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Recovery of Heritable, Transposon-Induced, Mutant Alleles of the *rf2* Nuclear Restorer of T-Cytoplasm Maize

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Manuscript received July 20, 1993
Accepted for publication December 2, 1993

ABSTRACT

T (Texas) cytoplasm is associated with a mitochondrial disruption that is phenotypically expressed during microsporogenesis resulting in male sterility. Restoration of pollen fertility in T-cytoplasm maize is controlled by dominant alleles at two unlinked, complementary, nuclear-encoded genes, *rf1* and *rf2*. As a first step in the molecular isolation of the *rf2* gene, 178,300 gametes derived from plants that carried the *Mutator*, *Cy* or *Spm* transposon families were screened for *rf2* mutant alleles (*rf2-m*) via their inability to restore pollen fertility to T-cytoplasm male-sterile maize. Seven heritable *rf2-m* alleles were recovered from these transposon populations. Pedigrees and restriction fragment length polymorphism (RFLP)-based analyses indicated that all seven *rf2-m* alleles were derived independently. The ability to obtain *rf2-m* derivatives from *Rf2* suggests that *Rf2* alleles produce a functional product necessary to restore pollen fertility to cmsT. Molecular markers flanking the *rf1* and *rf2* loci were used to decipher segregation patterns in progenies segregating for the *rf2-m* alleles. These analyses provided preliminary evidence of a weak, third restorer gene of cmsT that can substitute for *Rf1*.

POLLEN sterility in Texas (T)-cytoplasm maize is due, in part, to the disruption of mitochondrial biogenesis by the T-*urf13* mitochondrial gene. T-*urf13* is present only in the mitochondrial genome of T-cytoplasm maize (DEWEY *et al.* 1986; ROTTMANN *et al.* 1987; WISE *et al.* 1987a; FAURON *et al.* 1990) and encodes a 13-kD polypeptide (URF13) (DEWEY *et al.* 1987; WISE *et al.* 1987b), present as an oligomer in the inner mitochondrial membrane (DEWEY *et al.*, 1988; KORTH *et al.* 1991). The 13-kD URF13 protein functions (either directly or indirectly) to increase the sensitivity of maize to fungal pathotoxins (DEWEY *et al.* 1988; GLAB *et al.* 1990; HUANG *et al.* 1990; VON ALLMEN *et al.* 1991) and interferes with pollen development.

Restoration of pollen fertility in T-cytoplasm maize is a function of dominant alleles at two complementary restorer loci, *rf1* and *rf2* (reviewed by LAUGHNAN and GABAY-LAUGHNAN 1983). These nuclear-encoded fertility-restoring genes compensate for the mitochondrial disruption that is phenotypically expressed during microsporogenesis and/or microgametogenesis (DUVICK 1965; BECKETT 1971; GRACEN and GROGAN 1974; LAUGHNAN and GABAY-LAUGHNAN 1983). The restoration of male fertility to T cytoplasm is induced sporophytically, thus the genetic constitution of the diploid, sporophytic anther tissue determines if the pollen is fertile or sterile. (LAUGHNAN and GABAY-LAUGHNAN 1983).

The organization of the T-*urf13* complex is the result of numerous recombination events, one of which has duplicated a 5-kb DNA region 5′ to the *atp6* gene (DEWEY *et al.* 1986; WISE *et al.* 1987a). Five major transcripts of T-*urf13* and the cotranscribed gene *orf221* range between 3.9 and 1.5 kb (DEWEY *et al.* 1986; KENNELL *et al.* 1987; KENNELL and PRING 1989). An additional 1.6-kb T-*urf13* mitochondrial transcript is observed in T-cytoplasm plants that have been restored to fertility. This 1.6-kb transcript may represent an RNA processing derivative of one of the five major transcripts, and its appearance is accompanied by a 70–80% reduction of the URF13 protein. (DEWEY *et al.* 1986; KENNELL *et al.* 1987; KENNELL and PRING 1989). These two phenomena appear to require the action of a dominant allele at only *rf1* and not *rf2* (DEWEY *et al.* 1987). Although the absence of *Rf2* has no visible effect on T-*urf13* transcription, it is also essential for restoration of pollen fertility.

One of our long term objectives is to clone the fertility restorer gene, *rf2*, to aid in our understanding of its role in fertility restoration. As a first step in investigating the function of the *rf2* locus, we initiated a transposon tagging effort. Transposon tagging is now established as one of the most efficient methods for isolating genes for which the gene product is unknown. Because the insertion of a transposon into a gene (tagging) causes a mutation, it is possible to select for newly tagged alleles by screening large populations for newly generated mutant alleles. A critical component is a mutant phenotype like that of *rf2* (male sterility) that can unambiguously and inexpensively be distinguished from the wild type.

