Mutator-Induced Mutations of the *rf1* Nuclear Fertility Restorer of T-Cytoplasm Maize Alter the Accumulation of T-*urf13* Mitochondrial Transcripts

Roger P. Wise,*,†,‡ Carren L. Dill†,‡ and Patrick S. Schnable‡,§

**Field Crops Research, USDA-Agricultural Research Service, †Department of Plant Pathology, ‡Interdepartmental Genetics Program and §Departments of Agronomy and Zoology and Genetics, Iowa State University, Ames, Iowa 50011*

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ABSTRACT

Dominant alleles of the *rf1* and *rf2* nuclear-encoded fertility restorer genes are necessary for restoration of pollen fertility in T-cytoplasm maize. To further characterize fertility restoration mediated by the *Rf1* allele, 123,500 gametes derived from plants carrying the *Mutator* transposable element family were screened for *rf1*-mutant alleles (*rf1-m*) Four heritable *rf1-m* alleles were recovered from these populations. Three *rf1-m* alleles were derived from the progenitor allele *Rf1-IA153* and one was derived from *Rf1-Ky21*. Cosegregation analysis revealed 5.5- and 2.4-kb *Mu1*-hybridizing *Eco*RI restriction fragments in all of the male-sterile and none of the male-fertile plants in families segregating for *rf1-m3207* and *rf1-m3310*, respectively. Mitochondrial RNA gel blot analyses indicated that all four *rf1-m* alleles in male-sterile plants cosegregated with the altered steady-state accumulation of 1.6- and 0.6-kb T-*urf13* transcripts, demonstrating that these transcripts are *Rf1* dependent. Plants carrying a leaky mutant, *rf1-m7323*, revealed variable levels of *Rf1*-associated, T-*urf13* transcripts and the degree of pollen fertility. The ability to obtain *rf1-m* derivatives from *Rf1* indicates that *Rf1* alleles produce a functional gene product necessary for the accumulation of specific T-*urf13* transcripts in T-cytoplasm maize.

CYTOPLASMIC male sterility (cms) is a maternally inherited trait characterized by the inability to produce functional pollen. Cms is widely distributed in the plant kingdom and has been observed in approximately 140 plant species (HANSON *et al.* 1989). There are three groups of male-sterile cytoplasms in maize; C (Charrua), S (USDA), and T (Texas). These cytoplasms can be classified by the specific nuclear genes that restore pollen fertility to them. Pollen sterility in T cytoplasm has been attributed to the presence of the unique mitochondrial gene, T-*urf13*. T-*urf13* is present only in the mitochondrial genome of maize plants carrying T cytoplasm (DEWEY *et al.* 1986) and is altered by a deletion or frameshift in male-fertile revertants (ROTTMANN *et al.* 1987; WISE *et al.* 1987a; FAURON *et al.* 1990). The 13-kD URF13 polypeptide encoded by T-*urf13* (DEWEY *et al.* 1987; WISE *et al.* 1987b) resides as an oligomer spanning the inner mitochondrial membrane (KORTH *et al.* 1991). The URF13 protein binds host-selective pathotoxins produced by race T of *Cochliobolus heterostrophus* Drechsler [asexual stage *Bipolaris maydis* Nisikado and Miyake] and *Phyllosticta maydis* Arny and Nelson, thereby increasing the susceptibility of maize to disease (DEWEY *et al.* 1988; BRAUN *et al.* 1990; GLAB *et al.* 1990; HUANG *et al.* 1990; VON ALLMEN *et al.* 1991). The molecular mechanism by which URF13 causes pollen sterility is unknown. However, in T cytoplasm a rapid increase in mitochondrial numbers during tapetal development is quickly followed by mitochondrial dysfunction. It has been suggested that this phenomenon initiates early tapetal vacuolation and degeneration, which ultimately leads to pollen abortion (WARMKE and LEE 1977, 1978).

Restoration of pollen fertility in T-cytoplasm maize requires dominant alleles at two complementary nuclear restorer loci, *rf1* and *rf2*. Fertility restoration in T cytoplasm is determined by the genetic constitution of the diploid, sporophytic anther tissue rather than the haploid gametophytic pollen grain (LAUGHNAN and GABAY-LAUGHNAN 1983). Analysis of mitochondrial transcription has revealed an additional 1.6-kb T-*urf13* transcript in T-cytoplasm plants restored to fertility as compared to nonrestored siblings (DEWEY *et al.* 1986; KENNELL *et al.* 1987). It has been hypothesized that the modification of T-*urf13* transcription and the concurrent reduction of the URF13 protein require the action of only *Rf1* and not *Rf2* (DEWEY *et al.* 1987); however, other modifiers also appear to have an effect on T-*urf13* transcription depending on the nuclear background (KENNELL *et al.* 1987; ROCHEFORD *et al.* 1992).

As a further step in dissecting the molecular basis of fertility restoration, we have initiated a program to isolate the genetic components of this system. A series of *rf2-m* alleles were isolated (SCHNABLE and WISE 1994) and used to clone the *Rf2* gene (CUI *et al.* 1996). Here we report the identification of transposon-tagged mutant alleles of the *Rf1* nuclear restorer and their

This manuscript is dedicated to the memory of John Laughnan, who contributed significantly to our understanding of cytoplasmic male sterility and fertility restoration in maize.

Corresponding author: Roger P. Wise, USDA-ARS, Department of Plant Pathology, 351 Bessey Hall, Iowa State University, Ames, IA 50011. E-mail: rpwise@iastate.edu

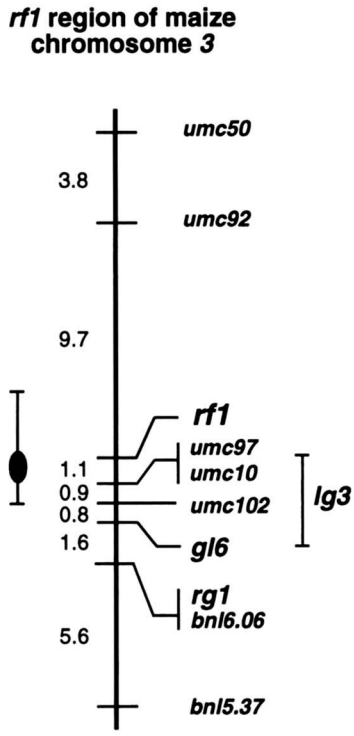

FIGURE 1.—Consensus genetic map of chromosome *3* in the vicinity of the *rf1* locus prepared using the JoinMap program (STAMM 1993) to combine data from seven populations and 729 individuals (WISE and SCHNABLE 1994). The *lg3* locus is positioned between *rf1* and *gl6*.

specific effects on T-*urf13* mitochondrial transcript accumulation.

MATERIALS AND METHODS

Genetics and scoring of male fertility: T-cytoplasm conditions pollen sterility in the absence of dominant alleles at either of two complementary nuclear restorer loci, *rf1* and *rf2*. Unless otherwise noted, plants used in this study were homozygous for *Rf2*. Plants were grown at either the Iowa State University Curtiss Research Farm in Ames, IA (summer season) or at the Hawaiian Research Ltd. facility on Molokai, HI (winter season). Under these environmental conditions, anthers on male-fertile plants exserted, whereas anthers on male-sterile plants usually did not, and were scored as F and S, respectively. However, it should be noted that some of the *rf1-m* alleles exhibited a leaky phenotype, in that plants carrying these alleles sometimes displayed partial fertility. These plants were scored as "S" if a low number of anthers exserted and "F" if a higher number of anthers exserted.

