and *rf* alleles will be transmitted through the pollen	W182BN (S-type cytoplasm) × individual plants of the F1, F2, and backcross generations
4	Grow out progeny of generation three crosses ear-to-row and score for tassel fertility. Since these plants are in S-type cytoplasm, pollen containing only *rf* alleles will abort	No crosses made. This is a testing generation

summer nursery, and at Homestead, Florida, in the winter nurseries of 1981 and 1982. On the third or fourth day of pollen shed, when the lateral branches of the tassels had begun to flower, tassels were cut off and placed on paper bags. This was done in the early morning before the anthers which would dehisce that day had exserted. After dehiscence, a sample of pollen was sprinkled into a drop of 50% glycerol/1% aceto-carmine on a glass slide, stirred, and a cover slip was applied. The next day, pollen morphology was examined with a light microscope at 100× magnification. Five hundred pollen grains were counted per slide and divided into two categories – those that appeared normal in size, shape, and staining, and those that appeared abnormal. Five tassels were sampled per row, except for the first experiment of August, 1981, when from 3–10 tassels were sampled. There was little sampling variation within tassels. In initial studies (results not shown), pollen was sampled from two florets, each from a different branch of a tassel. The percentage normal-appearing pollen was very consistent among florets (±2%).

Nucleic acid studies

The cms-S accessions were first screened for the presence of the S-1 and S-2 plasmid-like DNAs using the procedure of Kemble and Bedbrook (1979). Since time and resources did not permit the testing of all possible combinations of 25 cms-S accessions in 36 inbreds, only the 25 accessions in the inbred W182BN were analyzed, plus other inbred/cytoplasm combinations which had anomalous fertility ratings in Gracen and Grogan (1974). The mtDNA of the W182BN cms-S accessions was also digested with the restriction endonucleases BamHI and EcoRI and fractionated by electrophoresis on horizontal slab agarose gels for comparison of restriction pattern, using the procedure of McNay et al. (1984). For those cms-S accessions that appeared atypical in fertility restoration, digests were done with additional restriction endonucleases (e.g. SalI, XhoI, HindIII) and compared to cms-S accessions having standard restoration.

Results

Male fertility studies

Method 1. Tassels of inbreds

The 1980 and 1981 field ratings of the Cornell cytoplasm accessions are summarized in Table 1. Tassels of several inbred lines were more fertile in B and D than in the other cms-S accessions (e.g., inbreds A619, B8, C153, CrS4HLA, NY821, and SD10). The ME accession was more fertile than other S-group cytoplasms in the inbred W182BN, but not in any other inbred.

Method 2. Tassels of plants from F_2 and backcross generations

This was the first of the four methods to be used, and most of the initial results were negative – crosses between restorers and steriles gave 100% sterile progeny, even in F_2 and backcross generations. Analysis of the cytoplasms of the "restored" inbreds used as male showed that they did not contain the S plasmids, hence were probably not in S cytoplasm. Review of pedigree records indicated some mislabelling at the time the cytoplasms were first received by Cornell, and other errors evidently occurred at planting, when seed packets for two rows had been planted in reverse order. Most anomalous ratings of Gracen and Grogan (1974) could be attributed to mislabelled cytoplasms.

The positive results of this study came from crosses using NY821LERf as the source of restorer genes. The F_2 and both backcrosses of cms-S steriles with this line

Table 3. Tassel fertility of F_1 hybrids between cms-S steriles and partial restorers of fertility (Method 3)

Crosses using CO192 as male			Crosses using CrS4HLA as male		
Genotype	Cytoplasm	Fertility[a]	Genotype	Cytoplasm	Fertility
W182BNcms × CO192	B and D	5	Ay191-71cms × CrS4HLA	CA	1
			Ay191-71cms × CrS4HLA	B	5
W182BNcms × CO192	EK, G, H, I, IA, J, K, M, ML, PS, R, S, TA, TC, and W	3	NYD410cms × CrS4HLA	CA	1–3
			NYD410cms × CrS4HLA	D	5
W182BNcms × CO192	LBN	1	W64Acms × CrS4HLA	CA	2–3
			W64Acms × CrS4HLA	D	5

