Copyright © 1997 by the Genetics Society of America

The Nuclear Gene *Rf3* Affects the Expression of the Mitochondrial Chimeric Sequence R Implicated in S-Type Male Sterility in Maize

Gracia Zabala, Susan Gabay-Laughnan and John R. Laughnan

Department of Plant Biology, University of Illinois, Urbana, Illinois 61801

Manuscript received February 4, 1997
Accepted for publication July 10, 1997

ABSTRACT

The mitochondrial genomes of maize plants exhibiting S-type cytoplasmic male sterility (*cms-S*) contain a repeated DNA region designated R. This region was found to be rearranged in the mitochondria of all *cms-S* cytoplasmically revertant fertile plants in all nuclear backgrounds analyzed. A 1.6-kb mRNA transcribed from the R region in mitochondria of sterile plants was absent from all cytoplasmic revertants examined. The nuclear gene *Rf3*, which suppresses the *cms-S* phenotype, was found to have a specific effect on the expression of the R sequence; the abundance of the major R transcripts, including the *cms-S*-specific 1.6-kb mRNA, is decreased in mitochondria of restored plants. Nucleotide sequence analysis of R has revealed similarities to the R1 plasmid found in some South American maize races with RU cytoplasm, to the M1 plasmid found in one source of *Zea luxurians* teosinte, to the *atp9* mitochondrial gene and its 3′ flanking sequence, and also to a region 3′ to the *orf221* gene. The derived amino acid sequence of the R region predicts two open reading frames (ORFs). These ORFs contain the similarities to R1, M1, *atp9* and *orf221*. The present report reveals the chimeric nature of the R region, describes the complex effect of *Rf3* on the expression of the R sequence and implicates R in the sterile phenotype of *cms-S* maize.

CYTOPLASMIC male sterility (*cms*) is a common phenomenon in higher plants and is manifested as the inability of a plant to produce viable pollen; female fertility is unaffected. *cms* is a trait of economic importance in the production of hybrid seed as it eliminates the need for emasculation to control pollination, thus reducing labor costs. In maize, *cms* was originally classified into three groups, S (USDA), T (Texas) and C (Charrua), based on the nuclear genes that restore them to fertility (reviewed by Laughnan and Gabay-Laughnan 1983). *cms* is maternally inherited and, in the species where it has been studied, a correlation between *cms* and unique arrangements of the mitochondrial genome has been found. Analyses of such mitochondrial DNA (mtDNA) arrangements have revealed *cms*-specific differences in transcription and/or translation in *cms-T* maize (Dewey *et al.* 1986, 1988; Rottmann *et al.* 1987; Wise *et al.* 1987), in sorghum (Bailey-Serres *et al.* 1986; Tang *et al.* 1996), petunia (Young and Hanson 1987; Nivison and Hanson 1989; Rasmussen and Hanson 1989), sunflower (Siculella and Palmer 1988; Kohler *et al.* 1991), *Brassica napus* (Singh and Brown 1991; Singh *et al.* 1996), rice (Iwabuchi *et al.* 1993), *Phaseolus vulgaris* (Mackenzie *et al.* 1988) and radish (Makaroff *et al.* 1989). Despite these many studies, in no case can the expression of any of the *cms*-associated mtDNA regions explain the mechanism that brings about failure of pollen function.

Maize mtDNA consists of a "master" molecule, carrying perhaps the entire complexity of the genome, as well as a range of smaller molecules generated by recombination through large and small direct repeats (Lonsdale *et al.* 1983; Andre *et al.* 1992). In *cms-S* mitochondria, the genome structure is complicated further by the existence of two linear double-stranded DNA episomes, S1 (6397 bp) and S2 (5453 bp) (Pring *et al.* 1977; Levings and Sederoff 1983; Paillard *et al.* 1985). These episomes have identical terminal inverted repeats (IRs) of 208 bp (Levings and Pring 1978, 1979). Similar IR sequences are part of a 2-kb recombinational repeat, R, present in the high molecular weight *cms-S* mtDNA molecules at the junctions of regions designated σ-σ' and ψ-ψ' (Schardl *et al.* 1984); these R sequences will be referred to both as the fully integrated Rs and as the R repeat (Figure 1A). Recombination between the episomal and integrated IRs results in the rearrangement of the mitochondrial genome (Schardl *et al.* 1984). Some of the resulting recombinant molecules have an episome now integrated into the main mitochondrial genome and present as a linear end, flanked by a σ or ψ sequence (Figure 1B). The previously fully integrated R sequence, common to σ' and ψ', is now found as another linear end (Figure 1B). A portion of the R repeat shows strong nucleotide sequence similarity to the unique end of the R1 plasmid (Weissinger *et al.* 1982; Leaver *et al.* 1985; Houchins

This manuscript is dedicated to the memory of John R. Laughnan, who died July 15, 1994. He is deeply missed.

Corresponding author: Gracia Zabala, Department of Plant Biology, University of Illinois, 505 S. Goodwin Ave., Urbana IL 61801. E-mail: gracia_zabala@qms1.life.uiuc.edu

et al. 1986). R1 is one of a pair of linear double-stranded DNA plasmids found in some South American races of maize with RU fertile cytoplasm; R1 has 4.9 kb of similarity and 2.6 kb of unique sequence compared with S1 (WEISSINGER *et al.* 1982; LEVINGS *et al.* 1983) (Figure 1A). An R1-similar sequence is found adjacent to one copy of a recombinationally active repeat (5.2 kb) in normal (N) cytoplasm maize mtDNA (HOUCHINS *et al.* 1986) (Figure 1A). One source of *Zea luxurians* teosinte, collected by L. MAZOTI, contains two linear DNA plasmids, designated M1 and M2. The M1 plasmid appears to be very similar to the R1 plasmid (GRACE *et al.* 1994).

Unlike the maize T and C cytoplasms that are stable in field-grown plants, numerous spontaneous cytoplasmic revertants from the *cms-S* versions of different inbred lines and hybrids have been isolated. These cytoplasmically revertant plants are available for use in correlating mtDNA rearrangements with reversion to fertility (reviewed by GABAY-LAUGHNAN *et al.* 1995). Several different classes of mtDNA alterations have been observed (SCHARDL *et al.* 1985; SMALL *et al.* 1988) and the type of rearrangement depends on the nuclear background in which the reversion event occurred. For example, in the M825, M825/Oh07, 38-11, H95 and W182BN nuclear backgrounds, the S episomes and all of the linear mtDNA molecules that result from recombination with them are lost (LEVINGS *et al.* 1980; KEMBLE and MANS 1983; SCHARDL *et al.* 1985; ESCOTE 1986; ESCOTE-CARLSON *et al.* 1988; SMALL *et al.* 1988). On the other hand, revertants in the WF9 inbred nuclear background lose neither the free S episomes nor the linear high molecular weight mtDNA molecules that result from recombination with them (ESCOTE *et al.* 1985; ISHIGE *et al.* 1985). However, both the fully integrated and terminal R sequences are rearranged (SMALL *et al.* 1988; present report).

Nuclear restorer-of-fertility genes that suppress *cms* have been described for many plant species and, as more of these systems are studied at the molecular level, information on the effects of nuclear restorer genes is accumulating. Restorer genes have been demonstrated to affect transcription and/or translation of *cms*-associated mtDNA regions in *cms-T* maize (DEWEY *et al.* 1987; KENNELL *et al.* 1987; KENNELL and PRING 1989; WISE *et al.* 1996), in petunia (NIVISON and HANSON 1989), *B. napus* (SINGH and BROWN 1991; SINGH *et al.* 1996), rice (IWABUCHI *et al.* 1993), sorghum (TANG *et al.* 1996), radish (KRISHNASAMY and MAKAROFF 1994) and sunflower (MONEGER *et al.* 1994). Either of two restorer genes, *Fr* or *Fr2*, can accomplish restoration of *cms* in *P. vulgaris*. Restoration to fertility by *Fr2* affects expression of the *cms*-associated region posttranscriptionally (ABAD *et al.* 1995). Restoration to fertility by *Fr*, however, is unique in that it is a permanent condition not reversed by segregation of the *Fr* gene (MACKENZIE and CHASE 1990). The *cms*-associated region, not present in normal fertile lines, is lost upon restoration (JANSKA and MACKENZIE 1993). In this case, restoration more closely resembles cytoplasmic reversion to fertility. In only one case has the sequence of a restorer gene been determined. The *Rf2* gene of maize encodes a putative aldehyde dehydrogenase whose role in suppressing *cms-T* is still unknown (CUI *et al.* 1996).

Here we report that loss of restriction endonuclease fragments containing the terminal R regions was the only change observed in all stable *cms-S* revertants examined. Moreover, differences were observed in R sequence expression in *cms-S* sterile, cytoplasmically fertile revertant, and restored plants. All cytoplasmically revertant plants lacked a 1.6-kb R transcript present in all sterile plants. The *Rf3* nuclear gene of maize that restores fertility to *cms-S* plants was found to have a complex effect on the expression of the R sequence resulting in a decreased abundance of most of the major R transcripts. Nucleotide sequence analysis revealed the chimeric nature of R. The derived amino acid sequence of this region predicts two open reading frames (ORFs). The products of one or both of these ORFs are strong candidates for rendering pollen nonfunctional in S-type male-sterile plants.

MATERIALS AND METHODS

Plant material: The characteristics of the maize strains used in these studies are listed in Table 1. Cytoplasmic revertants and their sterile progenitors were isogenic. Plants restored to fertility by *Rf3* and their sterile controls were essentially isogenic except for the *Rf3* gene and the region immediately surrounding it.

Plants were field grown in summer nurseries at the University of Illinois in Urbana. The M825 inbred line was developed by JOHN R. LAUGHNAN at the University of Illinois, Urbana. Inbred line H95 was provided by EARL B. PATTERSON, University of Illinois, Urbana. The other inbred lines, as well as the S-type cytoplasms, were supplied by JACK BECKETT, University of Missouri, Columbia. South American races carrying the RU cytoplasms were provided by MAJOR M. GOODMAN, North Carolina State University, Raleigh. The cytoplasmic revertants studied arose spontaneously from plants carrying S-sterile cytoplasms grown in winter nurseries on Molokai or in summer nurseries in Urbana. A restored version of *cms-S* B37 was obtained from MARC ALBERTSEN, Pioneer Hi-Bred International, Johnston, Iowa. The *Rf3* gene from inbred line Tr was introgressed into WF9. The *cms-S* versions of WF9 were crossed by Tr and fertile plants were backcrossed with WF9 at least seven times. The *Rf3* gene from Tr was also introgressed into M825. The *cms-S* version of M825 was crossed by Tr and fertile plants were backcrossed with M825 at least five times.

DNA and RNA isolation from immature cobs: Mitochondria and mtDNA were isolated from immature cobs following the procedure of KEMBLE *et al.* (1980).

For mtRNA isolation, mitochondria were purified from immature cobs as described by STERN and NEWTON (1986) including the sucrose gradient mitochondrial fractionation step. After mitochondrial lysis, RNA was concentrated and separated from other nucleic acids by precipitation with LiCl as described by STERN and NEWTON (1986).