Genetic stocks: The origins and genotypes of many of the genetic stocks and inbred lines used in this study have been described previously (SCHNABLE and WISE 1994; WISE and SCHNABLE 1994). N-cytoplasm inbred lines were obtained as indicated: B37 (our Accession no. 302, Pioneer Hi-Bred, Johnston, IA, C74312), Mo17 (D. ROBERTSON, Iowa State University, 88 8180-3), W64A (Accession no. 142, D. PRING, USDA-ARS and University of Florida), and B73 (Accession no. 660, ROBERTSON). *Mutator* stocks were obtained from D. ROBERTSON (Accession no. 446). T-cytoplasm versions of the inbreds were obtained as indicated: W64A (Accession no. 144, PRING) and B37 (Accession no. 301 Pioneer Hi-Bred, JH86017059+). Testcrosses were used to establish that each of these inbred

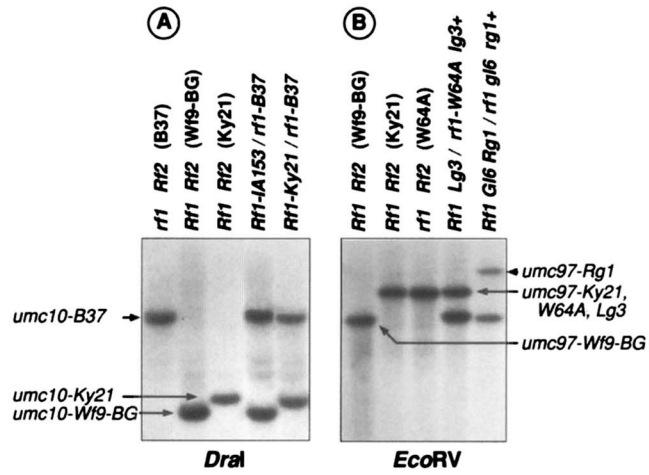

FIGURE 2.—RFLP patterns characteristic of lines used in *rf1* tagging. (A) *umc10/Dra*I. (B) *umc97/Eco*RV. *Rf1-IA153* and *Rf1-Ky21* are the progenitor alleles in the *Mutator* parent in cross 4, and *B37-rf1* is the male parent. In crosses 5, 6, and 7, the *Rf1* allele is in coupling with the *Rg1* and *Lg3* alleles.

lines has the genotype *rf1/rf1, Rf2/Rf2* (unpublished observation). The origin of our *Rg1 Rf1* stock has been described previously (WISE and SCHNABLE 1994). A *rf1 Lg3* stock was obtained from the Maize Genetics Coop (Accession no. 730, Maize Genetics Cooperation Stock Center, University of Illinois, 90 2929/2962-8). The origin of the *Rf1 Lg3* stock derived from Accession no. 730 is described below.

Allele tracking with visible and RFLP markers: In conjunction with our earlier efforts to transposon tag the *rf2* locus, we generated detailed genetic maps of the *rf1*- and *rf2*-containing regions of chromosomes *3* and *9*, respectively (Figure 1, WISE and SCHNABLE 1994). These maps contain both molecular and morphological markers and allow us to (1) map new male-sterile mutations, (2) distinguish transposon-tagged *rf1-m* alleles from the recessive *rf1* allele used to identify the tagged allele in our directed tagging experiment, and (3) facilitate backcrossing programs. We utilized three visible markers closely linked to *rf1* (*lg3, gl6,* and *rg1*) as contamination controls and to track various *rf1* alleles from generation to generation. Seedling leaves on plants homozygous for the *gl6* mutant allele are shiny (or "glossy") (EMERSON *et al.* 1935; SCHNABLE *et al.* 1994). Adult plants carrying the dominant mutant, *Rg1,* have regions of defective tissue between the veins of mature leaves; this results in a "ragged" appearance (BRINK and SENN 1931). The dominant *Lg3* allele conditions an altered ligule; plants carrying *Lg3* have broad upright leaves (H. S. PERRY, 1939, Maize Gen. Coop. News 13:7).

Our *Rf1 Rg1* stock was generated as a recombinant from an *Rf1 rg1+/rf1 Rg1* plant (WISE and SCHNABLE 1994). Hence, although the *Rf1 rg1+* chromosome carried by this plant was derived from the inbred line R213, the *Rf1 Rg1* stock has an *rf1*-linked *umc97*-RFLP marker that is distinguishable from those in coupling with both *Rf1-Ky21* (Ky21 is the source of the *Rf1* allele in R213) and *Rf1-IA153* (Figure 2).

To identify an *rf1* donor line with distinctive RFLP alleles flanking *rf1,* we obtained chromosome *3* RFLP fingerprints of five *rf1* inbreds [Line C (a color-converted version of W22), B37, Mo17, W64A, and B73]. These inbreds were analyzed for RFLPs by digestion with eight restriction endonucleases (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Dra*I, *Bcl*I, and *Bgl*II), followed by Southern blot analyses with RFLP markers that flank *rf1*. The results from this survey established that *rf1-B37* can easily be distinguished from the *Rf1-Ky21* or *Rf1-IA153* alleles present in our *Mutator* population by using the restric-

TABLE 1

Phenotypic ratios associated with the segregation of four *rf1-m* alleles in the progeny of cross 5

rf1-m allele	1993-94g progeny rows[b]	No. of non-ragged plants with the indicated phenotypes[a]				Total no. of plants	Ratio[c]	χ^{2d}
		S	"S"	"F"	F			
rf1-m3207	93g 2288–2292	18	0	1	3	22	19/3	13.76**
rf1-m3310	93g 2298–2302	12	0	0	7[e]	19	12/7	1.32
rf1-m7323	94g 1279–1282	4	5	5	1	15	14/1	11.27**
rf1-m7212	94g 1271–1274	7	5	4	2	18	16/2	10.89**

[a] Data are shown only for nonragged progeny of cross 5. Most ragged progeny were, as expected, male-fertile.
[b] Progeny from cross 5 and segregating for the indicated *rf1-m* allele.
[c] S, "S," or "F"/F.
[d] Chi-squared tested for a 1 S, "S," or "F":1 F ratio. **Highly significantly different from a 1:1 ratio at $P = 0.01$.
[e] These plants are likely the result of pollen contamination in cross 5 (see MATERIALS AND METHODS). The pollen in the mutant screening field was almost exclusively *rf1-B37 Gl6 rg1+* and *Rf1 Gl6 rg1+*. Therefore, some classes of contaminants could yield male-fertile, non-ragged progeny. Genetic crosses conducted with two of these male-fertile, non-ragged progeny involving *rf1-m3310* established that both plants lacked the *gl6* genetic marker. Hence, these male-fertile plants arose via pollen contamination.

tion enzyme *Dra*I in conjunction with the RFLP markers *umc10* and *umc92*. In subsequent generations, the restriction enzyme *Eco*RV was used in conjunction with the RFLP markers *umc97* and *umc50* to differentiate among chromosome segments derived from the *Rf1 Rg1*, *Rf1 Lg3*, and *rf1* (W64A) stocks (Figure 2).

Mapping of *Lg3* and the origin of the *Rf1 Lg3* stock: To effectively utilize the *Lg3* visible marker to track *rf1-m* alleles, we positioned it relative to *Rf1* and chromosome *3* RFLP markers (Figure 1). A single male-fertile, liguleless recombinant (plant 93 3101y-5 with the genotype **T** *Rf1 Lg3 Gl6 Lg2/rf1 lg3+ gl6 lg2*) was obtained from less than 125 progeny from cross 1 and used to develop our *Rf1 Lg3* stock.

Cross 1: **T** *Rf1 lg3+ Gl6 Lg2* (R213)/*rf1 Lg3 Gl6 Lg2* × **N** *rf1 lg3+ gl6 lg2/rf1 lg3+ gl6 lg2*.

Two mapping populations were developed from this stock. In the summer of 1992, LG3 population 1 was generated via cross 2.

Cross 2: **T** *rf1 lg3+ Gl6 Lg2/rf1 lg3+ Gl6 Lg2* (W64A) × **T** *Rf1 Lg3 Gl6 Lg2/rf1 lg3+ gl6 lg2*.