[a] The fertility scale is the same as that of Table 1

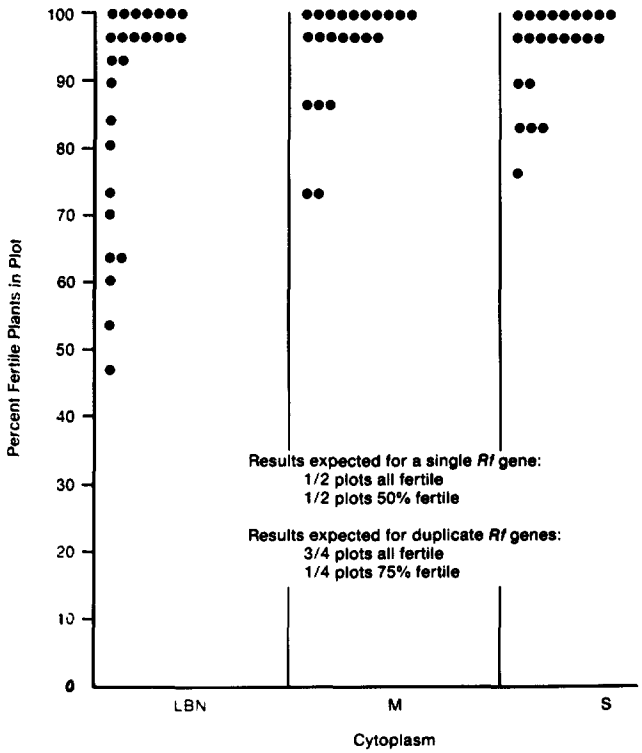

Fig. 1. Tassel fertility of cms-sterile × backcross-to-fertile (cms × BC_F) progeny rows. Each plot was approximately 60 progeny from a single pollination. cms × BC_F = W182BN(LBN, M, or S) × (W182BN × NY821LERf) NY821LERf

had a greater percentage of male-fertile plants than would be expected if NY821LERf carried only a single S-*Rf* gene. L cytoplasm in the inbred W182BN (now called LBN cytoplasm – see results of nucleic acid studies) was also significantly less restored to fertility by NY821LERf than M and S cytoplasms. Figure 1 summarizes the results of the BC_F (backcross to the fertile line) progeny and clearly shows both the greater-than-single-gene restoration capacity of NY821LERf and the relative sterility of LBN cytoplasm when compared to M and S. Only a limited number of cytoplasms could be studied using this method, because of the space and time required to investigate any one combination.

Method 3. Tassels of F_1 plants from crosses between steriles and partial fertiles

Results of this experiment are summarized in Table 3. The cms-S accessions in the cross W182BN × CO192 could be divided into three groups: B and D (fully fertile), most S-types (partially sterile), and LBN cytoplasm (fully sterile). Figs. 2a, 2b, and 2c show tassels typical of the three groups. Although ME cytoplasm was not part of this study, it would be expected to have tassels like those of B and D (i.e., fully fertile). In the special study of the restorative effect of CrS4HLA on the B and D accessions, B and D were more fertile in F_1 plants than was the control cytoplasm, CA (Table 3). The advantage of Method 3 was that differences in restoration capacity not evident in the inbreds themselves could be seen. B, D, CA, and LBN cytoplasms, for example, are all fully sterile in the inbred W182BN (Table 1), but in crosses to the partial restorer CO192, they were divided into three subgroups.

Method 4. Pollen phenotype of F_1 plants from crosses between S-steriles and inbreds carrying S-*Rf* genes

This method proved superior to the other three for quantitatively comparing many inbred/cytoplasm combinations. The difference between normal and cms-S-aborted pollen was quite clear (Fig. 2D). Figures 3 and 4 summarize the results using this method. Inbred MS64-7, the male in Fig. 3, appeared to have a single *Rf* gene for cms-S (50% pollen restoration), while inbred NY821LERf, the male in Fig. 4, gave greater than 50% pollen restoration, indicating that it had more than a single *Rf* gene.