Isolation of RNA from microspores: The following protocol is a modification of the one described by BEDINGER and EDGERTON (1990). Spikelets were stripped from spikes of four

to five preemergent tassels. In a chilled mortar with pestle, spikelets were ground in homogenization buffer (0.35 M sorbitol, 50 mM Tris-hydrochloride pH 8.0, 5 mM ethylenediaminetetraacetic acid, with 0.1% bovine serum albumin, 0.25 g/liter each spermine, spermidine and 2-mercaptoethanol added just before use). When homogenized, the sample was filtered through eight layers of cheesecloth and one layer of miracloth. The filtrate was passed through a 40μ Nitex nylon membrane (Tetko Inc.) where the microspores are retained but not the mitochondria and other organelles. Microspores were scraped from the nylon membrane and resuspended in 2 ml of homogenization buffer. Microspores were precipitated by centrifugation at 12,100 × *g* in an SS34 rotor for 20 min. The pellet was resuspended in 1 ml of lysis buffer [100 mM Tris pH 7.6, 50 mM EDTA, 100 mM NaCl, 1% SDS, 1.5% sarkosyl, 50 mM 2-mercaptoethanol and 1 mM aurintricarboxylic acid (ATA, ammonium salt, Sigma)]. The sample was then transferred to a small chilled sterile mortar and the tube was rinsed with an additional 1 ml of lysis buffer and transferred to the mortar. The combined 2-ml sample was ground with the pestle until a smooth consistency was reached. The sample was transferred with a sterile pasteur pipette to a tube where nucleic acids were separated with one phenol and two chloroform extractions. RNA was concentrated and separated from other nucleic acids by precipitation with LiCl and stored as described by STERN and NEWTON (1986).

Nucleic acid analysis: Restriction endonuclease digestion of mtDNA, Southern blotting, and hybridization procedures were as previously described (ESCOTE-CARLSON *et al.* 1988).

Formaldehyde/agarose gel electrophoresis, Northern blotting and hybridization of mitochondrial RNA were carried out as described by STERN and NEWTON (1986). Before hybridizing a blot with a new probe, the residual radioactive probe was stripped from the blot just before rehybridization as suggested by the supplier of the Biodyne Membrane (Pall BioSupport, East Hills, NY), the nylon membrane used in this work.

Mitochondrial DNA clones used in Southern and Northern analyses were the following: IS1E5, a single stranded 947-nucleotide *Sau*IIIA fragment of the R sequence of *cms-S* cloned into the *Bam*HI site of mp19 (SMALL *et al.* 1988) provided by Dr. I. SMALL (Laboratoire de Biologie Cellulaire INRA Versailles Cedex, France) (Figure 1C); R-1E, a clone containing bases 1–134 of the IR of the S episomes in the pSP64 transcription vector (LEVINGS and SEDEROFF 1983) provided by Dr. C. S. LEVINGS III (North Carolina State University, Raleigh) (Figure 1C); the cosmid clone CSA61 containing the σ-σ' DNA region of *cms-S* mitochondria (SCHARDL *et al.* 1984) provided by Dr. D. R. PRING (University of Florida, Gainesville). From the cosmid clone CSA61 we subcloned a 4.7-kb *Bam*HI fragment containing the R sequence into the *Bam*HI site of the pBS K^+ vector to generate the pZmS4.7 clone. From this, a 1.6-kb *Pst*I-*Bam*HI fragment was subcloned into pUC119 to generate the pZmS1.6 clone used to determine the DNA sequence of R (Figure 1C). The SR0.35 genomic clone, a *Sal*I-*Eco*RI fragment containing 324 nucleotides from the very 5′ end of the coding region of maize *atpA*, constructed by Dr. M. MULLIGAN (University of California, Irvine), was provided by Dr. K. NEWTON (University of Missouri, Columbia).

The 1.6-kb *Pst*I-*Bam*HI fragment of the pZmS1.6 clone (Figure 1C) was sequenced using the Sequenase system (U.S. Biochemical Co.). To obtain the complete sequence of both DNA strands, oligonucleotides (17–21 mers) were designed based on the DNA sequence information gathered by the use of the primer provided in the Sequenase kit and a reverse primer, Reverse 22 (5′AACAGCTATGACCATGATTACG3′) provided by Dr. T. JACOBS (University of Illinois, Urbana). The oligonucleotides were synthesized by Operon Technologies Inc. (Alameda, CA) and the Genetic Engineering Facility of the University of Illinois at Urbana. The IS1E5 clone that contains 947 bp (from 484 to 1431) internal to the pZmS1.6 clone (Figure 1C) was also sequenced in the same fashion as described for the pZmS1.6 clone. The resulting DNA sequence confirmed the results obtained from sequencing the pZmS1.6 clone.

RESULTS

Rearrangements of the R sequence at the ends of mitochondrial molecules: In *cms-S* mitochondria the R sequence is found repeated in different environments as a result of recombination between the two copies of the R repeat as well as recombination between the IRs of the S1 and S2 free episomes and the IR-similar sequence of the R repeat in the σ-σ' and ψ-ψ' target regions (Figure 1A) (SCHARDL *et al.* 1984). When mtDNA of nonrestored *cms-S* plants is digested with *Xho*I and hybridized to the R sequence-specific probe IS1E5 (see MATERIALS AND METHODS; Figure 1C), two DNA fragments containing the R repeat at the ends of linear molecules are detected. A 4.4-kb fragment contains the R region found at the σ' terminus and a 10.1-kb fragment contains the R sequence at the ψ' end (SCHARDL *et al.* 1985) (Figures 1B and 2, S lanes). The other IS1E5 hybridizing bands in the mtDNA from sterile plants are 13.0, 11.0, 7.4 and 5.4 kb in size corresponding to the σ-ψ', ψ-ψ', σ-σ' and ψ-σ' fully integrated R sequences, respectively (SCHARDL *et al.* 1985) (Figure 2, S lanes).

Except for two strains (Figure 2 WF9 CR_3 and data not shown), all cytoplasmic revertants contained R sequences in novel arrangements as shown by loss of bands or new bands hybridizing to R (Figure 2). In the two exceptions, R sequences were visible only after longer exposures (threefold that of autoradiograph shown in Figure 2), suggesting that the copy number of such sequences had been reduced to levels barely detectable by Southern analysis. The novel arrangements of R in the cytoplasmic revertants differed from one another depending on the nuclear background in which the reversion event occurred. Cytoplasmic revertants in the *cms-S* 38-11, M825 and H95 inbred nuclear backgrounds (Table 1), in addition to losing the S episomes, lost the linear molecules containing the terminal R sequences (Figure 2, 10.1- and 4.4-kb fragments) but some or all intact target regions were maintained (Figure 2, 5.4-, 7.4-, 11.0- and 13.0-kb fragments and our unpublished data; also, SCHARDL *et al.* 1985; SMALL *et al.* 1988). Cytoplasmic revertants in the WF9 background (Table 1), however, lost neither the linear molecules containing terminal S episomal sequences nor the S episomes themselves but did exhibit rearranged R termini and target regions (Figure 2, WF9 CR_1, CR_2, CR_3 and data not shown). Linear molecules containing terminal S episome sequences (Figure 1B) persisted in the WF9 cytoplasmic revertants as evi-

FIGURE 1.—(A) Maps comparing the R repeat in *cms-S* at the junctions of sites designated σ-σ' and ψ-ψ', the R1 similarity found next to one copy of the 5.2-kb repeat in normal cytoplasm, the R1 plasmid of RU mitochondria, and the S1 episome of *cms-S* mitochondria. The IR-similar region of the *cms-S* R sequence is represented as 209 bp in Figure 1 instead of 210 bp because the first base pair of this IR is a T instead of an A as found in the S1 and S2 IRs. The longer IR, with the 15 bp variant region, is also found in the R1-similar region of N cytoplasm next to one copy of the 5.2-kb repeat (α-R1) (HOUCHINS *et al.* 1986). Vertical dashed lines indicate the limits of the similarity between the episomal sequences. IR indicates inverted repeat sequences (open boxes). Identically hatched boxes represent regions of similarity. Not drawn to scale. (B) Maps of the linear high molecular weight mtDNA molecules of *cms-S* resulting from recombination with the S episomes. The R region of σ' or ψ' and the S1 or S2 episomes are at linear ends. The hatched boxes in R represent the sequence similar to the R1 plasmid. The open boxes are the sequenced portion of R with no similarity to any known sequence. IR, inverted repeats; X, *Xho*I. The 4.4- and 10.1-kb fragments result from digestion of the σ' and ψ' linear ends with *Xho*I, respectively. Not drawn to scale. (C) Map of the *cms-S* σ-σ' region integrated in the high molecular weight genome that has been sequenced. *cox*I, cytochrome oxidase subunit I gene. The relative size and approximate location of the pZmS1.6 clone used to sequence a portion of the σ-σ' R region is represented. Also, the location of clones R-1E (nucleotides 1–134 of the IR) and IS1E5 (nucleotides 484–1431 of R) are indicated. P, *Pst*I; B, *Bam*HI. Not drawn to scale.

denced by the fact that DNA fragments representing them were still detected in Southern blots of mtDNA digested with *Bam*HI (data not shown). Exhaustive study of different regions of the *cms-S* mitochondrial genome revealed no other rearrangements correlating with the male-sterile phenotype (G. ZABALA, C. LEJA, S. GABAY-LAUGHNAN and J. R. LAUGHNAN, unpublished results). Therefore, the only change detected in all cytoplasmic revertants of *cms-S* was the loss of intact R sequences at the termini of mtDNA molecules.

Expression of the *cms-S* R region: Since rearrangement of the R sequence was correlated with cytoplasmic reversion to fertility, the transcriptional activity of the R region in mitochondria of male-sterile and revertant plants with different nuclear backgrounds was compared. Using the single-stranded probe IS1E5 (see MATERIALS AND METHODS) in Northern blots, we detected a complex transcriptional profile of the R region in samples from sterile plants (signals at 12.0, 10.5, 4.4, 2.8, 1.6, 0.9, 0.8 and 0.4 kb) (Figure 3A; S lanes show RNAs down to 1.6 kb in size). All these transcripts are presumably synthesized off the DNA strand that contains *orf355* and *orf77* since the sequence of the single-stranded IS1E5 probe is identical to that DNA strand.