A single male-fertile, nonliguleless recombinant (plant 93g 2310-6) was obtained from less than 100 individuals derived from cross 2. In the summer of 1994, LG3 population 2 was generated via cross 3.

Cross 3: **T** *rf1 lg3+ Gl6 Lg2/rf1 lg3+ Gl6 Lg2* (W64A) × **T** *Rf1 Lg3 gl6 Lg2/rf1 lg3+ Gl6 Lg2*.

A second male-fertile, nonliguleless recombinant (plant 94g 1247-11) was obtained from among less than 190 progeny of cross 3. These two male-fertile, nonliguleless recombinants [in addition to the parental controls and three plants each of male-sterile, nonliguleless and male-fertile, liguleless progeny (from crosses 2 and 3)] were scored for chromosome *3* RFLP markers to position *Lg3* on the map. The recombination event that gave rise to plant 93g 2310-6 occurred between the *umc10* RFLP marker (from *rf1 lg3+ gl6 lg2*) and *Rf1*. The recombination event that gave rise to plant 94g 1247-11 occurred between *umc97* (from W64A) and *Rf1* (from the *Rf1 Lg3 gl6 Lg2* chromosome). These results indicate that the *lg3* locus is "south" of the *rf1* locus. Because the *lg3* locus has been mapped to the short arm of chromosome *3* (Y. CHI, J. FOWLER, and M. FREELING, 1994, Maize Gen. Coop. News. 68:16) and the *gl6* locus is on the long arm (SCHNABLE *et al.* 1994), *lg3* resides between *rf1* and *gl6* (Figure 1).

Creation of T-cytoplasm *Rf1 Rf2 Mutator* stocks: Most maize lines, including standard *Mutator* stocks, carry the N cytoplasm

TABLE 2

Phenotypes and genotypes associated with the segregation of two *rf1-m* alleles in the nonragged progeny of cross 5

rf1-m allele	1994g progeny rows[b]	*Dra*I/*umc10*-associated RFLP[c]	No. of nonragged plants with the indicated phenotypes[a]			Total no. of plants
			S	"S"	"F"	
rf1-m7323	1279–1282	Wf9-BG	0	3	1	4
		B37	2	0	0	2
Total						6
rf1-m7212	1271–1274[a]	Ky21	4	5	1	10
		B37	3	0	3	6
Total						16

[a] Data are shown only for nonragged progeny of cross 5. Most ragged progeny were, as expected, male-fertile.
[b] Progeny from cross 5 and segregating for the indicated *rf1-m* allele.
[c] The *Dra*I/*umc10* RFLPs associated with Wf9-BG and Ky21 indicates the presence of the *rf1-m7323* and *rf1-m7212* alleles, respectively. In contrast, the *Dra*I/*umc10* RFLP associated with B37 indicates the presence of the *rf1-B37* allele. The two RFLPs derived from the male-fertile, ragged *(Rf1 Rg1)* plants from cross 5 are not shown.

FIGURE 3.—Tassel phenotypes of fertile (*Rf1*) and sterile (*rf1-m*) progeny from crosses involving *rf1-m* alleles derived from *Rf1-IA153*. (A) Male fertile, ragged progeny (*Rf1-R213 Rg1*) from cross 6. (B–D) Male-sterile, nonragged progeny from cross 6 representing the *rf1-m3207*, *rf1-m3310*, and *rf1-m7323* alleles, respectively. Genotypes of the plants are indicated below each panel. The *rf1-m* alleles condition a sterile phenotype; however, they may appear leaky and shed some pollen over time (an "S" phenotype) as shown in D. Note the ragged leaves (conditioned by the *Rf1*-linked *Rg1* allele) on the fertile plants in A.

and are homozygous for *rf1* and *Rf2*. *Mutator* stocks (which are homozygous for *rf1* and *Rf2*) were crossed as males on T-cytoplasm Wf9-BG (which is homozygous for *Rf1* and *Rf2*). Subsequent crosses on T-cytoplasm Ky21 (also homozygous for *Rf1* and *Rf2*) or Wf9-BG were used to generate T-cytoplasm *Mutator* stocks that were homozygous for *Rf1* and *Rf2*. The resulting lines were intercrossed and then testcrossed to identify those lines which were homozygous for *Rf1*. *Mutator* activity was monitored throughout the backcrossing program via the ROBERTSON seedling test assay (ROBERTSON 1978, 1980).

DNA isolation, RFLP markers, and DNA gel blot analysis: Total DNA was isolated from fresh or lyophilized maize tissue (immature ears or young leaves) using a modified CTAB extraction (SAGHAI-MAROOF *et al.* 1984). RFLP markers and DNA gel blot analyses were described previously (WISE and SCHNABLE 1994). Probes specific to individual classes of *Mu* transposons (and the plasmids from which they were isolated) were as follows: *Mu1* (pMJ9), *Mu3* (pKD121), *Mu5* (pF280), *Mu7* (pSB9), *Mu8* (p713), and *MuDR* (pMuA2SX) (CHANDLER and HARDEMAN 1992).

Contamination controls: Measures to prevent pollen contamination in cross 5 have been described previously (SCHNABLE and WISE 1994). Briefly, male-sterile progeny from cross 4 were "shootbagged" only after their male-sterile phenotype was recognized. Because of this approach, some of the kernels borne on ears resulting from cross 5 might have arisen via pollen contamination. Since no *Rg1* or *gl6* pollen was present in the field used to screen for *rf1-m* alleles, valid progeny from this cross were identified via the *Rg1* and *gl6* visible markers and distinctive RFLP markers on chromosome *3*. Only progeny that carried the *Rg1* marker were advanced to subsequent generations.

Estimation of population sizes and maintenance of subpopulation pedigrees: The identities of subpopulations from cross 4 were maintained during machine planting. Population sizes in the observation plots were estimated by counting the number of plants in 5–10 rows per plot, averaging the number of plants per row, and multiplying by the total number of rows.

Mitochondrial RNA isolation and RNA gel blot analysis: Mitochondria were isolated from immature ear shoots (30–40 g fresh weight) as described by MCNAY *et al.* (1983) without DNAse or proteinase K. Mitochondria were lysed in 6 M guanidium thiocyanate, 2.0% sarkosyl, followed by phenol-choloroform extraction and centrifugation through a 5.7 M CsCl, 0.1 M Na_2EDTA cushion for 12–16 hr at 32,000 rpm (SAMBROOK *et al.* 1989). Husk or leaf tissue from the same individuals were utilized to prepare DNA for RFLP analysis to establish the genotypes of the plants. Four milligrams of mtRNA were denatured for 1 hr at 50° with glyoxal and dimethyl sulfoxide (DMSO). The denatured RNA was size fractionated through a 1.8% agarose gel (Seakem GTG, FMC, Rockland, ME) with 10 mM $NaHPO_4$, pH 7.0 as the running buffer and transferred to nylon (Hybond N, Amersham). RNA was crosslinked to the nylon membrane with 220 mJ of UV light emitted by 302-nm bulbs in a Stratalinker 2400 (Stratagene, La Jolla, CA).

Plasmid inserts and oligonucleotides were labeled with α-^{32}P-labeled dCTP by the random hexamer method (FEINBERG and VOGELSTEIN 1983) or 3′-end labeling with terminal deoxynucleotidyl transferase (Promega), respectively. Membranes were hybridized for 18–20 hr at 65–67° (55° for oligonucleotides) in 7% SDS, 1% BSA, 1 mM Na_2EDTA, 0.5 M $NaHPO_4$, pH 7.2 (CHURCH and GILBERT 1984). The membranes were washed 1 ×30 min and 1 ×60 min at 65° in 1× SSPE (20× = 3.6 M NaCl, 0.2 M NaH_2PO_4, 20 mM Na_2EDTA, pH 7.4), 20 min at 65° in 0.2× or 0.1× SSPE, and exposed to Kodak X-Omat AR film for 1–5 days at −80° with one or two Lightning Plus (Dupont) intensifying screens.