The results of Method 4 were clear and consistent. Most cms-S accessions were similar in their restoration

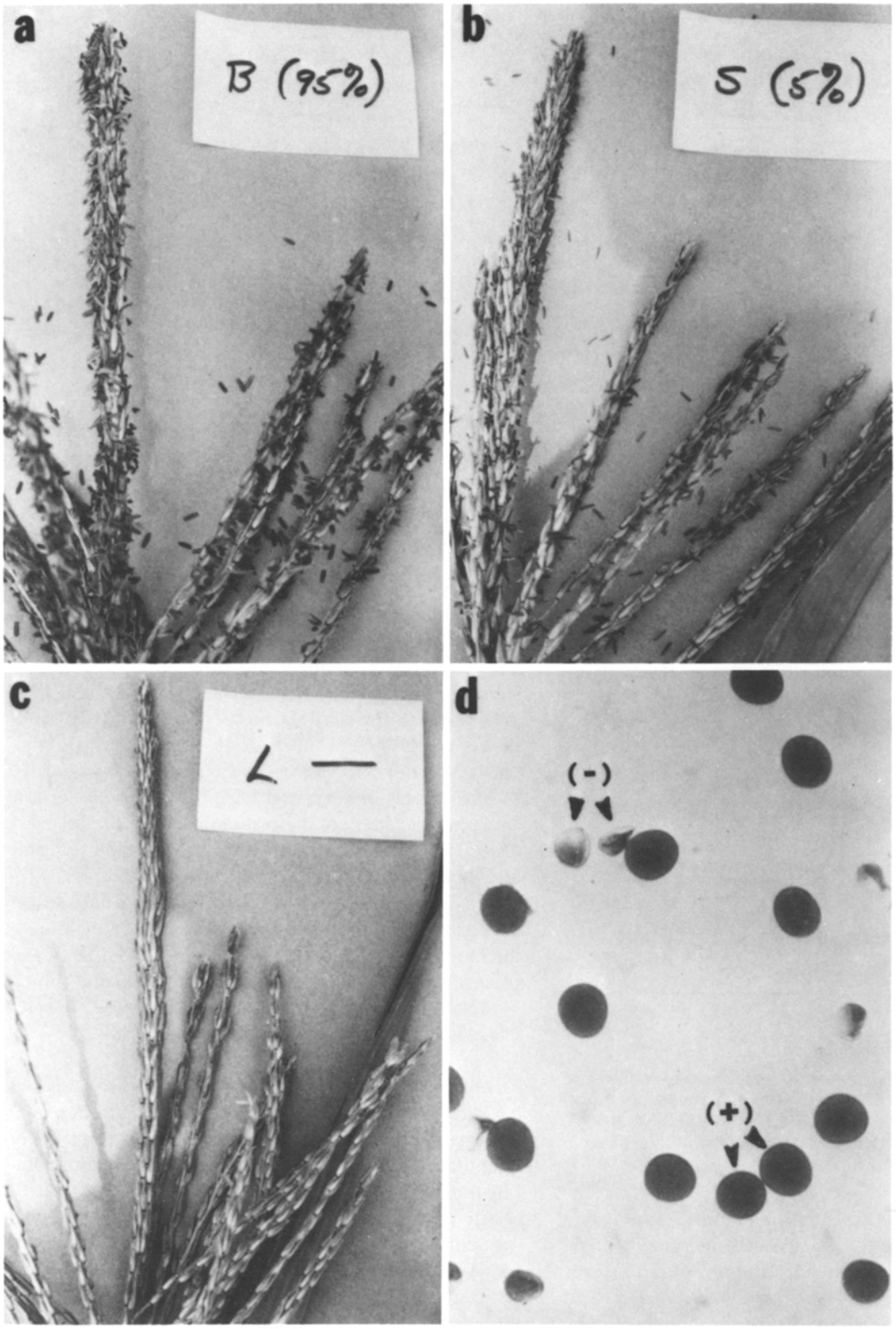

Fig. 2. **a, b, c** are tassels of F_1 hybrid progeny of the crosses W182BN cms × CO192 (Method 3). CO192, as a partial restorer of fertility, differentiates among S-group cytoplasms. Note the plump anthers in B cytoplasm (**a**), the thin anthers in S(USDA) cytoplasm (**b**), and the lack of anther exsertion in LBN cytoplasm (**c**). The percentages shown are the proportion of normal-appearing pollen in the anthers. **d** shows pollen from F_1 plants in CA cytoplasm heterozygous for *Rf* genes (Method 4). The difference between normal-appearing pollen (+) and S-aborted pollen (–) is clearly seen (note arrows). The pollen was stained with aceto-carmine and magnified about 100×