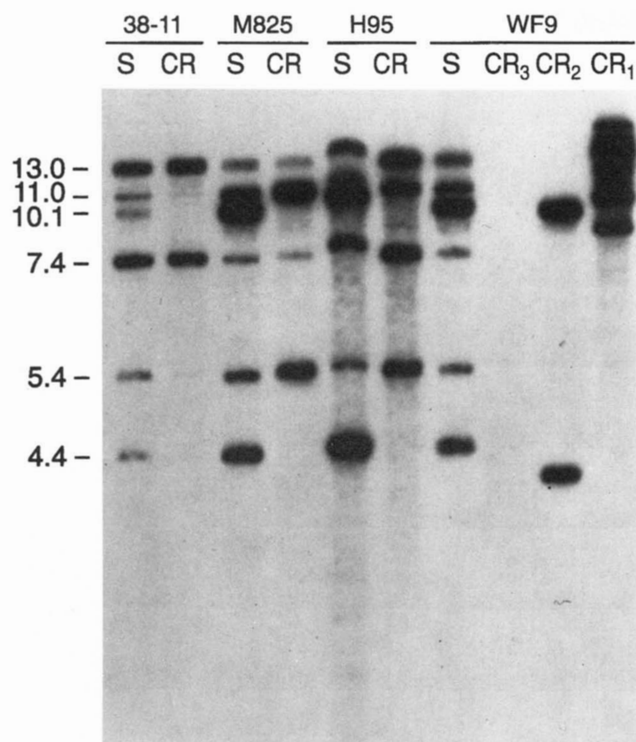

FIGURE 2.—Southern blot of mtDNAs from immature cobs digested with *Xho*I and hybridized to the R-specific probe IS1E5. Samples are as follows: mtDNA from sterile plants (S) in the nuclear backgrounds 38-11, M825, H95 and WF9; cytoplasmic revertants (CR), fertile plants isolated from the respective sterile strains 38-11: 81-115-2, M825: 84-323-3, H95: 82-82-1 and WF9: 85-2053-2 (CR1), 85H-629-4 (CR2), 81-47-15 (CR3). The sizes of the *Xho*I fragments are indicated in kilobases. The bands, from largest to smallest, correspond to σ-ψ', ψ-ψ', the ψ' linear end, σ-σ', ψ-σ' and the σ' linear end.

With the exception of WF9 cytoplasmic revertants, *e.g.*, CR_1, CR_2 and CR_3 (Figure 3A), all cytoplasmic revertants in the different nuclear backgrounds examined (Figure 3A, lanes M825 CR, H95 CR and 38-11 CR) expressed this region in a fashion similar to that of mitochondria from their sterile progenitors with one major exception. The 1.6-kb transcript present in the sterile strains was absent from the cytoplasmic revertants (Figure 3A, lanes M825 CR, H95 CR and 38-11 CR). In addition, the M825 and H95 cytoplasmic revertants exhibited increased amounts of the 12.0-, 10.5-, 4.4- and 2.8-kb transcripts (Figure 3A, lanes M825 CR and H95 CR) compared to their sterile progenitors (Figure 3A, lanes M825 S and H95 S).

The 38-11 revertant analyzed exhibited an overall decrease in the abundance of all R transcripts. The 1.6-kb RNA that seems to correlate with the sterile phenotype was absent from this revertant as well (Figure 3A, lane 38-11 CR). However, note that *cms-S* plants in this nuclear background already have low amounts of this transcript due to the low amounts of R sequences as linear ends (Figure 2, 38-11 S lane, 10.1- and 4.4-kb fragments). This observation supports the involvement of the terminal R sequences in the S male-sterile phenotype, since these plants are not "tight" steriles and one finds exserted anthers with some starch-filled pollen grains.

The lack of R transcription in CR_3 in the WF9 nuclear background was not unexpected since the R sequence was present at very low levels, as evidenced by the poor hybridization with IS1E5 on Southern blots (Figure 2, WF9 CR_3). Revertants CR_1 and CR_2, also in the WF9 nuclear background, in which all R sequences are rearranged showed transcriptional activity associated with the R region (Figure 3A) but the sizes of the transcripts were different from those found in samples from sterile plants (Figure 3A, lane WF9 S) and the steady-state transcript levels were decreased. The three WF9 revertants, CR_1, CR_2 and CR_3, lacked the 1.6-kb transcript. Thus, all of the cytoplasmic revertants we analyzed expressed the R region differently from their sterile progenitors and they all had in common the lack of the 1.6-kb transcript.

Transcription of the R-like sequences in N and RU cytoplasms: Fertile plants with N cytoplasm have adjacent to one copy of the 5.2-kb repeat a sequence related to the R1 plasmid (HOUCHINS *et al.* 1986). Since it has been shown previously that the R region of *cms-S* contains at least 1 kb of sequence resembling the unique end of the R1-similar region of N (Figure 1A) (LEVINGS *et al.* 1983; HOUCHINS *et al.* 1986), it was relevant to find out if the R1-similar regions of N and S cytoplasm exhibit the same expression. For the same reason, it is also important to know whether the R1 plasmid of the fertile RU cytoplasm is expressed in the same manner as is the R region of *cms-S*.

RNA isolated from fertile plants with N cytoplasm did not hybridize to the IS1E5 probe (Figure 3A, lane M825 N and data not shown). When HOUCHINS *et al.* (1986) analyzed the transcription of the R sequence found in N using a 4129-bp probe containing part of the 5.2-kb repeat plus the unique end of R1, a 2.85-kb transcript was detected. The fact that the IS1E5 probe, which contains 536 bp of the R1-similar sequence (from nucleotide position 485 to 1060 of Figure 8A), did not hybridize to any transcripts from N mitochondria supports the interpretation by HOUCHINS *et al.* (1986) of their results. The 2.85-kb transcript detected with the 4129-bp probe is transcribed from the 5.2-kb repeat portion of the sequence; the 5′ terminus of this transcript lies within the R1 sequence with transcription proceeding toward and into the 5.2-kb repeat. These results show that the majority of the R1 sequence is not transcribed in N cytoplasm as it is in *cms-S*. The hybridization observed with mtRNAs from RU cytoplasm was weak and differed from that in *cms-S* sterile and *cms-S* revertant plants (data not shown). Comparable results were obtained by GRACE *et al.* (1994). Therefore, the complex pattern of expression in mitochondria of S male-sterile and some S cytoplasmically revertant plants may be due to the particular arrangement of the R sequence in *cms-*

TABLE 1

Characteristics of maize plants used in this study

Nucleus	Cytoplasm	Free S episomes	Restorer genotype	Phenotype	Figure
B37	N	−	*rf3/rf3*	Fertile	5
B37	S	+	*rf3/rf3*	Sterile	4, 5
B37	S	+	*Rf3/rf3*	Fertile	4, 5
B37	S	+	*Rf3/Rf3*	Fertile	5
B37/WF9 F1	N	−	*rf3/rf3*	Fertile	6
B37/WF9 F1	S	+	*rf3/rf3*	Sterile	6
B37/WF9 F1	S	+	*Rf3/rf3*	Fertile	6
B37/WF9 F1	S	+	*Rf3/Rf3*	Fertile	6
H95	S	+	*rf3/rf3*	Fertile[a]	2, 3
H95	CR 82-82-1[b]	−	*rf3/rf3*	Fertile	2, 3
M825	N	−	*rf3/rf3*	Fertile	3, 6
M825	S	+	*rf3/rf3*	Sterile	2, 3, 6
M825	S	+	*Rf3/rf3*	Fertile	6
M825	CR 84-323-3[c]	−	*rf3/rf3*	Fertile	2, 3, 6
WF9	ML	+	*rf3/rf3*	Sterile	4, 7
WF9	ML	+	*Rf3/rf3*	Fertile	4
WF9	RD	+	*rf3/rf3*	Sterile	2, 3, 4, 7
WF9	RD	+	*Rf3/rf3*	Fertile	4
WF9	S	+	*rf3/rf3*	Sterile	4
WF9	S	+	*Rf3/rf3*	Fertile	4
WF9	CR 81H-51-1[d]	+	*rf3/rf3*	Fertile	7
WF9	CR 85-2053-2[e]	+	*rf3/rf3*	Fertile	2, 3, 7
WF9	CR 85H-629-4[e]	+	*rf3/rf3*	Fertile	2, 3
WF9	CR 81-47-15[e]	+	*rf3/rf3*	Fertile	2, 3
38-11	S	+	*rf3/rf3*	Sterile	2, 3
38-11	CR 81-115-2[f]	−	*rf3/rf3*	Fertile	2, 3

[a] Inbred H95 carries a restorer gene different from *Rf3*.
[b] CR 82-82-1 arose spontaneously in *cms-S* H95.
[c] CR 84-323-3 arose spontaneously in *cms-RD* M825.
[d] CR 81H-51-1 arose spontaneously in *cms-ML* WF9.
[e] CR 81-47-15, CR 85-2053-2 and CR 85H-629-4 arose spontaneously in *cms-RD* WF9.
[f] CR 81-115-2 arose spontaneously in *cms-S* 38-11.

S mitochondria or to the sequence differences between the R region of *cms-S*, the R1-similar region of N, and the unique end of the R1 and M1 plasmids.

Effect of restorer gene on the expression of the R sequence: If the R sequence is indeed responsible for S-type cytoplasmic male sterility, the nuclear restorer-of-fertility gene *Rf3* that restores fertility to S-type cytoplasm might be expected to affect its expression. Therefore, mRNAs from the mitochondria of several pairs of *cms-S* strains, essentially isogenic except for the presence of the nuclear restorer gene and closely linked sequences, were examined with the R probe IS1E5. The *Rf3* gene in the different nuclear backgrounds examined, B37, WF9, M825 and B37/WF9 (Table 1), affected expression of the R region in both immature cobs (Figures 4A and 5A) and microspores (see next section and Figure 6A and 7A). Also, as examined in the WF9 nuclear background, the *Rf3* gene in the different subtypes of S cytoplasm, S, RD and ML, affected R expression equally (Figure 4A). Therefore, the changes imposed on the transcriptional profile of the R region by the nuclear restorer gene *Rf3* were independent of both nuclear background and subtype of S.