RESULTS

Isolation of male-sterile mutants of the *rf1* fertility-restorer locus: During the 1993 and 1994 summer seasons, approximately 123,500 progeny derived from a cross between T-cytoplasm *Rf1 Rf2 Mutator* lines (see MATERIALS AND METHODS) and the inbred line B37 (cross 4) were screened for mutations at *rf1* using the approach previously described for isolating *rf2-m* alleles (SCHNABLE and WISE 1994).

Cross 4: **T** *Rf1 rg1+/Rf1 rg1+* (*Mutator*) × **N** *rf1 rg1+/rf1 rg1+* (B37)[1]

In the absence of mutation, progeny from cross 4 will

[1] The plants used in cross 4 and subsequent crosses are homozygous for *Rf2*. Both parents of cross 4 are homozygous for the wild type *rgl* + allele. *rgl* + refers to the normal wild type allele found in most maize lines. The mutant *Rgl* is dominant to *rgl*+. T and N refer to Texas and Normal cytoplasms.

TABLE 3

Phenotypic ratios associated with the segregation of four *rf1-m* alleles in the progeny of cross 6

rf1-m allele	1993-94g plants[b]	1994-95 progeny rows[c]	No. of plants with the indicated phenotypes[a]						
			Sterile, nonragged	Fertile, ragged	Fertile, nonragged	Sterile, ragged	Total no. of plants	χ^{2d}	Homogeneity χ^{2e}
rf1-m3207	93g 2291-1	94 4105-4106	9	11	0	0	20		
	93g 2290-3	94 4101-4104	7	18	0	0	25		
	93g 2290-3	94g1205-1214	47	40	3	2	92		
	94g 1197-1	95 1907	55	43 F 5 "F"	3 F 2 "F"	1 "S"	109		
	94g 1204-1	95 1908	19 S 1 "S"	22	0	3 S 1 "S"	46		
		Total[f]	138	139	8	7	292	0.01^{ns}	5.70^{ns}
rf1-m3310	93g 2299-3	94 4046-4047	13	11	1	0	25		
	93g 2300-1	94 4048-4050	7	16	1	1	25		
	93g 2300-5	94 4111-4112	9	11	0	0	20		
	93g 2300-5	94g1215-1222	30	41	0	0	71		
	94g 1190-4	95 1922	33	45 F 2 "F"	2	2 S 1 "S"	85		
	94g 1196-3	95 1923	42 S 1 "S"	38 F 11 "F"	1	13 S 8 "S"	114		
		Total[f]	135	175	5	25	340	1.18^{ns}	7.71^{ns}
rf1-m7323	94g 1279-10	95 1909	33 S 14 "S"	42 F 1 "F"	6 F 1 "F"	2 S 1 "S"	100		
	94g 1280-6	95 1910	32 S 3 "S"	44	5	1 S 3 "S"	88		
		Total[f]	82	87	12	7	188	0.53^{ns}	0.61^{ns}
rf1-m7212	94g 1271-9	95 1913	3	3	0	0	6[g]	0.0	

[a] For the purposes of these analyses, plants with the "S" phenotype were grouped with the S class, and those with an "F" phenotype with the F class.

[b] Selected male-fertile, ragged progeny from cross 5 carrying *rf1-m* alleles. Genotypes at the *rf1* locus were established using the diagnostic *DraI/umc10* RFLP (see text).

[c] Progeny of cross 6.

[d] Chi-squared tested for a 1 sterile:1 fertile ratio. ns indicates not significantly different from a 1:1 ratio at $P = 0.05$.

[e] Homogeneity chi squared test established that the data could be pooled across years and generations.

[f] Twenty-six seed derived from cross 6 were planted, but only six germinated and gave rise to mature plants.

have the genotype T-cytoplasm *Rf1/rf1-B37* and be male-fertile. However, if the *Rf1* locus was inactivated in a given progeny, that plant would be male-sterile. Ten such male-sterile plants were identified by this screen. Exceptional plants that carried inactivated *rf1* alleles would have the genotype T-cytoplasm *rf1-m/rf1-B37*. A DNA sample isolated from the immature second or third ear from each male-sterile plant was used to obtain an RFLP fingerprint of the alleles in coupling with each putative *rf1-m* allele. It was possible to determine these fingerprints because mutant plants carried the *rf1-m* chromosome heterozygous with the previously fingerprinted B37 chromosome. These fingerprints also identified the progenitor *Rf1* allele (*i.e., Rf1-Ky21,* from Ky21, or *Rf1-IA153,* from Wf9-BG) of each putative *rf1-m* allele. The exceptional male-sterile plants identified in this mutant screen were crossed as females by our *Rg1 Rf1* stock (cross 5).

Cross 5: **T** *rf1-m rg1+/rf1-B37 rg1+* × **T** *Rf1 Rg1/rf1 rg1+*.

If the female parent of a particular cross 5 carried a heritable *rf1-m* allele, all of the nonragged progeny from that cross would be expected to be male-sterile (except for rare crossovers). In contrast, if the female parent did not carry an *rf1-m* allele, then only half of the nonragged progeny would be expected to be male-sterile. Most of the nonragged progeny from cross 5 that were derived from three of the exceptional male-sterile plants isolated from cross 4, were in fact male-sterile, partially sterile, or only partially fertile (Table 1). This result is consistent with the segregation of new *rf1-m* alleles in cross 5. The appearance of partially sterile and partially fertile plants among families derived from cross 5 and segregating for *rf1-m7323* and *rf1-m7212* suggested that these mutants might be leaky (see below).

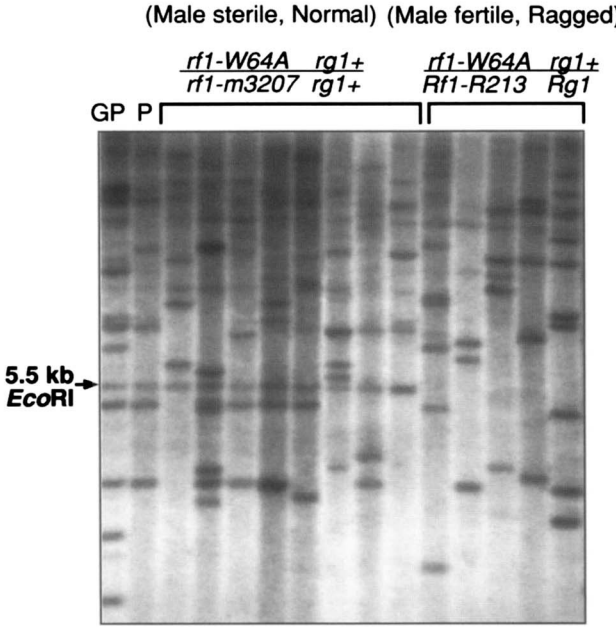

FIGURE 4.—Cosegregation of a 5.5-kb *Eco*RI *Mu1*-hybridizing restriction fragment in a subset of progeny derived from cross 6: **T** *rf1 rg1+/rf1 rg1+* (W64A) × **T** *rf1-m rg1+/Rf1 Rg1*. GP (Grandparent) corresponds to the original progeny of cross 4 carrying the *rf1-m3207* allele. P (Parent) corresponds to the parent carrying the *rf1-m3207* allele used in the cross 5 to generate the segregating population. All 62 male-sterile plants that were analyzed from rows 94 4101–4106 (Table 3) carried the 5.5-kb *Eco*RI *Mu1*-hybridizing fragment, whereas all 38 male-fertile plants from the same families did not.