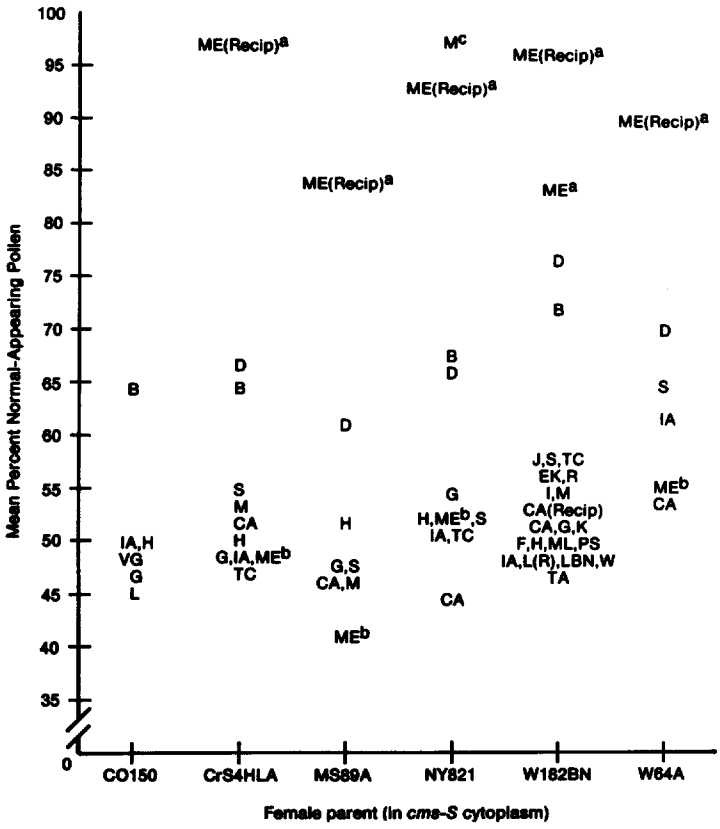

Fig. 3. Pollen phenotype of F_1 hybrids having MS64-7 as their common male parent. Capital letters indicate the cytoplasm of the female parent. ME^a = ME cytoplasms descended from 38-11(ME); ME^b = cytoplasms descended from N6(ME); M^c = an M cytoplasm that reverted to fertility; (Recip) = progeny of reciprocal crosses, where MS64-7 was the genotype of the female in sterile cytoplasm

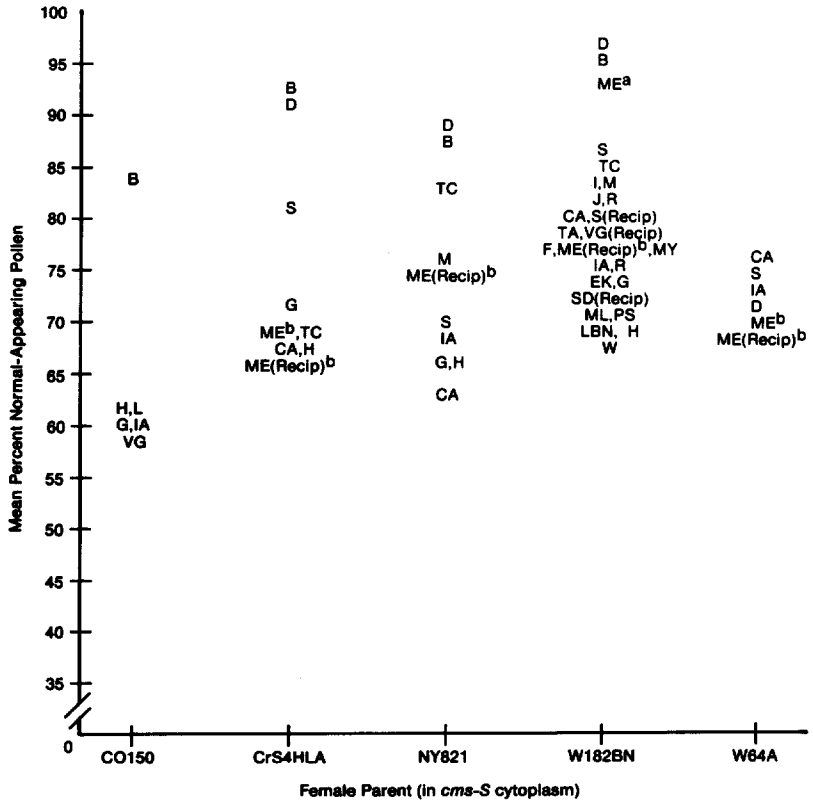

Fig. 4. Pollen phenotype of F_1 hybrids having NY821LERf as their common male parent. Capital letters indicate the cytoplasm of the female parent. ME^a = ME cytoplasms descended from 38-11(ME); ME^b = cytoplasms descended from N6(ME); (Recip) = progeny of reciprocal crosses, where NY821LERf was the genotype of the female parent in sterile cytoplasm