The results suggest that either the overall transcriptional activity of the region is decreased or the transcripts become less stable. In addition, the *Rf3* gene affects RNA processing, initiation or termination, since at least one of the transcripts (10.5 kb) present in the sterile plants was not detected in the restored versions and at least two novel transcripts (10.2 and 0.5 kb) were present in the *Rf3* strains that were not detected in *cms-S* strains lacking *Rf3* (Figures 4A and 5A). Transcript levels observed in *Rf3/rf3* heterozygotes (Figures 4A and 5A) were further decreased (or increased in the case of the 2.2- and 0.5-kb transcripts) in homozygous *Rf3/Rf3* plants (Figure 5A). This effect was specific to the expression of the R sequence in *cms-S* since no changes were observed in the expression of the *urf1, orf2, urf3, coxI, atp9* and *atpA* genes. Figure 5B shows transcripts hybridizing to the IR probe, R-1E, (see MATERIALS AND METHODS and Figure 1C). The 4.9-, 3.7-, 2.9- and 1.4-kb transcripts result from the expression of *urf1, orf2* and *urf3* from the S1 and S2 episomes (TRAYNOR and LEVINGS 1986; ZABALA *et al.* 1987; ZABALA and WALBOT 1988) and were unaffected by *Rf3* (Figure 5B, lanes *Rf3/rf3* and *Rf3/Rf3*). This finding of specificity is significant

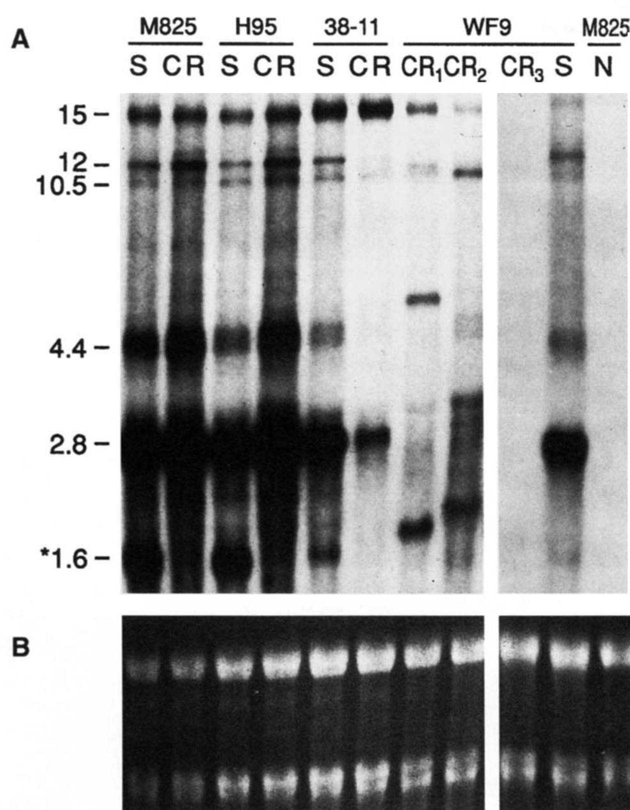

FIGURE 3.—(A) Northern blot of mtRNAs from immature cobs hybridized to the probe IS1E5 specific to the R sequence. None of the RNA bands shown in the CR_1 and CR_2 WF9 revertants is 1.6 kb in size since the two lanes for those RNA samples ran askew (see Figure 3B). (B) A portion of the ethidium bromide-stained gel used for the Northern blot shown in A. Samples are as follows: mitochondria from sterile plants (S) in the nuclear backgrounds M825, H95, 38-11, WF9; cytoplasmic revertants (CR), fertile plants isolated from the respective sterile strains M825: 84-323-3, H95: 82-82-1, 38-11: 81-115-2, WF9: 85-2053-2 (CR1), 85H-629-4 (CR2), 81-47-15 (CR3); M825 normal cytoplasm (N) fertile plants. The sizes of relevant transcripts are indicated in kilobases. S lanes show RNAs down to the 1.6 kb size. The 15-kb band could be random DNA molecules that coprecipitate with LiCl-RNA complexes. (B) A portion of the ethidium bromide-stained gel used for the Northern blot shown in A.

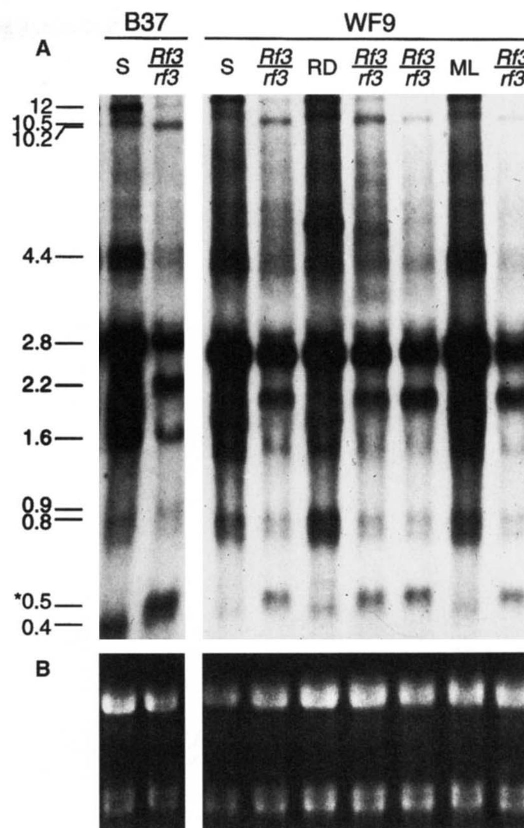

FIGURE 4.—Northern blot of mtRNAs from immature cobs hybridized to the probe IS1E5 specific to the R sequence. (A) S, RD, ML and VG are mtRNA samples from S sterile plants with different *cms-S* subtypes; *Rf3/rf3* is a mtRNA sample from fertile plants with sterile cytoplasm restored by the nuclear restorer gene *Rf3*. The nuclear backgrounds are B37 and WF9. Sizes of the transcripts affected by the nuclear restorer gene *Rf3* are indicated in kilobases. The starred sizes (left) represent novel transcripts in the restored lines. (B) A portion of the ethidium bromide-stained gel used for the Northern blot shown in A.

because *urf1*, *orf2* and *urf3* are episomal sequences as is the first 1 kb of R, and it has been shown that their transcripts may initiate in the IR (TRAYNOR and LEVINGS 1986). The *atpA* transcripts were also unaffected in *Rf3* heterozygous or homozygous plants (Figures 6B and 7B). Likewise, transcripts resulting from the expression of *coxI* and *atp9* were unaffected by the *Rf3* gene (data not shown). *CoxI* is in the σ region adjacent to the IR of one copy of R (Figures 1C and 9A) (ISAAC *et al.* 1985) and might thus have been expected to be affected by *Rf3*.

Expression of the R repeat in microspores: The initial experiments on the expression of the R sequence were conducted using mtRNA samples isolated from immature cobs of male-sterile, cytoplasmically revertant, and restored plants. Because pollen abortion in *cms-S* anthers takes place postmeiotically, it was relevant to examine the expression of the R region in developing microspores. Unlike *cms-T* and *cms-C* maize where restoration of the *cms* defect is sporophytic, restoration to fertility in *cms-S* is gametophytic; the genotype of a pollen grain determines whether it functions (*Rf*) or aborts (*rf*).

To examine R expression in the male gametes, microspores were isolated from immature tassels (see MATERIALS AND METHODS). Total RNA isolated from microspores was used in the Northern blots shown in Figures 6 and 7. It was striking to see the level of expression obtained for the R sequence in microspore RNA samples from *cms-S* plants (Figures 6A and 7A). These radiolabeled signals were obtained after an overnight exposure and show intensities similar to those obtained from blots with purified mtRNA during the same exposure times.

Notably, there was a greater relative abundance of the 1.6-kb transcript in all microspore RNA samples

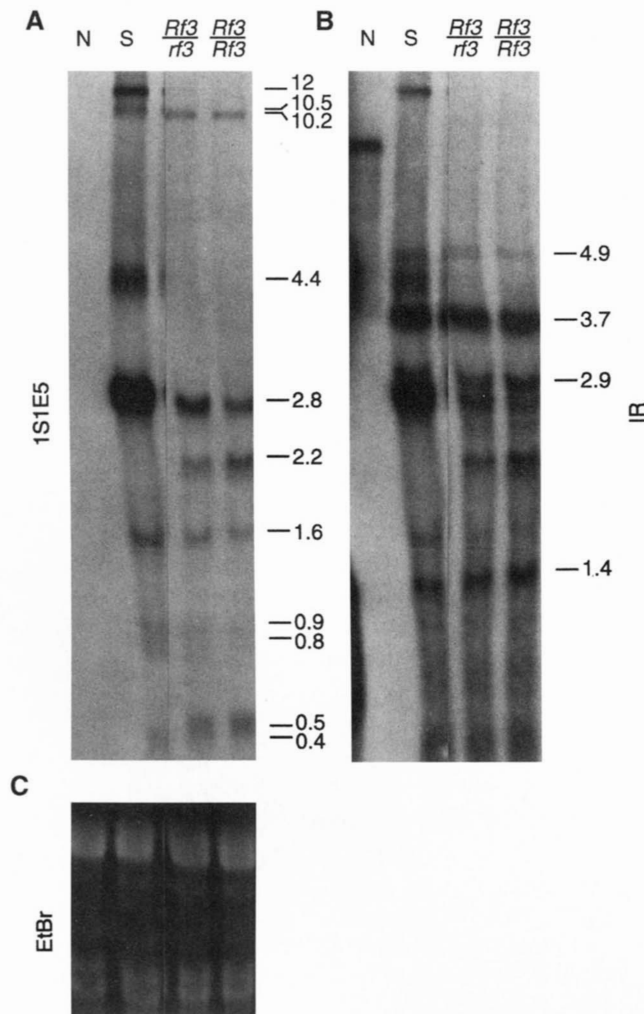

FIGURE 5.—Northern blot of mtRNAs from immature cobs hybridized to the R probe IS1E5 (A) and the 134-bp-long IR-specific probe R-1E (B). (C) A portion of the ethidium bromide-stained gel used for the Northern blots shown in A and B. Samples are as follows: mtRNA samples from B37 plants with normal (N) cytoplasm; B37 *cms-S* sterile plants *rf3*/*rf3* (S); B37 fertile plants with S-sterile cytoplasm restored by *Rf3*/*rf3* and *Rf3*/*Rf3* nuclear genes. The sizes of the transcripts affected by the nuclear restorer gene *Rf3* are indicated in kilobases in A. The sizes of the transcripts derived from S1 and S2 episomes and not affected by the restorer genes are indicated in kilobases to right of B.

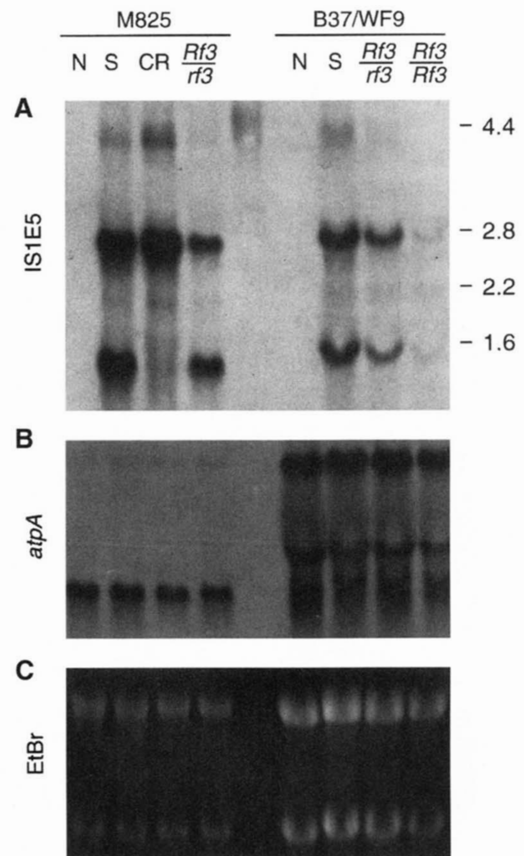

FIGURE 6.—(A) Northern blot of total RNA from microspores hybridized to the R probe IS1E5. (B) Northern blot shown in A stripped and reprobed with SR0.35, specific to the *atpA* gene. (C) A portion of the ethidium bromide-stained gel used for the Northern blots shown in A and B. Samples are as follows: normal cytoplasm (N); *cms-S* cytoplasm (S); cytoplasmic revertant (CR) 84-323-3; *Rf3*/*rf3*, nuclear restored heterozygous fertile plants; *Rf3*/*Rf3*, nuclear restored homozygous fertile plants; M825 and B37/WF9 nuclear backgrounds.

from sterile plants compared to the same transcript in equally loaded mtRNA samples from cob tissues of sterile plants (data not shown). In addition, it seems that for most of the microspore RNA samples from sterile plants the abundance of the 1.6-kb transcript was equal to or higher than that of the 2.8-kb transcript (Figures 6A and 7A, S lanes). The opposite was observed in the mtRNA samples obtained from immature cobs of equivalent plant samples (Figures 3 and 4).