Based upon the crossing scheme used to generate these mutants, a given *rf1-m* allele would be expected to be in coupling phase linkage with Ky21- or Wf9-BG-derived RFLP markers (which are easily distinguished from those derived from B37). Progeny from cross 5 and segregating for the four putative *rf1-m* alleles were genotyped at the *rf1* locus via the diagnostic *Dra*I/*umc10* RFLP marker (Figure 2). Based on these analyses, it was established that *rf1-m7212* is derived from *Rf1-Ky21* and *rf1-m7323, rf1-m3310* and *rf1-m3207* are derived from *Rf1-IA153,* the *Rf1* allele carried by Wf9-BG.

Two of the putative *rf1-m* alleles (*rf1-m7212* and *rf1-m7323*) were further tested at this generation via RFLP analysis. If a male-sterile mutant is heritable, half of the male-sterile, nonragged plants derived from cross 5 will carry the RFLPs associated with the appropriate progenitor inbred line (Ky21 or Wf9-BG, as indicated above) and half will carry RFLPs associated with B37. In contrast, if a male-sterile mutant is not heritable, the male-sterile, nonragged plants derived from cross 5 would not be expected to carry RFLPs derived from Ky21 or Wf9-BG (except for rare crossovers). Among the six nonragged, male-sterile progeny of cross 5 segregating for *rf1-m7323* that were analyzed, four carried the diagnostic *Dra*I/*umc10* RFLP marker derived from Wf9-BG (Table 2). Similarly, among the 16 nonragged, male-sterile progeny of cross 5 segregating for *rf1-m7212* that were analyzed, 10 carried the diagnostic *Dra*I/*umc10* RFLP marker derived from Ky21 (Table 2). Hence, these results establish that these two mutants were heritable, because plants carrying them and heterozygous with an *rf1* allele, were at least partially male-sterile. In addition, the finding that all of the partially sterile and partially fertile plants within the family segregating for *rf1-m7323,* carried the *rf1-m7323* allele (and all of the fully sterile plants carried the *rf1-B37* allele), suggests that *rf1-m7323* is a leaky mutant. In contrast, within the family segregating for *rf1-m7212,* the incompletely penetrant phenotype is not correlated with a particular *rf1* allele. This result suggests that in this family, the partially fertile phenotype is controlled by a factor not closely linked to the *rf1* locus.

To aid in the further characterization of these four mutant alleles, progeny of cross 5 with the genotype, *rf1-m rg1+/Rf1 Rg1* were identified using the visible and RFLP markers, ragged and *umc10,* respectively, as described above. Plants having this genotype were crossed as males on a T-cytoplasm version of the inbred line, W64A (cross 6) and as females by our *Lg3 Rf1* stock (cross 7). Fertile, liguleless, nonragged progeny from cross 7 were selfed to generate plants carrying homozygous *rf1-m* alleles for T-*urf13* transcript analyses described below (cross 8).

Cross 6: **T** *rf1 rg1+/rf1 rg1+* (W64A) × **T** *rf1-m rg1+/Rf1 Rg1.*

Cross 7: **T** *rf1-m rg1+ lg3+/Rf1 Rg1 lg3+* × **T** *Rf1 rg1+ Lg3/rf1 rg1+ lg3+.*

Cross 8: **T** *rf1-m rg1+ lg3+/Rf1 rg1+ Lg3* selfed.

One to three hundred progeny from cross 6 and involving each putative *rf1-m* allele were scored for visible traits. Heritable male-sterile mutations would be expected to segregate 1:1 (male-sterile:male-fertile). In contrast, rows derived from nonheritable male-sterile "mutants" would consist entirely of male-fertile plants. Among progeny of cross 6 segregating for *rf1-m* alleles, male-sterile plants would be normal, whereas male-fertile siblings would be ragged (except for rare crossovers) (Figure 3). As shown in Table 3, progeny of cross 6 carrying all four of the *rf1-m* alleles segregated approximately 1:1 (male-sterile, nonragged:male-fertile, ragged), and thus, represent heritable mutations (although few data were available for *rf1-m7212,* the RFLP analyses conducted on the progeny of cross 5 provided additional support for this conclusion).

Progeny from cross 6 segregating for *rf1-m3310* displayed more male-sterile, ragged plants than expected when grown in our 1995 summer nursery (but not in our 1994 summer nursery). The 24 unexpected male-sterile, ragged plants observed in the 1995 summer nursery were assayed for their diagnostic *Eco*RV/*umc97* RFLPs. All 24 of these plants displayed an RFLP finger-

TABLE 4

Summary of the *Rf1* progenitor alleles, tassel phenotypes, and T-*urf13* transcript patterns associated with four *rf1-m* alleles

rf1-m allele	*Rf1* progenitor[a]	Tassel phenotype[b]	No. of plants with the indicated phenotypes and genotypes that were assayed for T-*urf13* transcripts		Unique T-*urf13* transcripts in all *rf1-m* plants[e]
			S, "S", or "F" plants carrying *rf1-m* alleles[c]	Fertile plants carrying *Rf1* alleles[d]	
rf1-m3207	*Rf1-IA153*	Tight tassel, very few or no anthers exsert	12	12	−1.6 −0.6
rf1-m3310	*Rf1-IA153*	Tight tassel, very few or no anthers exsert	6	5	−1.6 −0.6
rf1-m7323	*Rf1-IA153*	Leaky, variable numbers of anthers exsert with viable pollen	17	9	±1.6 ±0.6
rf1-m7212	*Rf1-Ky21*	Tassel not as tight as *rf1-3207* or *rf1-m3310*, but no anthers exsert. Segregation for a factor conferring partial fertility, but independent of the *rf1* locus	14	13	−1.6 −0.6 +1.4[f]

[a] *Rf1* alleles were classified based on RFLPs obtained from DNAs digested with *Dra*I and probed with *umc10* (Figures 1 and 2).

[b] Tassel phenotypes are based on two to four generations of observations (see Figure 3).

[c] Selected nonragged or nonliguleless progeny from crosses 5 through 8 with the genotype *rf1-m/rf1+* or *rf1-m/rf1-m* (confirmed by RFLP analysis with the *Dra*I/*umc10* or *Eco*RV/*umc97* marker).

[d] Selected ragged *(Rg1)*- or liguleless *(Lg3)*-progeny from crosses 5 through 8 with *Rf1* alleles (confirmed by RFLP analysis with the *Dra*I/*umc10* or *Eco*RV/*umc97* marker).

[e] A − indicates the absence of, a +, the presence of, and a ±, indicates that that variable accumulation of the designated transcripts is observed in plants carrying *rf1-m* alleles.

[f] Carrying an independent fertility (see Figure 5).

print consistent with an *rf1-W64A rg1+/Rf1 Rg1* genotype. This result indicates that male-sterile phenotype associated with these ragged plants was probably not under the genetic control of the *rf1* locus. Instead, it is thought that certain environmental conditions present in our 1995 summer nursery adversely affected the male fertility of these weaker ragged plants (particularly in family 95 1923).

In the progeny derived from cross 6 and segregating for *rf1-m7323,* 29 of the 94 nonragged plants displayed partial or full fertility, *i.e.,* "S," "F," or F phenotypes (Table 3, Figure 3C). These nonragged plants would be expected to have the genotype *rf1-m7323/rf1-W64A*. Since the *rf1-m7323* allele was shown to be heritable via the RFLP analyses conducted on the progeny of cross 5 as described above, it is likely that most of these phenotypes are conditioned by the leaky *rf1-m7323* allele (as suggested above). To confirm this hypothesis, these plants were assayed for their diagnostic *Eco*RV/*umc97* RFLPs. In 28 of these 29 cases where a partially fertile or fertile phenotype was observed, the diagnostic *Eco*RV/*umc97* RFLP marker indicated the presence of the *rf1-m7323* allele. This result provides further evidence that *rf1-m7323* is a leaky allele.