capacity, giving 50% normal-appearing pollen in the crosses to MS64-7 (Fig. 3). As expected, most reciprocal crosses gave the same results. Thus W182BN(CA)× MS64-7 and MS64-7(CA)×W182BN produced identical F_1 progeny, the tassels of which contained 50% normal-appearing pollen. A few accessions, however, differed significantly in restoration percentage:

B and D cytoplasms were consistently more fertile than most other S accessions.

ME cytoplasm was more male-fertile when W182BN(ME) and MS64-7(ME) were used as female parents, but not when ME cytoplasm in other inbreds was used. Thus MS64-7(ME)×CrS4HLA was highly male-fertile (98% normal pollen) while the reciprocal cross CrS4HLA(ME)×MS64-7 gave typical results for cms-S, 50% normal-appearing pollen. Pedigree records showed that the abnormal ME's came from the 38-11(ME) accession, while the ME's giving standard results were descended from N6(ME). It appeared that the Cornell collection contained two different cytoplasms labelled "ME". Subsequent nucleic acid studies confirmed this suspicion.

S(USDA) cytoplasm had significantly ($P<0.05$) more normal-appearing pollen than most other cms-S types in the crosses W182BN(S)×MS64-7 and CrS4HLA(S) ×MS64-7 (Fig. 3). This was an indication that the S(USDA) accession itself was not representative of the group as a whole. Subsequent nucleic acid studies confirmed this.

An M cytoplasm revertant was identified in the cross NY821(M)×MS64-7 (the point labelled M^c in Fig. 3). This was a cytoplasmic revertant to fertility of the type previously described by Laughnan and Gabay (1978). Laughnan and Gabay identified their revertants from the tassel phenotype of inbred plants; in contrast, the M cytoplasm revertant was discovered by observation of pollen morphology in F_1 progeny heterozygous for *Rf3* (Method 4). Instead of the expected 50% normal-appearing pollen, these plants had nearly 100% normal pollen. Nucleic acid studies confirmed the apparent disappearance of the S-1 and S-2 plasmids and rearrangement of high-molecular-weight mtDNA characteristic of many cms-S cytoplasmic revertants (Levings et al. 1980)[2]. The reversion probably occurred in the female parent, NY821(M), but was not identified until the F_1 generation.

Nucleic acid studies

The simple test for the S-1 and S-2 plasmids (Kemble and Bedbrook 1979) was very helpful in uncovering

[2] Laughnan et al. (1984) have recently reported that the S-1 and S-2 molecules do not disappear in five revertants to fertility in the nuclear background of inbred Wf9. The association of S-1 and S-2 with reversion to fertility is thus more uncertain than was conjectured in Levings et al. (1988)