The restorer gene *Rf3* affected the expression of the R sequence in microspores (Figure 6A, B37/WF9 lanes *Rf3*/*rf3* and *Rf3*/*Rf3*; Figure 7A, WF9 lane *Rf3*/*rf3*) in the same fashion as it did in immature ears with one exception. The 2.2-kb transcript that showed an increase in abundance with increasing *Rf3* dosage in mitochondrial samples from cob tissues (Figure 4A, *Rf3* lanes and Figure 5A, lanes *Rf3*/*rf3* and *Rf3*/*Rf3*) did not exhibit the same response in the microspores. There were no significant differences between samples from the microspores of sterile plants (Figures 6A and 7A, S lanes) and plants carrying *Rf3* (Figure 6A, M825 lane *Rf3*/*rf3*, B37/WF9 lanes *Rf3*/*rf3* and *Rf3*/*Rf3* and Figure 7A, WF9 lane *Rf3*/*rf3*) with respect to this transcript. As was the case with mtRNAs extracted from immature cobs, the abundance of the major 4.4-, 2.8- and 1.6-kb R transcripts in sterile (*rf3*/*rf3*) plants decreased in heterozygous restored (*Rf3*/*rf3*) plants (Figure 6A, M825 and B37/WF9; Figure 7A, WF9) and further decreased in homozygous restored (*Rf3*/*Rf3*) plants (Figure 6A, B37/WF9). Note that in Figure 7A lane *Rf3*/*rf3* is overloaded compared to the sterile progenitor, S sample in lane 4; see Figure 7C.

Nucleotide sequence organization of the σ' R region

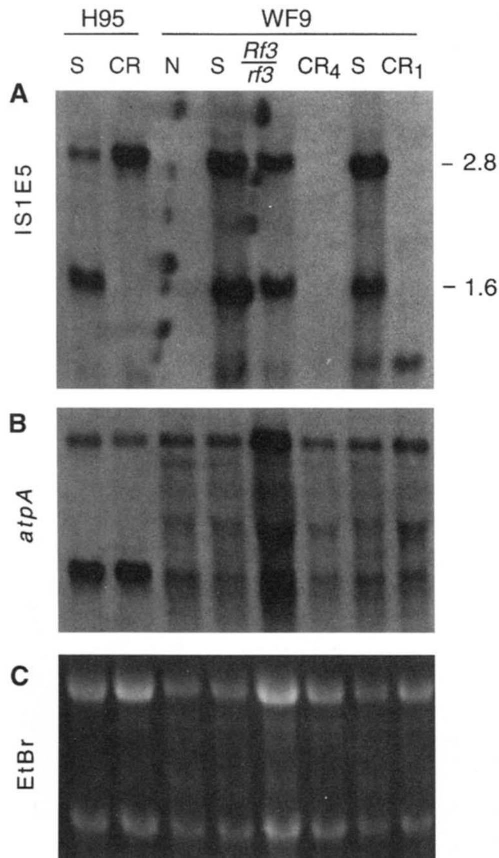

FIGURE 7.—Northern blot of total RNA from microspores hybridized to the R probe IS1E5 (A). (B) Northern blot shown in A stripped and reprobed with SR0.35, specific to the *atpA* gene. (C) A portion of the ethidium bromide-stained gel used for the Northern blots shown in A and B. Samples are as follows: normal cytoplasm (N); *cms-S* cytoplasm (S); cytoplasmic revertants: (CR) 82-82-1, (CR4) 81H-51-1, (CR1) 85-2053-2; *Rf3/rf3*, nuclear restored heterozygous fertile plant; H95 and WF9 nuclear backgrounds.

of *cms-S* mitochondria: The preceding data implicate the R region in the *cms-S* phenotype. All cytoplasmic revertants analyzed contained rearranged R sequences at the ends of linear mtDNA molecules. The expression of R observed in male-sterile progenitors was altered by cytoplasmic reversion to fertility as well as by restoration to fertility by the nuclear gene *Rf3*. Consequently, it was relevant to determine the DNA sequence of this region. A 1.6-kb *Pst*I-*Bam*HI fragment from a cosmid clone derived from *cms-S* mitochondria in the WF9 nuclear background (SCHARDL *et al.* 1984) containing the σ-σ' region of the mitochondrial genome was sequenced as described in MATERIALS AND METHODS. Figure 8A shows the DNA sequence of the 1.6-kb *Pst*I-*Bam*HI fragment plus the 134 bp of the IR upstream of the *Pst*I site. The σ' region of *cms-S* was previously sequenced only up to the *Hae*III site at position 617 in Figure 8A (HOUCHINS *et al.* 1986).

Comparison of our DNA sequence with that obtained by HOUCHINS *et al.* (1986) from the σ' region of S cytoplasm shows almost complete identity for 722 bp. There is a base pair difference, G for C at position 429, and we did not find a duplication of the base pairs from nucleotide position 596 to 603. Our sequence more closely resembles the R1 region of N cytoplasm (HOUCHINS *et al.* 1986) than did theirs.

We have sequenced 1110 bp of σ' beyond the *Hae*III site. This nucleotide sequence was compared to other DNA sequences in GenBank using BLAST (ALTSCHUL *et al.* 1990) and we found that the similarity to the R1 sequence of N (HOUCHINS *et al.* 1986) ends at position 1060 (Figure 8A). Up to nucleotide 722 (Figure 8A) the R sequence is identical to the R1-like sequence of N except for two base pair differences. Downstream from base pair 722, to base pair 1060, resemblance between the two sequences is absent in two regions while in two other regions it is 92% and 94%, respectively. Therefore, the first 1060 bp of the R region of *cms-S* exhibit similarity to three nonoverlapping segments of the R1-like sequence of N cytoplasm. Downstream from this R1-similar region, there is a stretch of 336 bp with no resemblance to any published sequence. From position 1397 to 1443 there are 47 bp with 94% sequence identity to the *atp9* coding sequence as determined in *cms-T* maize mitochondria (DEWEY *et al.* 1985) (Figure 8B). The mitochondrial gene *atp9* encodes the ATP9 subunit of the mitochondrial F1-F0 ATPase complex. Fifteen base pairs further downstream there is 179 bp of similarity to a region 3′ to the *orf221* gene in *cms-T* (originally designated *orf25*; DEWEY *et al.* 1986). While this sequence has been published only for *cms-T* (DEWEY *et al.* 1986), it has been determined that the region 3′ to *orf221* is nearly identical in *cms-T* and *cms-S* (STAMPER *et al.* 1987). The similarity to this *orf221* sequence extends from position 1458 to 1636 of the *cms-S* R region, corresponding to position 2641–2822 of the sequence 3′ to *orf221* in *cms-T* (DEWEY *et al.* 1986). The similarity between these segments of DNA is 76% (Figure 8B) and includes a short stretch of 39 bp that has 94% sequence identity. Within the 179 bp of similarity to the *orf221* 3′ region there are two additional stretches with sequence similarity to the *atp9* gene. One, 39 bp long (from position 1466 to 1504 in Figure 8B), has 94% identity to the 3′ end of the *atp9* coding region and its 3′ flank. Of these 39 bp of *atp9* similarity, 26 bp are repeated in the previously mentioned 47 bp with *atp9* similarity located upstream of the *orf221* 3′ similar region. The other stretch, 63 bp (from position 1536 to 1598 in Figure 8B), has 83% similarity to the 3′ flanking region of *atp9*. Altogether there are three short stretches with *atp9* similarity: 47, 39 and 63 bp. This observation may explain why, when hybridization to Southern blots of *cms-S* maize mtDNA with an *atp9* probe is carried out, the probe hybridizes to all *atp9*- and R-containing fragments (data not shown; WANG and GENGENBACH 1988). It also explains why similarity to the 3′ noncoding region of *atp9* was detected near the rearranged region of mtDNA of a WF9 cytoplasmic

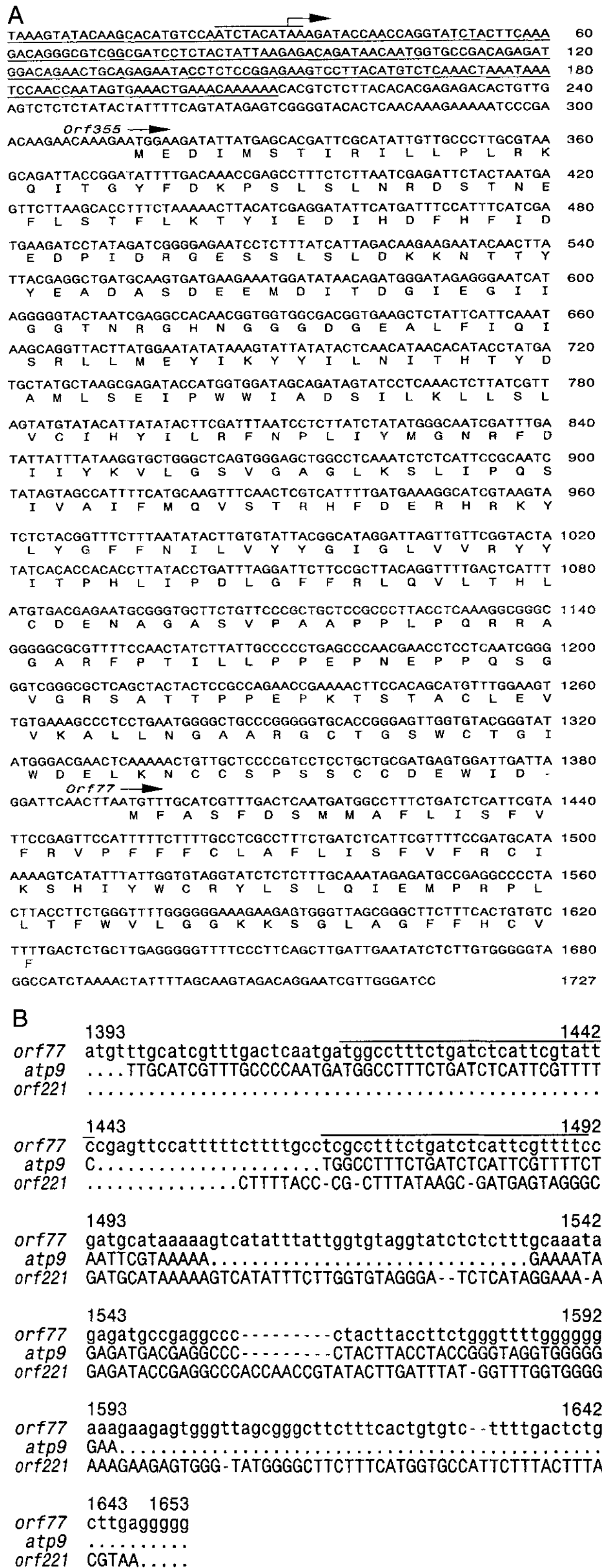

FIGURE 8.—(A) The nucleotide and deduced amino acid sequences of the R repeat from σ-σ′ of *cms-S* maize. First arrow indicates potential transcription initiation site. The sequence of the IS1E5 clone is contained from bp 485 to 1431. Arrows following *orf355* and *orf77* indicate the positions of the beginning methionine codons for these two ORFs. –, the positions of the termination codons. (B) Alignment of *orf77* DNA sequence with similar regions of the *atp9* gene and the region *orf221* 3′ region of *cms-T*. The overlined bases in *orf77* indicate a repeated region.

revertant (NEBIOLO *et al.* 1990). A schematic diagram of the R region, showing similarities to known mtDNA sequences, is presented in Figure 9A.