Cosegregation of male sterility with *Mutator*-specific hybridization probes and identification of a *Mu1* element associated with the *rf1-m3207* and *rf1-m3310* alleles: Because these *rf1-m* alleles were isolated from *Mutator* populations (cross 4), they may be associated with *Mu* element insertions. DNA flanking such *Mu* elements would have a high probability of representing *rf1* sequences. Families resulting from cross 6 segregating for male-sterile, nonragged (*rf1-W64A rg1+/rf1-m rg1+*) and male-fertile, ragged (*rf1-W64A rg1+/Rf1 Rg1*) plants (Table 3) were assayed for diagnostic RFLPs as described above. Approximately equal numbers of DNA samples from male-sterile and male-fertile individuals from these families (and carrying the appropriate RFLPs) were digested with *Hin*dIII and *Eco*RI. Southern blots containing these DNAs were hybridized with *Mu* transposon probes (CHANDLER and HARDEMAN 1992). A 5.5-kb *Mu1*-hybridizing *Eco*RI restriction fragment cosegregated with *rf1-m3207* in 100 progeny from rows 94 4101–4106 derived from cross 6 (Table 3, Figure 4). Likewise, a 2.4-kb *Mu1*-hybridizing *Eco*RI restriction fragment cosegregated in 100 progeny from rows 94 4046–4050, 94 4111–4112, and 94g 1215–1222 segregating for *rf1-m3310.* These results suggest that these

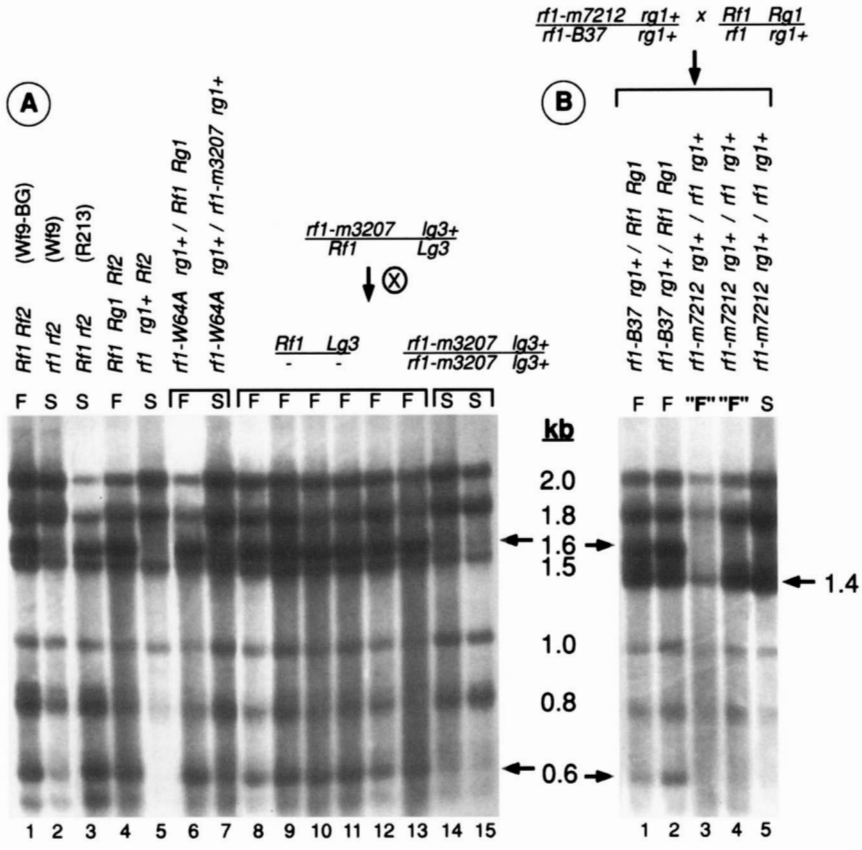

FIGURE 5.—Effect of *rf1-m* alleles on T-*urf13* transcript accumulation. Mitochondrial RNA was denatured with glyoxal and DMSO, size fractionated through 1.8% agarose (Seakem GTG) in 10 mM $Na(H)PO_4$ and transferred to Hybond N (Amersham). The membranes were hybridized with the T-*urf13* specific probe T-st308 (Figure 6A). F, a male-fertile plant; "F," partially fertile; and S, male-sterile. (A) Lane 1: Wf9-BG (*Rf1 Rf2*) carries the *Rf1-IA153* allele, the progenitor of *rf1-m3207*. Lane 2: Wf9 (*rf1 rf2*) carries a recessive *rf1* allele. Lane 3: R213 (*Rf1 rf2*) carries the *Rf1* allele present in the *Rf1 Rg1* (lane 4) and *Rf1 Lg3* stocks. Lane 5: *rf1 rg1+* carries the *rf1* allele that is heterozygous with the *Rf1* allele in the *Rf1 Rg1* stock (see B). Lanes 6 and 7: progeny of plants carrying *rf1-m3207* backcrossed to W64A. Lanes 8–15: selfed progeny of plants carrying *rf1-m3207*. The 1.6- and 0.6-kb T-*urf13* transcripts are significantly reduced in plants carrying *rf1-m3207* (derived from the *Rf1-IA153* allele) regardless of whether the *rf1-m* allele is homozygous or heterozygous with reference *rf1+* alleles. The transcript accumulation patterns in plants carrying *rf1-m3310* appear identical to those carrying *rf1-m3207* (data not shown). (B) Lanes 1 and 2: progeny of plants carrying the *Rf1-R213* allele in our *Rf1 Lg3* stock. Lanes 3–5: progeny of plants carrying the *rf1-m7212* allele. Plants carrying *rf1-m7212* did not accumulate the 1.6- and 0.6-kb T-*urf13* transcripts; however, they did accumulate a 1.4-kb transcript, indicative of the weakly penetrant *Rf8* gene. Some of the *rf1-m7212* plants that accumulate the 1.4-kb transcript are partially fertile (lanes 3 and 4).

two mutations may be due to *Mu* element insertions. To date, a cosegregating restriction fragment has not been identified in progeny from rows 95 1909–1910 carrying *rf1-m7323* or from progeny of crosses 5 and 6 carrying *rf1-m7212* when DNA gel blots were hybridized with sequences corresponding to *Mu1, Mu3, Mu5, Mu7, Mu8,* or *MuDR.*

***rf1-m* alleles alter the accumulation of T-*urf13* mitochondrial transcripts:** It had previously been observed that an additional 1.6-kb T-*urf13* transcript accumulates in restored T-cytoplasm plants (carrying both the *Rf1* and *Rf2* alleles) or plants carrying the *Rf1* allele only. Hence, it is thought that the 1.6-kb transcript accumulation is associated with *Rf1* but not *Rf2* (DEWEY *et al.* 1986, 1987; KENNELL *et al.* 1987). Eleven clones and oligonucleotides, representing 1388 bp of contiguous sequence of the T-*urf13* region (KENNELL *et al.* 1987; WISE *et al.* 1987a), were used as hybridization probes to dissect T-*urf13* transcript accumulation in cms-T plants carrying the newly isolated *rf1-m* alleles as compared to their wild-type siblings. Mitochondrial RNAs (mtRNA) were individually isolated from 89 T-cytoplasm plants derived from crosses 5 through 8, and carrying the *rf1-m3207, rf1-m3310, rf1-m7323, and rf1-m7212* alleles (as determined by RFLP and visible marker analyses). Six to 17 male-sterile and five to 13 male-fertile siblings from each family were assayed by northern blot analysis for the presence of *Rf1*-associated, T-*urf13* transcripts (Table 4). Ten mitochondrial transcripts, ranging in size from 3.9 to 0.6 kb, hybridized to T-st308 (Figure 5A), a 274-bp *Sau*3A-*Taq*I clone internal to T-*urf13* (Figure 6A). Of particular interest to this study are the 2.0-, 1.8-, 1.6-, 1.0-, and 0.6-kb transcripts. Of the 10 T-*urf13* transcripts, the 1.6- and 0.6-kb transcripts were