Although approximately a dozen transposon systems have been identified in maize (reviewed by PETERSON

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1988), only three have been widely used for tagging and cloning: *Mutator* (*Cy*), *Spm* (or *En*)[1] and *Ac*. In this study we compared the efficiencies of the first two of these systems. *Mutator* lines exhibit a mutation rate 50-fold higher than the spontaneous rate and the rate observed in lines carrying other transposable element systems (ROBERTSON and MASCIA 1981; ROBERTSON 1983). Most new *Mutator*-induced mutants arise via the insertion of *Mu1* transposable elements which are present in 10–50 copies per genome (BARKER *et al.* 1984; BENNETZEN *et al.* 1984; BROWN *et al.* 1989; BENNETZEN 1984), although other *Mu* elements also cause insertion mutations at reasonable rates, *e.g.*, *Mu3* and *Mu8* (OISHI and FREELING 1988; BUCKNER *et al.* 1990; FLEENOR *et al.* 1990). The high transposition rate of *Mu* elements makes the *Mutator* transposable element system an efficient tool for gene tagging and cloning (reviewed by WALBOT 1992).

The *Cy* transposon system has been shown by both genetic (SCHNABLE and PETERSON 1989) and molecular (SCHNABLE *et al.* 1989) criteria to be related to *Mutator*. Although not as widely used for transposon tagging as *Mutator*, mutants from *Cy* populations have facilitated the cloning of several genes (MENSSEN *et al.* 1990; BENSEN *et al.* 1993).

The *Spm* transposon system has also been widely used for gene tagging and cloning (PAZ-ARES *et al.* 1986; CONE *et al.* 1986; WIENAND *et al.* 1986; SCHMIDT *et al.* 1987; SULLIVAN *et al.* 1991). Although the mutation rates observed in *Spm* lines are generally considerably lower than those in *Mutator* lines (CONE *et al.* 1988; ROBERTSON and MASCIA 1981; ROBERTSON 1983), it may prove possible to exploit several genetic characteristics of the *Spm* system to increase the *Spm*-induced mutation rate at target loci. For example, because *Spm* has a marked tendency to engage in intra-chromosomal transpositions (MCCLINTOCK 1962; PETERSON 1970; NOWICK and PETERSON 1981), genetic constructs that place an *Spm* element in the vicinity of the target loci may prove advantageous (NELSON and KLEIN 1984).

As a prelude to cloning the *rf2* locus, we have isolated a collection of *rf2* mutant alleles from three different transposon families (*Mutator*, *Cy* and *Spm*). In this report, we describe the genetic analysis of these mutants and the use of molecular markers to ensure their authenticity. These experiments were also designed to determine whether *rf2* or *Rf2* alleles are functional, *i.e.*, whether *rf2* promotes male sterility or whether *Rf2* suppresses male sterility. Our success in obtaining *rf2* derivatives from *Rf2* alleles strongly suggests the latter. In addition, we present preliminary evidence for a third nuclear restorer gene which can substitute for *Rf1*.

MATERIALS AND METHODS

Genetic markers and nomenclature: Abbreviated genetic/restriction fragment length polymorphism (RFLP) maps of chromosomes *3* and *9* are shown in Figure 1. The *c1*, *sh1*, *bz1* and *wx1* loci condition readily scorable endosperm characteristics (NEUFFER *et al.* 1968) and serve as genetic markers linked to the *rf2* locus. Although the genotypes at these visible marker loci are not listed for all crosses in this report, these markers did aid us in tracking the mutagenized chromosomes over the course of this transposon tagging experiment. The *c1* locus gene product is necessary for anthocyanin pigmentation of the aleurone layer of the maize kernel. The *c1-m5* allele conditions a colorless aleurone with colored spots (MCCLINTOCK 1963). The colorless aleurone phenotype is a consequence of an *Spm* element insertion within this allele (MCCLINTOCK 1963; CONE *et al.* 1986). The colored spots are clonal sectors carrying revertant alleles derived via the excision of *Spm* from *c1-m5*.