Putative ORFs in R: The derived amino acid sequence of the R region contains two ORFs. The largest one, a chimeric 355-codon ORF (*orf355*), extends from position 314 to 1378, at which point there is a TAG stop codon. *orf355* begins 313 bp into the region of similarity to the R1-like sequence of N cytoplasm (210 bp of which is the IR) and contains the other two regions of R1 similarity plus 336 bp of unknown origin. *orf355* could potentially encode a 39.9-kD polypeptide. The hydropathy plot deduced from *orf355* indicates that the translation product could have four transmembrane domains (Figure 10). Computer searches of the predicted translation products of *orf355* within the nonredundant GenBank CDS translations, PDB, SwissProt, SPupdate and PIR databases using the BLAST program (ALTSCHUL *et al.* 1990) detected no significant similarities.

Eleven base pairs downstream from the stop codon of *orf355*, from position 1393 to 1626, there is a chimeric 77-codon ORF (*orf77*) that could encode a polypeptide of 9.1 kD. *orf77* contains the three regions with *atp9* similarity (Figure 9B). Those codons in *orf77* with similarity to the *atp9* coding region are in the same frame as they are in the *atp9* gene. The region of similarity between *orf77* and the region 3′ to *orf221* in *cms-T* maize extends from position 66 of *orf77* to a point 10 bp into its 3′ flanking region. This region therefore includes two of the three regions with *atp9* similarity. *orf77* could code for a polypeptide that is mostly hydrophobic (Figure 10), particularly the first half containing the homology to the *atp9* coding sequence (Figure 9B). The predicted translation product of *orf77* showed partial similarity to the ATP9 protein.

DISCUSSION

We have isolated numerous *cms-S* cytoplasmic revertants, enabling us to study the molecular changes in the mtDNA brought about by cytoplasmic reversion to fertility. We have observed a class of DNA rearrangements common to all revertants, regardless of nuclear background, involving the R sequence of *cms-S* mitochondria. In the mtDNA of S male-sterile plants, the R region is repeated and fully integrated at two locations; it is also found at the ends of linear mtDNA molecules (SCHARDL *et al.* 1984). In all *cms-S* cytoplasmic revertants examined, the terminal R regions underwent rearrangement.

We determined the DNA and derived amino acid

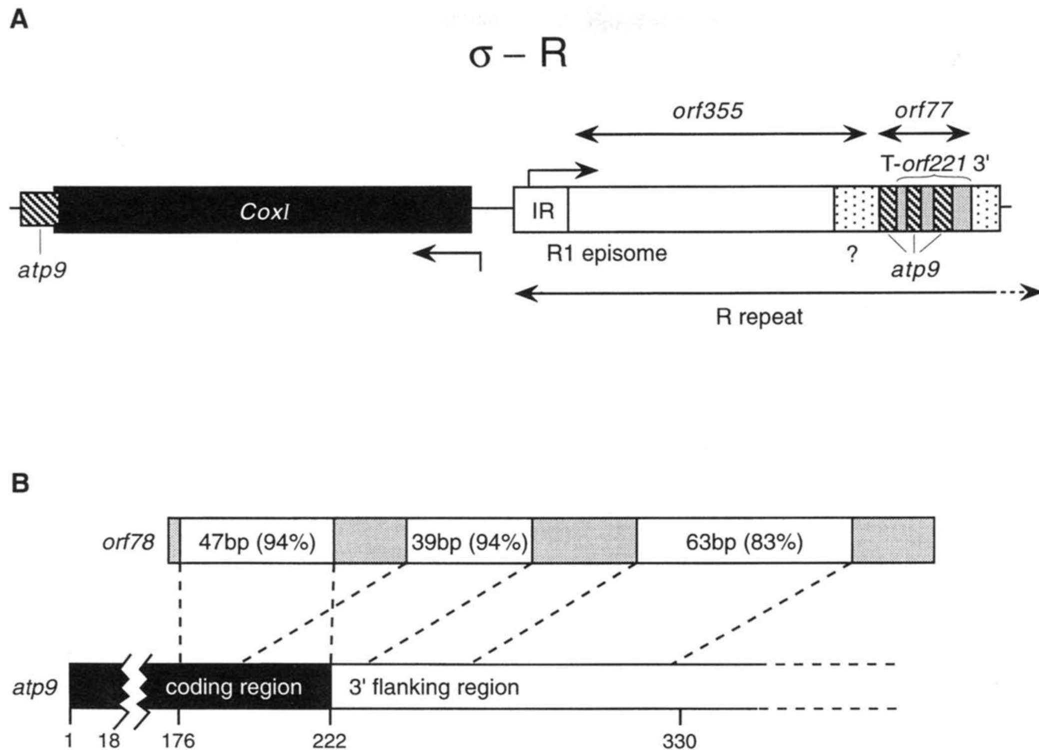

FIGURE 9.—(A) Diagrammatic representation of the σ-R region of *cms-S* maize mitochondria. The 5′ end of the *coxI* gene is 174 bp from nucleotide 1 of the IR of the R sequence. The R sequence with similarity to the R1 plasmid (1060 bp long) is represented by open boxes, the *atp9* gene by hatched boxes and the 3′ end of *orf221* in *cms-T* (179 bp long) by the light shaded box that includes two of the *atp9* similar regions. A portion of the R sequence with no similarity to any other published mitochondrial sequences is included in *orf355* (larger dotted box). *orf77* spans the *atp9* and *cms-T orf221* 3′ end similarities. A potential transcription initiation site is indicated with an arrow at position 32 of the IR. Similarity to the *atp9* gene is also found at the 3′ end of the *coxI* gene (larger hatched box). Not drawn to scale. (B) Diagrammatic representation of *orf77* showing the regions with similarity to the *atp9* gene and its 3′ flanking region. Drawn to scale.

sequences of one of the R repeats of *cms-S*. It is a chimeric region containing two ORFs, *orf355* and *orf77*. These ORFs appear to be the result of a complex series of recombination events. R contains similarity to three nonoverlapping segments of a sequence found in the R1 region of N mtDNA that is in turn similar to the R1 plasmid of some South American races with RU cytoplasm (WEISSINGER *et al.* 1982; LEVINGS *et al.* 1983) and to the M1 plasmid of Mazoti cytoplasm *Z. luxurians* teosinte (GRACE *et al.* 1994). Downstream from the R1-like region there is a stretch of 336 bp with no resemblance to any published mtDNA sequence. This chimeric region containing the R1 similarities and the 336 bp of unknown origin makes up *orf355*. Following this, there are three regions with resemblance to the *atp9* coding and/or 3′ flanking sequences. The latter two stretches are contained within a segment with similarity to a region 3′ to *orf221*. The three *atp9* regions are part of *orf77*. In the first region there is a series of 26 bp that are tandemly repeated in the second. This region translates to AFLISFVF, which represents the carboxy terminus of the ATP9 protein. The second copy of the repeat occurs within the segment with similarity to the region 3′ to *orf221*. As shown by KENNELL and PRING (1989), the sequence 3′ to *orf221* in *cms-T* includes the transcript terminus predicted by SCHUSTER *et al.* (1986). Therefore, the equivalent portion of R from nucleotide 1581 to 1607 is a possible transcript terminus.

Chimeric genes created by rearrangements of coding and noncoding segments of mtDNA have been found to be associated with *cms* in a number of species, including *cms-T* maize (DEWEY *et al.* 1986), sorghum (BAILEY-SERRES *et al.* 1986; TANG *et al.* 1996), petunia (YOUNG and HANSON 1987), rice (IWABUCHI *et al.* 1993) and *B. napus* (SINGH and BROWN 1991; SINGH *et al.* 1996). It is possible that in S cytoplasm a chimeric gene(s) was created by the recombination of a sequence with similarity to the R1 plasmid and an unknown mtDNA sequence (*orf355*), and/or by recombination of *atp9* and the sequence with resemblance to the region 3′ to *orf221* (*orf77*). Maize *cms-S* is therefore the fourth case in which ORFs associated with *cms* show sequence similarities to *atp9* (see TANG *et al.* 1996).

In both petunia and *cms-T* maize the *cms*-associated regions are located near recombination repeats (reviewed by HANSON 1991). In the case of *cms-S* maize the region of the mitochondrial genome correlated with male sterility is part of a recombination repeat. HANSON (1991) has pointed out that the association of recombination repeats with *cms*-correlated regions may not be

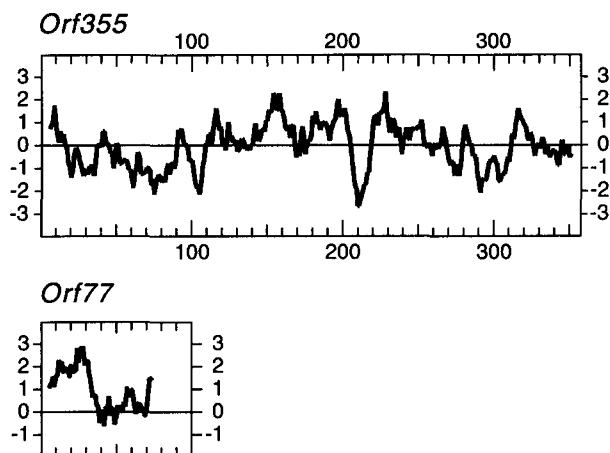

FIGURE 10.—Hydropathy plots of the proteins potentially encoded by (A) *orf355*, the largest ORF of the R repeat and (B) *orf77*. Values for hydropathic index (*y* axis) calculated according to KYTE and DOOLITTLE (1982) are plotted against amino acid residue numbers (*x* ordinate). Hydrophobic domains are represented by positive hydropathic values.

coincidental but may be indicative of the mechanism involved in the creation of these chimeric regions.

S-type male sterility could result from a partial impairment of mitochondrial function as a result of the expression of either or both of the putative ORFs of R. Northern analysis showed that all but one of the RNAs hybridizing to the R-specific probe IS1E5 also hybridized to the IR probe R-1E (LEVINGS and SEDEROFF 1983). Since this is the case, the sequence between the regions covered by the two probes should be expressed and be part of all transcripts hybridizing to both probes. Out of the seven transcripts hybridizing to the IR- and R-specific probes, only the relative abundance of the 1.6-kb transcript is correlated with S-type male sterility. In all cases, this transcript, present in the sterile progenitors, was absent from the cytoplasmic revertants. If transcript initiation takes place within the IR of R, it is possible that both ORFs are tandemly expressed in the 1.6-kb transcript.