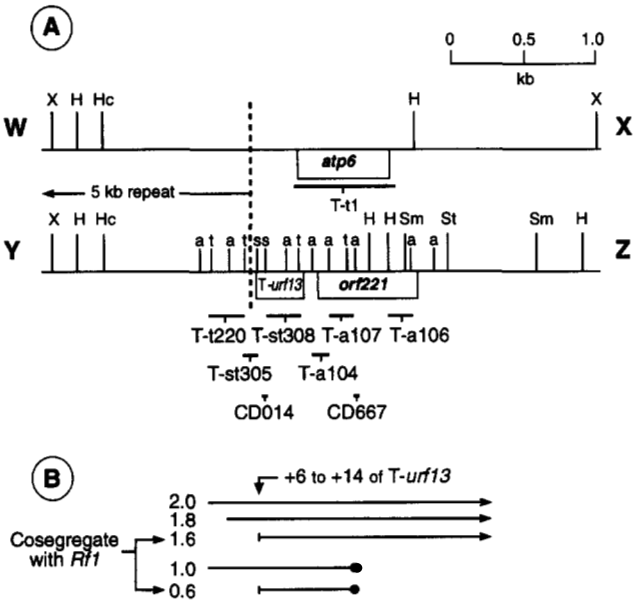

FIGURE 6.—The T-*urf13* region. (A) Map of the T-*urf13* region of T-cytoplasm maize mitochondria (adapted from WISE *et al.* 1987a). The WX configuration, containing *atp6*, and the YZ configuration, carrying T-*urf13* and *orf221*, represent separate subgenomic circles. The T-cytoplasm specific 5-kb repeat carries shared promoter regions for *atp6* and T-*urf13*. Representative restriction sites and the positions of nine of the 12 probes used in T-*urf13* transcript analysis are shown; a, *Alu*I; H, *Hind*III; Hc, *Hin*dI; s, *Sau*3a; Sm, *Sma*I; St, *Sst*II; t, *Taq*I; X, *Xho*I. (B) Model of the major transcripts influencing fertility restoration in T-cytoplasm maize and their processing derivatives. The 1.6-kb transcript is most likely a derivative of the 1.8- or 2.0-kb transcript and the 0.6-kb transcript is most likely a derivative of the 1.0-kb transcript. Both the 0.6- and 1.0-kb transcripts terminate within the T-a107, *orf221*-specific sequence.

absent or much less abundant in plants carrying *rf1-m3207, rf1-m3310, rf1-m7323* (derived from the *Rf1-IA153* allele) or *rf1-m7212* (derived from the *Rf1-Ky21* allele). The accumulation of the 1.6- and 0.6-kb transcripts is reduced regardless of whether the *rf1-m* allele is homozygous or heterozygous with an *rf1+* allele (Figures 5, A and B). All other transcripts are present regardless of the genotype at the *rf1* locus. An 0.8-kb transcript was observed in some plants; however, this transcript did not cosegregate with fertility or *rf1-m* alleles. Transcript patterns similar to those observed with T-st308 were observed when the mtRNA gel blots were hybridized with CD014, a 21-base oligonucleotide complementary to position 1235–1255 of the TURF 2HB sequence, corresponding to the 5′ end of the T-*urf13* reading frame (DEWEY *et al.* 1986).

To further investigate the origin of the 1.6- and 0.6-kb transcripts in families carrying *rf1-m* alleles, 11 overlapping and contiguous probes from T-t220, a 360-bp *Taq*I clone on the 5′ side of T-*urf13*, to T-a106, a 144-bp *Alu*I clone at the 3′ end of *orf221*, were hybridized to gel blots containing the mtRNA samples described above (Figure 6A). As diagrammed in Figure 6B, the 2.0- and 1.8-kb transcripts were detected with all probes from T-t220 to T-a106. In contrast, the 1.6-kb, *Rf1*-specific transcript was detected only with probes from CD014 to T-a106. Likewise, the 1.0-kb transcript hybridized to probes T-t220 through T-a107, but the 0.6-kb, *Rf1*-specific transcript was detected only with probes CD014 through T-a107. The 1.0- and 0.6-kb transcripts are not detected by CD667, an oligonucleotide immediately 3′ to T-a107, and complementary to nucleotides 1961–1990 of the TURF 2HB sequence (Figure 6B; DEWEY *et al.* 1986), indicating that these two transcripts terminate within *orf221*. No differences were observed in mitochondrial transcript accumulation among male-sterile *rf1-m* alleles and their male-fertile siblings when the membranes were hybridized with T-t1, a maize mitochondrial *atp6* clone that shares the same promoter region with T-*urf13* (DEWEY *et al.* 1986; KENNELL and PRING 1989) (data not shown).

Interestingly, the 17 plants carrying *rf1-m7323*, the putative leaky allele, displayed variable levels of steady-state 1.6- and 0.6-kb transcripts. This variability was not strictly correlated with the number of anthers exserted (*i.e.*, S *vs.* "S" *vs.* "F" phenotypes) (Table 5, Figure 7). Four of the five plants that displayed a high level of transcript accumulation (*i.e.*, "+++") exserted anthers; however, seven of the 11 plants that displayed a low level of transcript accumulation (*i.e.*, "+") also exserted anthers.

Unexpectedly, many nonragged progeny from cross 5 that carried the *rf1-m7212* allele were partially male-fertile ("S" or "F," Table 1). These plants did not accumulate the 1.6-kb *Rf1*-associated T-*urf13* transcript but instead accumulated a 1.4-kb transcript (Figure 5B). However, although nonragged plants carrying the *rf1-m7212* allele from cross 6 displayed the same pattern of transcript accumulation, they were male-sterile. Parallel investigations of *Rf8*, a newly described late and partial restorer of T-cytoplasm that is unlinked to *Rf1*, indicate that partial-fertility restoration by this factor is associated with the accumulation of a 1.4-kb T-*urf13* transcript (C. L. DILL, R. P. WISE and P. S. SCHNABLE, unpublished results). The presence of *Rf8*, or a similar genetic element, would account for the nonragged, male-fertile progeny of cross 5 involving *rf1-m7212*.

Independence of the four *rf1-m* alleles: Several lines of evidence suggest that the four *rf1-m* alleles arose independently. First, *rf1-m3207* and *rf1-m3310*, both identified in 1993, can be differentiated by their respective 5.5- and 2.4-kb tightly linked, *Mu1*-hybridizing *Eco*RI restriction fragments. Second, *rf1-m7212* and *rf1-m7323* can be differentiated from the first two since they were isolated from a unique *Mutator*-derived population screened in 1994. Third, *rf1-m7212* can be differentiated from the other three since it was the only mutant identified that had the *Rf1-Ky21* progenitor allele.

TABLE 5

Comparison of male-fertility and 1.6-kb *T-urf13* transcript levels associated with the *rf1-m7323* allele in the progeny of crosses 5 through 8

Relative level of the 1.6-kb *T-urf13* transcript[c]	No. of plants carrying the *rf1-m7323* alele with the indicated phenotypes[a]			No. of plants carrying an *Rf1* allele with a male-fertile phenotype[b]
	S	"S"	"F"	F
+	3	6	1	0
++	0	1	1	0
+++	1	2	2	0
++++	0	0	0	9

[a] Progeny from crosses 5 through 8 and segregating for the *rf1-m7323* allele. These plants were either homozygous for *rf1-m7323* or heterozygous with an *rf1+* allele. Genotypes were established using diagnostic *Dra*I/*umc10* or *Eco*RV/*umc97* RFLP markers.

[b] Progeny from crosses 5 through 8 and containing an *Rf1* allele. The presence of the *Rf1* allele was verified using diagnostic DraI/umc10 or *Eco*RV/*umc97* RFLP markers.

[c] +, the level of hybridizing transcript was similar to those observed for the *rf1-W64A* allele (see Figure 6); ++, a slightly higher than that observed for *rf1+* alleles; +++, a level slightly lower than that observed for *Rf1* alleles; and ++++, a level equal to that observed for an *Rf1* allele.