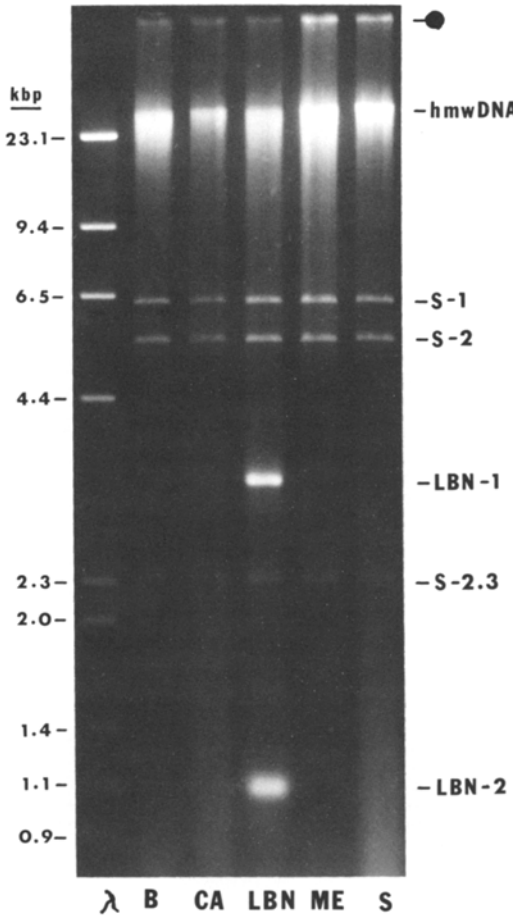

Fig. 5. Double-stranded RNAs unique to LBN cytoplasm. Electrophoresis on a 0.9% agarose gel of total mitochondrial nucleic acids from B, CA, LBN, ME, and S(USDA) cytoplasms after digestion with RNase "Mix" in the presence of 2X SSC to eliminate single-stranded RNA (Sisco et al. 1984). Nuclear genotype of all cytoplasms was W182BN. -● = origin; hmwDNA is high-molecular-weight "chromosomal" mitochondrial DNA; S-1, S-2, and S-2.3[3] are double-stranded linear DNAs; LBN-1 and LBN-2 are double-stranded RNAs. λ = a mixture of bacteriophage lambda DNA digested with HindIII and ΦX174 RF DNA digested with HaeIII (New England Biolabs)

[3] There is no consistent terminology for the 2.3 kb linear plasmid found in maize mitochondria. Kemble and Bedbrook (1980) were the first to report this molecule, finding it in normal, cms-S, and cms-C maize mitochondria. They named the molecule 'T', because they thought that cms-T lacked the plasmid. Then others (Thompson et al. 1980; Koncz et al. 1981) reported that this plasmid was present in cms-T, also, but reduced to 2.0 kb. The terminology for the molecule suggested by Koncz et al. (1981) is used in this paper, namely S2.3, C2.3, T2.0, and N2.3 for the homologous species in S, C, T, and normal cytoplasms. In more recent papers, the terms "2.35 kb molecule" (Kemble and Thompson 1982) and "n" for the 2.3 kb species and "t" for the 2.0 kb species (Kemble et al. 1983) have been used. The molecule is reported to be linear and to possess a terminally-attached protein (Kemble and Thompson 1982) and to share homology with the S-2 plasmid (Thompson et al. 1980; Koncz et al. 1981). There are also "normal" (non-sterilizing) cytoplasms in which this molecule is 2.0 rather than 2.3 kb (McNay et al. 1983). The 2.3 kb linear plasmid has received less attention than S-1 and S-2, probably because it does not seem to be associated with male-sterility

errors in cytoplasm labelling. Our data supported previous observations (Pring et al. 1977; Kemble et al. 1980) that all cms-S accessions have the S-1 and S-2 plasmids except the revertants to fertility described by Laughnan and Gabay (1978). Putative S cytoplasms lacking S-1 and S-2 were not discarded without corroborating evidence, since it was conceivable that a cytoplasm having the S restoration pattern could lack S-1 and S-2. Abnormal fertility restoration and pedigree records were used to identify errors in the cytoplasm collection.

In the initial test of undigested mtDNA, one inbred/cytoplasm combination, W182BN(L), had two prominent bands not found in other cytoplasms (Fig. 5). These bands, named LBN-1 and LBN-2, were double-stranded RNA and have been described elsewhere (Schuster et al. 1983; Sisco et al. 1984). The cytoplasm of W182BN(L) was renamed LBN, to distinguish its unique RNA species (Sisco et al. 1982 c).

More revealing than the simple test for the S-1 and S-2 plasmids were mtDNA patterns produced by restriction endonuclease digestions. Digestion of mtDNA with the endonucleases BamHI and EcoRI divided the cms-S accessions into three additional subgroups designated CA, ME, and S(USDA). The CA subgroup included CA, EK, F, G, H, IA, J, K, L, M, N6(ME), ML, MY, PS, R, SD, TA, VG, and W cytoplasms. B, D, and LBN mtDNA also had this pattern, although their fertility restoration was different from that of the CA subgroup. The digestion pattern of CA is shown in Fig. 6.

The ME subgroup consisted of 38-11(ME) and its descendants on the female side. The 38-11(ME) pattern is illustrated by the ME lanes of Fig. 6. The descendants of N6(ME) did not have this pattern, but were instead like the CA subgroup.