Genetic stocks: Most maize lines have the genotype *rf1/rf1 Rf2/Rf2* (DUVICK 1965). Allelism tests have confirmed that our three transposon stocks (*c1-m5*, *Mutator* and *Cy*) match this standard genotype (data not shown). The origin of our *bz1 wx1-m8* stock has been described previously (WISE and SCHNABLE 1994). This stock is homozygous for *Rf2-McC* and segregating for *rf1-McC* and *rf1-LC*. Two inbreds (WF9-BG and Ky21), that carry *Rf1* and *Rf2*, were obtained from D. PRING (USDA-ARS, University of Florida). Although the standard WF9 inbred carries *rf1-WF9* and *rf2-WF9*, the *rf2*

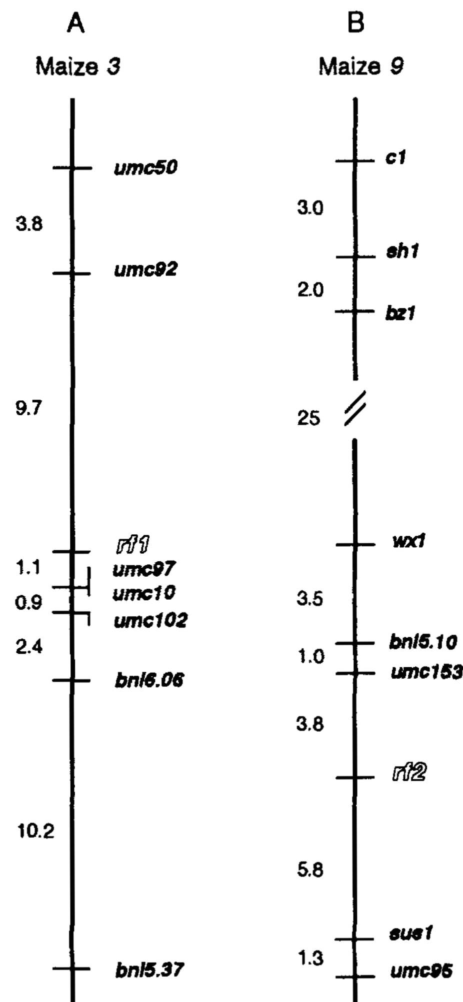

FIGURE 1.—Genetic/RFLP maps in of the regions of chromosomes *3* and *9* that include *rf1* (A) and *rf2* (B). The maps are based on WISE and SCHNABLE (1994).

[1] The terms *Spm* and *En* are synonymous (PETERSON 1965); in this report we use *Spm* because our transposon stocks are derived from those of MCCLINTOCK.

TABLE 1

Origin, size, and mutation rates in transposon populations screened for male-sterile mutations of maize

Population[a]	Subpopulation	Transposon donor source[b]	No. of *rf2-m* alleles isolated	Population size	Mutation rate no./100,000 gametes
Mutator[c]	YA	Mu^4 outcross (1220)	1	8,500	
	P	Mu^2 outcross (1120)	1	12,000	
	G	Mu^1 outcrosses (1212, 1215, 1218, 1219)	3	5,000	
	OB	Mu^2 outcross (1118)	0	12,000	
	B	Mu^2 outcross (1121, 4938)	0	9,700	
	M	*Mu* outcrosses (1207, 1216, 1222, 1224)	0	3,100	
Mutator population total			5	50,300	9.9
Cy[c]	OA/BB	1230–1234, 3919–3921	1	28,000	
Cy population total			1	28,000	3.6
Spm[c]	*Cl'*	Revertants from *c1-m5*	0	20,000	
	cl-m5	"Control"	1	80,000	
Spm population total			1	100,000	1.0

[a] See text for the crosses used to develop the screening populations.
[b] Transposon donor sources are indicated by our pedigree numbers. *Mu* outcross, Mu^2 outcross, and Mu^4 outcross are defined by ROBERTSON (1983).
[c] *Mutator, Cy* and *Spm* populations were derived from crosses 1, 2 and 3, respectively.

reference allele (DUVICK 1965), a T-cytoplasm *Rf1-IA153 Rf2-IA153* version of WF9 (termed in this report, WF9-BG) has been produced by B. GENGENBACH (University of Minnesota) using the inbred line IA153 as an *Rf1 Rf2* donor (personal communication). Ky21 (normal (N) cytoplasm) carries, in addition to *Rf1-Ky21* and *Rf2-Ky21*, *Wc*, a dominant endosperm marker we used as a contamination control.