An indication of a possible initiation site comes from the work of TRAYNOR and LEVINGS (1986). They mapped the 5′ ends of transcripts of two episomal genes, *urf1* and *orf2*, to nucleotide position 32 within the 208-bp terminal inverted repeats. Nucleotide 32 lies within a sequence element ATCTACATAA that compares favorably with the maize mitochondrial promoter consensus NNYNRYRTAT proposed by RAPP *et al.* (1993) at every position except for the final T. It has been demonstrated that this element is essential for promoter function in an *in vitro* transcription system (RAPP *et al.* 1993). Therefore, it is possible that a promoter of the R region is located at position 32 of the IR within the R repeat. It may be relevant to male sterility if transcription initiation takes place in the IR and *orf77* is included in the 1.6-kb *cms*-specific transcript. *orf77* contains three segments of the *atp9* gene in the same reading frame. Therefore, the transcripts and/or translation products could compete with the functional *atp9* RNA or ATP9 protein, somehow resulting in male sterility. Whether one or both of the putative ORFs is (are) translated is still an unanswered question.

Why the cytoplasmic revertants in the different nuclear backgrounds fail to generate the 1.6-kb transcript is not understood as yet. In WF9 revertants that all retain the S episomes, rearrangement of the R sequence at all of its sites within the mitochondrial genome changed its expression from that observed in the sterile progenitors. In contrast, revertants in all other nuclear backgrounds, where the S episomes are absent and the fully integrated R sequences are not rearranged, may fail to synthesize the 1.6-kb transcript by a combination of two possible causes. First, the promoter in the IR may be used inefficiently when R is integrated. This may seem unlikely but it is possible that, for example, the promoter at the IR could be recognized only by the RNA polymerase encoded in the S2 episome. When reversion results in loss of the episomes, this RNA polymerase is absent and this promoter may not be utilized. Second, the larger transcripts with initiation sites upstream of the IR may not be processed to the 1.6-kb transcript. Our data suggest that the larger transcripts that exhibit increased abundance upon reversion may be the result of additional upstream initiation sites rather than diverse termination sites at the 3′ end. In support of this view, and as mentioned above, is the fact that there is a well-defined termination site toward the 3′ end of *orf77*. Whatever the case, it is clear that the 1.6-kb transcript is most abundant in nonrestored *cms-S* plants with intact R termini in their mtDNA; its presence correlates with sterility.

Rf3, or closely linked gene(s), affect the expression of the R region resulting in a decrease in the abundance of its major transcripts. This decrease could be due to a reduced rate of transcription or to an increased rate of transcript degradation. The *Rf3* region may also affect processing since in its presence one transcript is no longer detectable while two novel transcripts are found instead. While most transcripts hybridizing to the R probe in *cms-S* plants decreased in abundance with increased dosage of the *Rf3* gene, two transcripts increased in amount with increasing Rf3 dosage in mitochondria of cob tissue. These results are suggestive of a complex effect of the *Rf3* region on the expression of the R sequence. If we compare the effects of the *Rf3* region in maize with those of restorer genes in other systems, it seems that it may combine a processing activity as observed for the restorers of *B. polima* male-sterile cytoplasm (SINGH and BROWN 1991; SINGH *et al.* 1996), rice *cms* (IWABUCHI *et al.* 1993) and sorghum *cms* (TANG *et al.* 1996) and a reduction in transcript accumulation as observed in *cms-T* maize (DEWEY *et al.* 1987; KENNELL *et al.* 1987; KENNELL and PRING 1989) and in petunia (NIVISON and HANSON 1989). The changes in RNA

abundance observed in restored *cms-S* strains may be the consequence of a reduction in translation rather than a decrease in transcription initiation. This has been suggested for the *Rf1* gene of *cms-T* maize (KENNELL and PRING 1989) and the *Rf* of petunia (PRUITT and HANSON 1991).

Relevant to the argument that the R repeat is the site of the *cms-S* mutation is the specificity of the effect of the *Rf3* gene on the expression of R. The *Rf3* gene did not affect the expression of other episomal sequences (*urf1, orf2* and *urf3*) nor of the mitochondrial genes examined (*coxI, atpA* and *atp9*). If the IR were the site of a major promoter for the R sequence, and *Rf3* affects this IR initiation site, one would expect that *Rf3* would also affect transcription of the episomal sequences encoding *urf1, orf2* and *urf3*. Our results suggest that the *Rf3* region may not decrease transcript levels by affecting transcription initiation or promoter function but rather by increasing RNA degradation or reducing translation.

Interestingly, the *cms-S*-associated sequence is expressed in mitochondria of both the sporophyte (immature cobs) and gametophyte (microspores) generations of the maize plant. However, its expression seems to be more prominent and detrimental only postmeiotically. Our results show that *Rf3* functions to reduce R transcripts in both generations. Therefore, gametophytic restoration seems to be a function of the fact that the mtDNA determinant of *cms-S* becomes critical in the developing pollen grain.

To date, the published data do not completely explain the molecular basis of cytoplasmic male sterility in any plant species. The R region of *cms-S* maize plays a key role in the intramolecular recombination of the mitochondrial genome since it is a recombinational repeat and has the ability to recombine with the S episomes. We are now in a position to determine the translation product(s) derived from the 1.6-kb transcript and how it (they) brings about pollen abortion in *cms-S* plants. Also, we can now study how the *Rf3* gene results in the many changes in the expression of the R sequence reported herein.

We thank CHARLES S. LEVINGS III, DARYL PRING, KATHLEEN NEWTON and IAN SMALL for providing mitochondrial clones; DEBORAH KATZ-DOWNIE for assistance in subcloning the CSA61 cosmid clone; PAIGE GOODLOVE (the Genetic Engineering Facility of the University of Illinois) for help on DNA sequencing; and MARC ALBERTSEN, JACK BECKETT, BURLE GENGENBACH, MAJOR GOODMAN and EARL PATTERSON for providing seed of some of the stocks used in these studies. We also thank ANDREW BENT for a critical reading of the manuscript and EUGENE KUZMIN, who assisted in the comparative analysis of the R sequence. This work was supported by a grant from the U.S. Department of Agriculture-NRICGP (91-37301-6288) to G.Z. and a Department of Energy (DE-FG02-90ER20003) grant to J.R.L. and S.G.-L.

LITERATURE CITED

ABAD, A. R., B. J. MEHRTENS and S. A. MACKENZIE, 1995 Specific expression in reproductive tissues and fate of a mitochondrial sterility-associated protein in cytoplasmic male-sterile bean. Plant Cell **7:** 271–285.

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS and D. J. LIPMAN, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

ANDRE, C., A. LEVY and V. WALBOT, 1992 Small repeated sequences and the structure of plant mitochondrial genomes. Trends Genet. **8:** 128–132.

BAILEY-SERRES, J., D. K. HANSON, T. D. FOX and C. L. LEAVER, 1986 Mitochondrial genome rearrangement leads to extension and relocation of the cytochrome c oxidase subunit I gene in sorghum. Cell **47:** 567–576.

BEDINGER, P. A., and M. D. EDGERTON, 1990 Developmental staging of maize microspores reveals a transition in developing microspore proteins. Plant Physiol. **92:** 474–479.

CUI, X., R. P. WISE and P. S. SCHNABLE, 1996 The *rf2* nuclear restorer gene of male-sterile T-cytoplasm maize. Science **272:** 1334–1336.

DEWEY, R. E., C. S. LEVINGS III and D. H. TIMOTHY, 1986 Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell **44:** 439–447.

DEWEY, R. E., A. M. SCHUSTER, C. S. LEVINGS III and D. H. TIMOTHY, 1985 Nucleotide sequence of Fo-ATPase proteolipid (subunit 9) gene of maize mitochondria. Proc. Natl. Acad. Sci. USA **82:** 1015–1019.

DEWEY, R. E., J. N. SIEDOW, D. H. TIMOTHY and C. S. LEVINGS III, 1988 A 13- kilodalton maize mitochondrial protein in *E. coli* confers sensitivity to *Bipolaris maydis* toxin. Science **239:** 293–295.

DEWEY, R. E., D. H. TIMOTHY and C. S. LEVINGS III, 1987 A mitochondrial protein associated with cytoplasmic male sterility in the T cytoplasm of maize. Proc. Natl. Acad. Sci. USA **84:** 5374–5378.

ESCOTE, L. J., 1986 Reorganization of mitochondrial genomes of *cms-S* maize associated with reversion to fertility and changes in nuclear background. Ph. D. Thesis, University of Illinois, Urbana-Champaign.

ESCOTE, L. J., S. J. GABAY-LAUGHNAN and J. R. LAUGHNAN, 1985 Cytoplasmic reversion to fertility in *cms-S* maize need not involve loss of linear mitochondrial plasmids. Plasmid **14:** 264–267.

ESCOTE-CARLSON, L. J., S. GABAY-LAUGHNAN and J. R. LAUGHNAN, 1988 Reorganization of mitochondrial genomes of cytoplasmic revertants in *cms-S* inbred line WF9 in maize. Theoret. Appl. Genet. **75:** 659–667.

GABAY-LAUGHNAN, S., G. ZABALA and J. R. LAUGHNAN, 1995 S-type cytoplasmic male sterility in maize, pp. 395–432 in *Advances in Cellular and Molecular Biology of Plants*, edited by C. S. LEVINGS III and I. K. VASIL. Kluwer Academic Publishers, Amsterdam.

GRACE, K. S., J. O. ALLEN and K. J. NEWTON, 1994 R-type plasmids in mitochondria from a single source of *Zea luxurians* teosinte. Curr. Genet. **25:** 258–264.

HANSON, M. R., 1991 Plant mitochondrial mutations and male sterility. Annu. Rev. Genet. **25:** 461–486.

HOUCHINS, J. P., H. GINSBURG, M. ROHRBAUGH, R. M. K. DALE, C. L. SCHARDL *et al.*, 1986 DNA sequence analysis of a 5.27-kb direct repeat occurring adjacent to the regions of S-episome homology in maize mitochondria. EMBO J. **5:** 2781–2788.

ISAAC, P. G., V. P. JONES and C. J. LEAVER, 1985 The maize cytochrome *c* oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male sterile plants. EMBO J. **4:** 1617–1623.

ISHIGE, T., K. K. STOREY and B. G. GENGENBACH, 1985 Cytoplasmic fertile revertants possessing S1 and S2 DNAs in S male-sterile maize. Japan. J. Breed. **35:** 285–291.

IWABUCHI, M., J. KYOZUKA and K. SHIMAMOTO, 1993 Processing followed by complete editing of an altered mitochondrial *atp6* RNA restores fertility of cytoplasmic male sterile rice. EMBO J. **12:** 1437–1446.