The S(USDA) subgroup included the I, S(USDA), and TC accessions. This digestion pattern is shown in the S lanes of Fig. 6.

B and D cytoplasms, which were more male-fertile than other cms-S accessions, could not be differentiated from the CA subgroup on the basis of restriction patterns of mtDNA resulting from digestion with 16 endonucleases: BamHI, BclI, BglI, BglII, ClaI, EcoRI, HindIII, KpnI, NruI, PvuI, PvuII, SacI, SacII, SalI, XbaI, and XhoI.

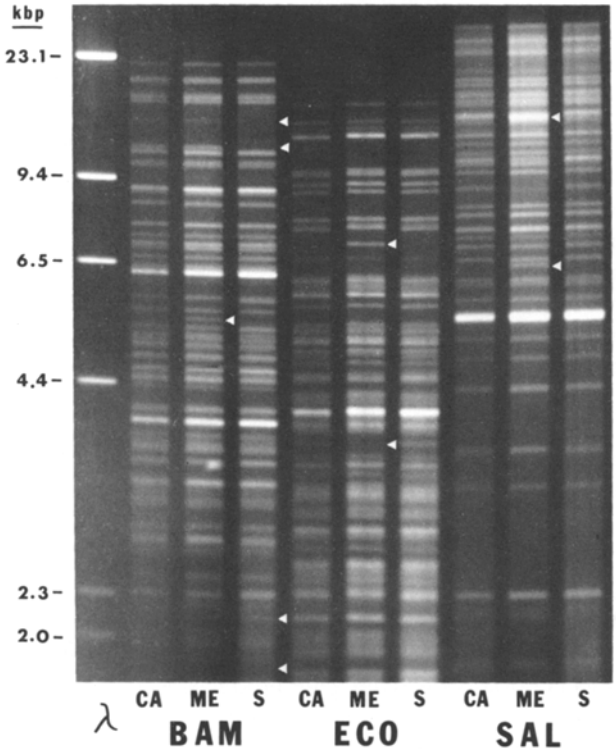

Fig. 6. Variation in restriction digests of mtDNA from CA, ME, and S(USDA) cytoplasms. Electrophoresis on a 0.7% agarose gel of mtDNA after digestion with BamHI, EcoRI, and SalI. Nuclear genotype was W182BN. *White arrows* to the *right* of certain lanes show where patterns differ from that of CA cytoplasm. λ = bacteriophage lambda DNA digested with HindIII (New England Biolabs)

Discussion

Cms-S has at least 5 subgroups

From studies of fertility restoration and mitochondrial nucleic acids (mtNA), the 25 cms-S accessions could be divided into five subgroups.

1 B and D subgroup. These two cms-S accessions were consistently more fertile than the standard (CA) types, as was evident in inbreds (Method 1), crosses with partial restorers (Method 3), and analysis of pollen morphology (Method 4). B and D could be duplicate accessions of a single atypical cms-S cytoplasm, because the fertility restoration of B and D cytoplasms is similar (Figs. 3 and 4) and because both cytoplasms originated from plant introductions from Turkey (Beckett 1971). Restriction enzyme analysis of mtDNA using 16 endonucleases nevertheless failed to differentiate between the B and D accessions and the accessions of the CA subgroup. This was the only example in which differences in fertility could not be correlated with variation in mitochondrial nucleic acids. It is very unlikely that the differential restoration of B and D is due to residual nuclear effects (from the sources of B and D cytoplasms), because B and D have remained different from other S-types in many inbred lines, even after 8 to 15 backcrosses.

2 CA subgroup. This subgroup included 18 of the 25 accessions: CA, EK, F, G, H, IA, J, K, L, M, ML, MY,

PS, R, SD, TA, VG, and W cytoplasms. Their fertility restoration and BamHI mtDNA restriction patterns were indistinguishable. Because these cytoplasms are the most representative in fertility restoration and mtDNA of the 25 accessions, we suggest that CA cytoplasm be used in the future as a standard for the cms-S group.