The inbred line R213 was derived from a cross between WF9 (*rf1, rf2*) and Ky21 (D. N. DUVICK, 1959 *Maize Genetics Coop. Newsl.* **33:** 95) and carries *Rf1-R213* and *rf2-R213*, which based on pedigrees are equivalent to *Rf1-Ky21* and *rf2-WF9*, respectively. T-cytoplasm R213 is therefore male sterile, and was maintained by crossing it by the N-cytoplasm version of R213. N-cytoplasm R213 was maintained by selfing. N- and T-cytoplasm versions of the inbred line, R213, were obtained from M. ALBERTSEN (Pioneer Hybrid) and D. PRING. R213 does not carry the *Wc* allele. Although R213 is generally thought to be homozygous for *Rf1* and *rf2*, crosses of phenotypically identical sublines of R213 to *rf1/rf1* lines have established that some R213 sublines carry *rf1* (data not shown). Because of this heterogeneity in R213, RFLP markers were used to confirm that the male sterility that segregated in crosses involving R213 was indeed the result of the segregation of *rf2* alleles.

Production of the *Mutator*-derived and *Cy*-derived screening populations: Stocks with elevated *Mutator* activity (as assayed by the *Mutator* seedling test, ROBERTSON 1978) were obtained from D. S. ROBERTSON (Iowa State University) and used as shown in cross 1 to develop a population suitable for screening for *Mutator*-induced mutations at *rf2*. The *Mutator* population is composed of six sub-populations each derived from a different *Mutator* source (Table 1). The *Cy* population was developed in a similar manner via cross 2.

Cross 1: **T** *Rf1/Rf1 rf2 wc1/rf2 wc1* (inbred R213) × **N** *rf1/rf1 Rf2 wc1/Rf2 wc1 Mutator.*

Cross 2: **T** *Rf1/Rf1 rf2 wc1/rf2 wc1 (inbred R213)* × **N** *rf1/rf1 Rf2 wc1/Rf2 wc1 Cy.*

Production of the *Spm*-derived screening population: There is some evidence to suggest that mutations occur at a higher rate when the *Spm* donor is used as the male parent as compared to the reciprocal cross (NELSON and KLEIN 1984). Our crossing strategy exploited this potential advantage. Cross 3 was used to isolate *Spm* germinal excision events (revertants) from *c1-m5*. Rare (5%) germinal revertants could be identified because they condition "colored" kernels. R213 is homozygous for *r* and the male parent of cross 3 is homozygous for *R*. Because *r* is epistatic to *C* for aleurone pigmentation (*R/r/r, C/c/c* kernels are mottled, while *R/r/r, c1-m5/c/c* kernels are spotted), classification of spotted *vs.* colored/mottled kernels was more difficult than in *R/R* lines but possible. Approximately 20,000 of these colored/mottled kernels were isolated for use in the tagging program. A population of 80,000 spotted kernels (*c1-m5/c1*) served as a control for the test of whether selection for excision events from *c1-m5* increases the rate of *Spm* insertion in *Rf2*.

Cross 3: **T** *Rf1/Rf1 c Sh Bz1 Wx1 rf2/c Sh Bz1 Wx1 rf2* (inbred R213) × **N** *rf1/rf1 c1-m5 Sh Bz1 wx1-m8 Rf2/c sh Bz1 wx1 Rf2.*

Estimation of population sizes: Population sizes presented in Table 1 were estimated by determining kernel mass from each cross and dividing that value by the average kernel weight for that population. Because these values estimate kernel numbers and germination was close to, but less than 100%, these values overestimate the actual number of plants in the various populations.

Screening the transposon-derived populations: In the absence of mutation, the progeny kernels from crosses 1, 2 and 3 will produce exclusively male-fertile plants because, although they have T cytoplasm, they carry one copy of each of the two dominant nuclear restorer factors, *Rf1* and *Rf2* (LAUGHNAN and GABAY-LAUGHNAN 1983). Because the female parent in each cross (R213) carried T cytoplasm, the progeny resulting from this cross also had T cytoplasm and could therefore express either *rf1/rf1*- or *rf2/rf2*-conditioned male sterility. If the *Rf2* locus were inactivated by a transposon insertion in a given progeny, that plant would be expected to be male sterile. Male-sterile plants could also arise as the result of a mutation of *Rf1* in the female parent of crosses 1–3 (*i.e.*, R213). However, allelism tests (with R213, which is *Rf1 rf2*, and *wx1-m8*, which is *rf1 Rf2*) could often distinguish between these two classes of mutations. In other instances, linkage between the male-sterile mutant and RFLP markers linked to *rf2* were used to confirm allelism with *rf2*. Because the male-sterile plants from the *Mutator* and *Cy* populations were not directly tested for their *rf1* constitution, *rf1-m* alleles would not be recovered; they would be discarded as non-heritable male steriles.