JANSKA, H., and S. A. MACKENZIE, 1993 Unusual mitochondrial genome organization in cytoplasmic male sterile common bean and the nature of cytoplasmic reversion to fertility. Genetics **135:** 869–879.

KEMBLE, R. J., R. E. GUNN and R. B. FLAVELL, 1980 Classification of normal and male sterile cytoplasms in maize. II. Electrophoretic analysis of DNA species in mitochondria. Genetics **95:** 451–458.

KEMBLE, R. J., and R. J. MANS, 1983 Examination of the mitochondrial genome of revertant progeny from S-*cms* maize with cloned S1 and S2 hybridization probes. J. Mol. Appl. Genet. **2:** 161–171.

KENNELL, J. C., and D. R. PRING, 1989 Initiation and processing of

atp6, T-*urf13* and *ORF221* transcripts from mitochondria of T cytoplasm maize. Mol. Gen. Genet. **216:** 16–24.

KENNELL, J. C., R. P. WISE and D. R. PRING, 1987 Influence of nuclear background on transcription of a maize mitochondrial region associated with Texas male sterile cytoplasm. Mol. Gen. Genet. **210:** 399–406.

KOHLER, R. H., R. HORN, A. LOSSL and K. ZETSCHE, 1991 Cytoplasmic male sterility in sunflower is correlated with the co-transcription of a new open reading frame with the *atpA* gene. Mol. Gen. Genet. **227:** 369–376.

KYTE, J., and R. F. DOOLITTLE, 1982 A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:** 105–132.

KRISHNASAMY, S., and C. A. MAKAROFF, 1994 Organ-specific reduction in the abundance of a mitochondrial protein accompanies fertility restoration in cytoplasmic male-sterile radish. Plant Mol. Biol. **26:** 935–946.

LAUGHNAN, J. R., and S. GABAY-LAUGHNAN, 1983 Cytoplasmic male sterility in maize. Annu. Rev. Genet. **17:** 27–48.

LEAVER, C. J., P. G. ISAAC, J. BAILEY-SERRES, I. D. SMALL and D. K. HANSON, 1985 Recombination events associated with the cytochrome *c* oxidase subunit I gene in fertile and cytoplasmic male sterile maize and sorghum, pp. 111–122 in *Achievements and Perspectives of Mitochondrial Research*, edited by E. QUAGLIARIELLO, E. C. SLATER, F. PALMIERI, C. SACCONE and A. M. KROON. Elsevier, Amsterdam.

LEVINGS, C. S., III, and D. R. PRING, 1978 The mitochondrial genome of higher plants. Stadler Genet. Symp. **10:** 77–93

LEVINGS, C. S., III, B. D. KIM, D. R. PRING, M. F. CONDE, R. J. MANS *et al.*, 1980 Cytoplasmic reversion of *cms-S* in maize: association with a transpositional event. Science **209:** 1021–1023.

LEVINGS, C. S., III, and D. R. PRING, 1979 Molecular bases of cytoplasmic male sterility in maize, pp. 171–193 in *Physiological Genetics*, edited by J. SCANDALIOS. Academic Press, New York.

LEVINGS, C. S., III, and R. R. SEDEROFF, 1983 Nucleotide sequence of the S-2 mitochondrial DNA from the S cytoplasm of maize. Proc. Natl. Acad. Sci. USA **80:** 4055–4059.

LEVINGS, C. S., III, R. R. SEDEROFF, W. W. L. HU and D. H. TIMOTHY, 1983 Relationships among plasmid-like DNAs of the maize mitochondria, pp. 363–371 in *Structure and Function of Plant Genomes*, edited by O. CIFERRI and L. DURE. Plenum Publishing, New York.

LONSDALE, D. M., T. P. HODGE, C. M.-R. FAURON and R. B. FLAVELL, 1983 A predicted structure for the mitochondrial genome from the fertile cytoplasm of maize, pp. 445–456 in *Plant Molecular Biology*, edited by R. B. GOLDBERG. Alan R. Liss, New York.

MACKENZIE, S. A., and C. D. CHASE, 1990 Fertility restoration is associated with loss of a portion of the mitochondrial genome in cytoplasmic male- sterile common bean. Plant Cell **2:** 905–912.

MACKENZIE, S. A., D. R. PRING, M. J. BASSETT and C. D. CHASE, 1988 Mitochondrial DNA rearrangement associated with fertility restoration and cytoplasmic reversion to fertility in cytoplasmic male sterile *Phaseolus vulgaris* L. Proc. Natl. Acad. Sci. USA **85:** 2714–2717.

MAKAROFF, C. A., I. J. APEL and J. D. PALMER, 1989 The *atp6* coding region has been disrupted and a novel reading frame generated in the mitochondrial genome of cytoplasmic male-sterile radish. J. Biol. Chem. **264:** 11706–11713.

MONEGER, F., C. J. SMART and C. J. LEAVER, 1994 Nuclear restoration of cytoplasmic male sterility in sunflower is associated with the tissue-specific regulation of a novel mitochondrial gene. EMBO J. **13:** 8–17.

NEBIOLO, C. M., Y. WANG, I. H. REMICK and B. G. GENGENBACH, 1990 Organization and expression of a "novel" mitochondrial DNA fragment in a maize WF9 fertile revertant and its cytoplasmic male sterile progenitor. J. Cell Biol. **111:** 454A (Abstract).

NIVISON, H. T., and M. R. HANSON, 1989 Identification of a mitochondrial protein associated with cytoplasmic male sterility in petunia. Plant Cell **1:** 1121–1130.

PAILLARD, M., R. R. SEDEROFF and C. S. LEVINGS III, 1985 Nucleotide sequence of the S-1 mitochondrial DNA from the S cytoplasm of maize. EMBO J. **4:** 1125–1128.

PRING, D. R., C. S. LEVINGS III, W. W. L. HU and D. H. TIMOTHY, 1977 Unique DNA associated with mitochondria in the "S"-type cytoplasm of male-sterile maize. Proc. Natl. Acad. Sci. USA **74:** 2904–2908.

PRUITT, K. D., and M. R. HANSON, 1991 Transcription of the *Petunia* mitochondrial CMS-associated *Pcf* locus in male sterile and fertility-restored lines. Mol. Gen. Genet. **227:** 348–355.

RAPP, W. D., D. S. LUPOLD, S. MACK and D. B. STERN, 1993 Architecture of the maize mitochondrial *atp1* promoter as determined by linker-scanning and point mutagenesis. Mol. Cell. Biol. **13:** 7232–7238.

RASMUSSEN, J., and M. R. HANSON, 1989 A NADH dehydrogenase subunit gene is co-transcribed with the abnormal *Petunia* mitochondrial gene associated with cytoplasmic male sterility. Mol. Gen. Genet. **215:** 332–336.

ROTTMANN, W. H., T. B. BREARS, T. P. HODGES and D. M. LONSDALE, 1987 A mitochondrial gene is lost via homologous recombination during reversion of *cms-T* maize to fertility. EMBO J. **6:** 1541–1546.

SCHARDL, C. L., D. M. LONSDALE, D. R. PRING and K. R. ROSE, 1984 Linearization of maize mitochondrial chromosomes by recombination with linear episomes. Nature **310:** 292–296.

SCHARDL, C. L., D. R. PRING and D. M. LONSDALE, 1985 Mitochondrial DNA rearrangements associated with fertile revertants of S-type male-sterile maize. Cell **43:** 361–368.

SCHUSTER, W., R. HIESSEL, P. G. ISAAC, C. J. LEAVER and A. BRENNICKE, 1986 Transcript termini of messenger RNAs in higher plant mitochondria. Nucleic Acids Res. **14:** 5943–5954.

SICULELLA, L., and J. D. PALMER, 1988 Physical and gene organization of mitochondrial DNA in fertile and male-sterile sunflower. CMS-associated alterations in structure and transcription of the *atpA* gene. Nucleic Acids Res. **16:** 3787–3799.

SINGH, M., and G. G. BROWN, 1991 Suppression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. Plant Cell **3:** 1349–1362.

SINGH, M., N. HAMEL, R. MENASSA, X-Q. LI, B. YOUNG *et al.*, 1996 Nuclear genes associated with a single brassica CMS restorer locus influence transcripts of three different mitochondrial gene regions. Genetics **143:** 505–516.

SMALL, I. D., E. D. EARLE, L. J. ESCOTE-CARLSON, S. GABAY-LAUGHNAN, J. R. LAUGHNAN *et al.*, 1988 A comparison of cytoplasmic revertants to fertility from different *cms-S* maize sources. Theoret. Appl. Genet. **76:** 609–618.

STAMPER, S. E., R. E. DEWEY, M. M. BLAND and C. S. LEVINGS III, 1987 Characterization of the gene *urf13*-T and an unidentified reading frame, ORF 25, in maize and tobacco mitochondria. Curr. Genet. **12:** 457–463.

STERN, D. B., and K. J. NEWTON, 1986 Isolation of plant mitochondrial RNA. Methods Enzymol. **118:** 488–496.

TANG, H. V., D. R. PRING, L. C. SHAW, R. A. SALAZAR, F. R. MUZA *et al.*, 1996 Transcript processing internal to a mitochondrial open reading frame is correlated with fertility restoration in male-sterile sorghum. Plant J. **10:** 123–133.

TRAYNOR, P. L., and C. S. LEVINGS III, 1986 Transcription of the S-2 maize mitochondrial plasmid. Plant Mol. Biol. **7:** 255–263.

WANG, Y., and B. GENGENBACH, 1988 Unique mtDNA restriction fragment in an S cytoplasm fertile revertant line retaining the S1 and S2 plasmid. Maize Genet. Coop. Newslet. **62:** 102–103.

WEISSINGER, A. K., D. H. TIMOTHY, C. S. LEVINGS III, W. W. L. HU and M. M. GOODMAN, 1982 Unique plasmid-like mitochondrial DNAs from indigenous maize races of Latin America. Proc. Natl. Acad. Sci. USA **79:** 1–5.

WISE, R. P., C. L. DILL and P. S. SCHNABLE, 1996 *Mutator*-induced mutations of the *rf1* nuclear fertility restorer of T-cytoplasm maize alter the accumulation of T-*urf13* mitochondrial transcripts. Genetics **143:** 1383–1394.

WISE, R. P., D. R. PRING and B. G. GENGENBACH, 1987 Mutation to male fertility and toxin insensitivity in Texas (T)-cytoplasm maize is associated with a frameshift in a mitochondrial open reading frame. Proc. Natl. Acad. Sci. USA **84:** 2858–2862.

YOUNG, E. G., and M. R. HANSON, 1987 A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell **50:** 41–49.

ZABALA, G., C. O'BRIEN-VEDDER and V. WALBOT, 1987 S2 episome of maize mitochondria encodes a 130-kilodalton protein found in male sterile and fertile plants. Proc. Natl. Acad. Sci. USA **84:** 7861–7865.

ZABALA, G., and V. WALBOT, 1988 An S1 episomal gene of maize mitochondria is expressed in male sterile and fertile plants of the S-type cytoplasm. Mol. Gen. Genet. **211:** 386–392.

Communicating editor: J. A. BIRCHLER