3 LBN subgroup. This cytoplasm, identified in W182BN(L), was peculiar in being somewhat less fertile than other cms-S accessions and in having large quantities of two double-stranded RNA species (dsRNAs). Because the dsRNAs were seen only in W182BN(L) and its descendants on the female side, L cytoplasm with the dsRNAs was given a new designation – LBN cytoplasm. Later studies showed, however, that the amount of dsRNAs varied with nuclear genotype. Of 10 inbreds backcrossed into LBN cytoplasm, only two, W182BN and 2132, contained large quantities of the dsRNAs (Sisco et al. 1984). It is possible that other "L" cytoplasms in the Cornell collection, such as CO150(L), would have the dsRNAs if backcrossed to compatible nuclear genotypes such as W182BN. A backcrossing program is now underway using CO150(L) as the cytoplasm source and W182BN as the recurrent parent to see if the dsRNAs will appear in the newly-converted W182BN(L). It was clear, in any case, that other cms-S accessions with the W182BN and 2132 nuclear genotypes did not have the abundant dsRNAs of LBN. LBN cytoplasm may have the greatest commercial potential of any of the cms-S accessions studied, because it confers an extra degree of sterility on cms-S inbreds. Cms-S has not been widely used for hybrid production because it fully and reliably sterilizes very few inbreds (Table 1). Inbreds which are partially male-fertile in most cms-S accessions might be fully sterile in LBN cytoplasm.

4 ME subgroup. Cornell's two sources of ME cytoplasm, 38-11(ME) and N6(ME), apparently were different. Remnant seed of the original accessions was not available for testing, but all inbreds descended from 38-11(ME) were atypical in fertility and had a unique mtDNA restriction pattern, while inbreds whose cytoplasms descended from N6(ME) were like members of the CA group, both in fertility and in mtDNA restriction patterns. The most probable explanation was that N6(ME) was mislabelled. Beckett (1971) had found ME to be unusually fertile in many inbred nuclear backgrounds with the single exception of N6. ME was so different from other cms-S accessions in his studies that he suggested putting it in a separate subgroup.

5 S(USDA) subgroup. S(USDA), TC, and I cytoplasms from the Cornell collection shared a common BamHI restriction pattern that differentiated them from other cms-S accessions. The cms-S cytoplasm used to determine the sequence of S-2 (Levings and Sederoff 1983) also matched this pattern. S(USDA) and TC cytoplasms are probably identical. TC is said to have originated from a mixture of T ("Texas") and S ("Connecticut") cytoplasms – thus the name "TC". The N6 and 38-11 sources of TC cytoplasm received by Cornell had only the cms-S component (Beckett 1971). The accession designated "I" may also be the same as S(USDA). Both S(USDA) and I cytoplasms are said to have originated from stocks containing the *iojap* gene (Beckett 1971).

These five represent the minimum number of subgroups within cms-S. Duvick (1972) notes that there are over a hundred accessions of S-type cytoplasms available. Only 25 of these are represented in the Cornell collection. Even among these 25 accessions, there may be more than five subgroups. Recent work by Carlson and Kemble (1984), using the same source of seed as this study, has shown that the J accession is like the S(USDA) subgroup in lacking a 1.94 kb plasmid in its mitochondria. Our restriction data, however, places the J accession in the CA subgroup. The J accession may therefore represent a unique sixth subgroup. The EK accession is sometimes associated with plant bifurcation and dwarfing. This may be due to residual nuclear effects, since the source plant for EK cytoplasm was itself bifurcated, and the bifurcation phenotype is transmissible through pollen (A. L. Hooker and H. L. Everett, unpublished data). Our data places EK within the CA subgroup.

Pollen phenotype is the most efficient means of differentiating fertility differences among cms-S subgroups

The differences in fertility between cms-S subgroups were consistent among inbreds in all four methods used, but Method 4 was the fastest and most quantitative. Although Duvick (1965) reported that in certain inbred backgrounds a small percentage of *rf*-containing pollen may be functional, we were unable to detect this in inbreds used in our study. The higher percentage of normal-appearing pollen caused by NY821LERF, for example (Fig. 4), is correlated with a higher percentage of fertile plants in crosses (Fig. 1). The explanation for the $>50\%$ normal pollen is that there is more than one S-*Rf* locus (Gabay-Laughnan and Laughnan 1979).

Cms-S fertility variation is correlated with mitochondrial nucleic acid differences

Results of this study support the hypothesis that the various CMS systems of maize are due to variations in mitochondrially-encoded gene products. Mitochondrial nucleic acid variation was correlated with fertility differences in the LBN, ME and S(USDA) subgroups.

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