FIGURE 2.—Selection of *rf2-m8904*. Approximately 20,000 plants from the *Spm* population derived from the cross of T cytoplasm R213 by an *Spm* stock (cross 3, see text) are shown near the completion of the screen for male-sterile plants. At the time of this exposure, essentially all male-fertile plants had been detasseled and male-sterile plants (see inset) had been crossed as described in the text (cross 7).

During the summers of 1990 and 1991, the populations resulting from these crosses were screened for mutants that resulted in male sterility. To ensure success in isolating *rf2* mutants from crosses 1–3, large populations of mature plants were screened (approximately 100,000/summer season). To make the screening effort more manageable, two planting dates, two to three weeks apart, were utilized. The identities of subpopulations from crosses 1–3 were maintained during machine planting. Rows were limited to approximately 400 plants, thereby making it easier to locate previously identified male-sterile plants for pollinating and harvesting purposes. This row length also resulted in increased attention levels by the field crew which was charged with identifying rare male-sterile plants from the screening populations.

The failure of anthers to exert is the phenotype of an *rf2/rf2* (or *rf1/rf1*) plant with T cytoplasm (DUVICK 1965), see Figure 3A. Therefore, plants that exerted their anthers did not carry an *rf2-m* allele and were detasseled. In contrast, plants that failed to exert their anthers putatively carried an *rf2-m* allele and were selected as putative mutants and crossed by one of several stocks homozygous for *Rf2* (Figure 2). To identify these putative mutants, each tassel in a given row was checked daily while plants in that row were flowering. Tassels on which greater than 30% of the flowers had exerted their anthers were clipped off with long-handled loppers, thus eliminating them from further consideration (Figure 2). Plants that had non-shedding, "tight" tassels were classified as putative male-steriles. Ear shoots (which contain the female flowers) on these putative male-sterile plants were covered with a dated shoot bag. The shoot bags prevented the female flowers from being pollinated by stray pollen. On the days following shoot bagging the tassels of putative male-sterile plants were reexamined. If a given plant still appeared to be male sterile by the time its female flowers were ready for pollination, its ear shoot was prepared for pollination ("cut back"). Such prepared plants were marked with a red tag and recorded as pollination candidates. A green tag with the plant's approximate location within its row was placed at the head of the respective row. These tags made it possible to quickly locate male-sterile plants on subsequent days. If a red-tagged plant still appeared to be male sterile the next day, it was pollinated (cross 5) and photographed. If, at any time after pollination, a given plant began to shed, its tassel was cut off and the pollinated ear was removed from the plant. Each summer, the screening took a crew of 4–6 individuals approximately 4–6 weeks. Immature second ears were harvested from each male-sterile plant that was pollinated. DNA isolated from these tissues was used to obtain an RFLP fingerprint of the alleles in coupling with the putative mutant *rf2* allele. It was possible to determine these fingerprints because mutant plants carried the *rf2-m* chromosome heterozygous with the previously fingerprinted R213 chromosome. These RFLP fingerprints served as valuable references for subsequent generations.

Contamination controls: It was not feasible to shoot bag (*i.e.*, protect silks from pollen contamination) the entire population of 178,300 plants prior to flowering. Therefore, putative male-sterile plants were shoot-bagged as soon as their exceptional phenotype was suspected. However, because male-sterile mutant plants could not be identified prior to tassel flowering, some silks on putative mutant plants may have been sib contaminated before they were shoot bagged. As will be shown below (crosses 5 and 7), the *Wc* or *bz1* visible markers, or distinctive RFLP markers in the pollen parent, served as contamination controls. (*Wc* and *bz1* were appropriate contamination markers because no plants in the screening plot carried these alleles). In addition, because flowering of the maize ear begins in florets just below the middle of the ear, kernels for propagation purposes were selected from the base or tip of pollinated ears resulting from crosses 5 and 7. Such kernels are more likely to have arisen via the controlled pollination rather than earlier contamination events.