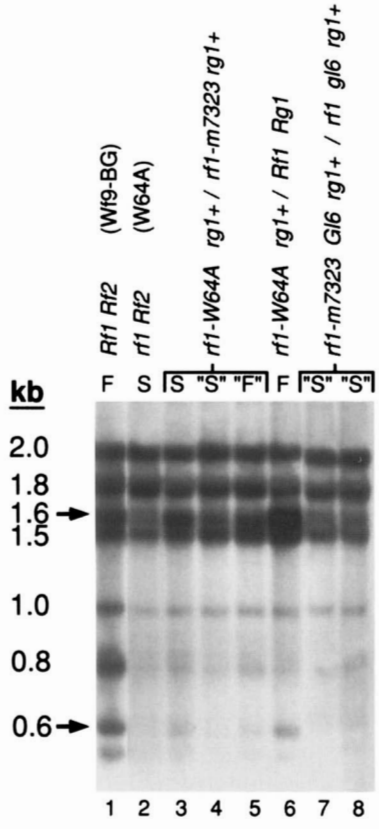

FIGURE 7.—T-*urf13* transcript accumulation from plants carrying *rf1-m7323* alleles. Mitochondrial RNA was denatured with glyoxal and DMSO, size fractionated through 1.8% agarose in 10 mM $Na(H)PO_4$, transferred to Hybond N, and hybridized with T-st308. Lane 1: Wf9-BG (*Rf1 Rf2*) carries the *Rf1-IA153* progenitor allele in *rf1-m7323*. Lane 2: W64A (*rf1 Rf2*) carries the recessive *rf1* allele that is heterozygous with the *rf1-m7323* allele in the progeny of cross 6. Lanes 3–6: progeny of cross 5. Lane 7–8: Progeny from cross 5 carrying the *rf1-m7323* allele that exhibited a "S" phenotype. Note the 1.6- and 0.6-kb T-*urf13* transcripts present in mtRNA isolated from plants carrying an *Rf1* allele.

DISCUSSION

It has been thought that *Rf1* is a functional allele (LEVINGS and BROWN 1989; LEVINGS 1993), even though it is quite rare in the maize gene pool [only seven inbred lines that carry *Rf1* have been identified (GABAY-LAUGHNAN and LAUGHNAN 1993)]. The mutation rate from *Rf1* to *rf1* reported in this study (4/123,500) supports the view that *Rf1* is functional. Given these two observations, the *Rf1* allele could represent a neomorph, encoding a novel gene product functioning in the processing of T-*urf13* transcripts.

Previous reports established that T-cytoplasm inbred lines that carry *Rf1* differ from those that are homozygous for *rf1*, in that the former accumulate 1.6- and 0.6-kb T-*urf13* transcripts and the latter do not (DEWEY *et al.* 1986, KENNELL *et al.* 1987). Although this was taken to suggest that *Rf1* was responsible (directly or indirectly) for the accumulation of these transcripts, it was formally possible that, instead, a locus linked to *Rf1* was responsible. The finding that all four *rf1-m* alleles generated in this study cosegregated with the reduced ability of T-cytoplasm mitochondria to accumulate the *Rf1*-associated, 1.6- and 0.6-kb T-*urf13* transcripts (relative to their *Rf1* progenitor) demonstrates that these transcripts are indeed *Rf1*-dependent.

It has been hypothesized that the accumulation of the 1.6- and 0.6-kb T-*urf13* transcripts are a result of differential RNA processing (DEWEY *et al.* 1986, 1987; KENNELL *et al.* 1987) in that *Rf1*-specific cleavage occurs between nucleotides +6 and +14 of the T-*urf13* open reading frame (DEWEY *et al.* 1987; KENNELL and PRING 1989). One interpretation of the RNA processing model has been that in plants that carry *Rf1*, a larger T-*urf13* transcript is cleaved at a site internal to the open reading frame into the 1.6- and 0.6-kb transcripts. This hypothesis would predict that the 0.6-kb transcript would hybridize to a probe 5′ to the (+6 to +14) pro-

cessing site, since the 1.6-kb transcript hybridizes to all of the probes from CD014 to T-a106. However, analyses of mtRNA from families segregating for four *rf1-m* alleles, have established that the *Rf1*-specific 0.6-kb transcript was not detected when identical membranes were hybridized with T-t220 and T-st305, two probes directly upstream of the (+6 to +14) site of T-*urf13* (data not shown). Moreover, these analyses demonstrated that the 0.6-kb transcript does hybridize with T-a104 and T-a107, two probes on the 3′ side of T-st308. Therefore, it appears likely that the 0.6-kb transcript is a derivative of the 1.0-kb transcript, since both transcripts terminate within T-a107. The observation that both the 1.6- and 0.6-kb transcripts accumulate in concert within individual plants, further suggests that the *Rf1*-encoded gene product functions in the processing of both the 1.8- or 2.0-kb and 1.0-kb substrate RNAs at the (+6 to +14) site of the T-*urf13* reading frame (DEWEY *et al.* 1987; KENNELL and PRING 1989).

An 80% reduction of the 13-kD URF13 protein has been associated with the presence of *Rf1* (FORDE and LEAVER 1980; DEWEY *et al.* 1987). However, a causal relationship between accumulation of *Rf1*-associated T-*urf13* transcripts and the concurrent reduction of the 13-kD URF13 protein has not been established. Based on the observed levels of hybridizing 2.0- and 1.8-kb transcripts, there is no significant reduction in the levels of steady-state T-*urf13* transcripts available for translation. Therefore, the end result of *Rf1* function may involve trans-inhibition of translation by truncated RNAs, and not regulation of transcription, per se.

The ability to follow *rf1-m* alleles (via visible or RFLP markers) in plants segregating for full or partial fertility was invaluable in differentiating genotypic effects attributed to the *rf1* locus, as in *rf1-m7323,* or an independent factor, such as that observed in *rf1-m7212.* The unmasking of additional loci elsewhere in the genome by creating mutations in known restorers has allowed the functional analysis of *rf1* and *rf2.* In summary, four mutant alleles of the *rf1* locus on chromosome *3* were identified utilizing the protocol that was successful for the isolation of complementary *rf2-m* alleles on chromosome *9* (SCHNABLE and WISE 1994). Two notable modifications of the previous protocol were utilized. First, we preselected *rf1/rf1* inbreds that displayed diagnostic RFLPs, allowing the quick elimination of any contaminant male-sterile plants prior to advancing their progeny to future generations. In addition, the *Rg1* and *Lg3* linked-visible markers allowed us to use phenotypic selection to identify those progeny from various crosses with a high probability of having the desired genotypes for subsequent confirmation with RFLPs. This preselection substantially reduced the number of progeny that required RFLP analysis. Subsequent to selection with visible markers, closely linked RFLP markers were invaluable in deciphering genotypes of segregating families carrying *rf1-m* alleles.

In this study, we have demonstrated that *rf1-m* alleles alter the ability of mitochondria to accumulate the 1.6- and 0.6-kb T-*urf13* transcripts, providing rigorous genetic evidence that *Rf1* functions in T-*urf13* mitochondrial RNA processing. At least one other nuclear gene, *Rf8,* has the ability to alter T-*urf13* transcript accumulation (C. L. DILL, R. P. WISE and P. S. SCHNABLE, unpublished results). Neither of these genes can restore pollen fertility in the absence of a dominant *Rf2* allele, even though the *Rf2* allele has no known effect on T-*urf13* transcription or transcript accumulation. Indeed, the *Rf2* restorer gene appears to be an aldehyde dehydrogenase and may interact with the URF13 protein on an indirect level (CUI *et al.* 1996). Further functional analyses of the role of these restorer genes in pollen development and nuclear-cytoplasmic communication will be facilitated by our collection of *rf1-* and *rf2-m* alleles.

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