Test for the presence of *Spm*: To ascertain whether a plant carried *Spm*, it was crossed by the *bz1 wx1-m8* line (cross 4). Because the *wx1-m8* allele contains a *dSpm* element (MCCLINTOCK 1961; SCHWARZ-SOMMER *et al.* 1984), it conditions a mutant kernel phenotype. However, because *dSpm* elements are capable of transposition only when a functional *Spm* element is present in the genome, *wx1-m8* serves as a "reporter allele" for the presence of *Spm*; kernels exhibit sectors of wild-type tissue on an otherwise mutant endosperm only when *Spm* is introduced from the female parent of cross 4. A plant was deemed to lack *Spm* if ten out of ten random *wx1/wx1-m8* kernels from the appropriate cross 4 exhibited a stable waxy phenotype. The probability of a false-negative result from this

assay is 0.001 (0.5^{10}). In contrast, if one or more of the tested kernels exhibited wild-type sectors, the female parent of cross 4 was deemed to carry *Spm*.

Cross 4: **T** *wx1 rf2-m (or Wx1 Rf2)/wx1 rf2-m* X **N** *wx1-m8 Rf2/wx1-m8 Rf2.*

DNA isolation and DNA gel blot analysis: Total DNA was isolated from fresh or lyophilized maize tissue (immature second ears or young leaves) using a modified CTAB extraction (SAGHAI-MAROOF *et al.* 1984), and DNA gel blot analysis was carried out as previously described (WISE and SCHNABLE 1994).

Allele tracking: To differentiate among putative mutant alleles and the standard recessive allele (*rf2-R213*) in segregating families, it was necessary to use flanking markers to track the otherwise indistinguishable alleles. For this reason, we have mapped *rf1* and *rf2* relative to a set of linked RFLP and visible markers (WISE and SCHNABLE 1994). In addition to the visible markers on chromosome *9* that were discussed above, RFLP markers *wx1*, *bnl5.10*, *umc153*, *sus1* and *umc95* were used in tracking the *rf2-m* alleles (Figure 1B) and *bnl5.37*, *bnl6.06*, *umc102*, *umc10*, *umc97*, *umc92* and *umc50* were used to track *rf1* alleles (Figure 1A). *rf2* and *rf1* are positioned between *umc153* and *sus1* and between *umc97* and *umc92*, respectively.

Scoring male fertility/sterility: Plants were grown at either the Iowa State University Curtiss Research Farm in Ames, Iowa (summer season) or at the Hawaiian Research Ltd., facility on Molokai, Hawaii (winter season). Tassels on which all florets exerted anthers were classified as male fertile (F). Similarly, tassels on which none of the florets exerted anthers were classified as fully male sterile (S). The F (Figure 3B) and S (Figure 3A) phenotypes are quite distinct, but in some families, partial sterility was observed. Plants exhibiting this phenotype typically exerted a variable number of anthers several days later than their fertile siblings. Plants which exerted only a few anthers were designated "S"; those that exerted more anthers, but still, many fewer than fertile plants, were designated "F." Our designations probably relate to the Roman numeral designations of DUVICK (1956) as follows: S = I, "S" = IIIA; "F" = IIIB; and F = V.

FIGURE 3.—Tassel phenotypes identified in the *rf2* mutant screen (progeny of crosses 1–3, see text). Mutations at *rf2* result in a male-sterile phenotype similar to that observed in Panel A. Most progeny from crosses 1–3 did not carry a mutant *rf2* allele and were therefore fully male fertile (panel B). Two additional classes of male-sterile phenotypes were identified: the open-glume phenotype (panel C) and the basal anther phenotype (panel D). The reference allele of the *rf2* locus (*rf2-Ref*) is equivalent to *rf2-R213*.

RESULTS

Recovery of putative *rf2-m* alleles: Transposon tagging requires that large numbers of progeny be screened for the appropriate mutant phenotype because mutation rates range from 0.1×10^{-5} to 40×10^{-5} (CONE *et al.* 1988; ROBERTSON 1985). Screening these large populations (typically 100,000–1,000,000 plants) for traits such as male sterility, which are expressed only at maturity, is obviously daunting. In an effort to identify an efficient approach to tagging mature plant traits such as male sterility, we compared the tagging efficiency of the *Mutator/Cy* system to that of *Spm*. In the hopes of making the *Spm* system competitive with the *Mutator* and *Cy* transposon systems (which have the highest mutation rates of all maize transposon systems, ROBERTSON and MASCIA 1981) we utilized a transposon donor allele (*c1-m5*) linked to the target gene (*Rf2*) and enriched for transposition events by the selection of germinal revertants from the transposon donor allele (see MATERIALS AND METHODS section).

Plants carrying an active transposable element system (*Spm*, *Cy* or *Mutator*) and homozygous for wild type alleles of the *rf2* locus were crossed as males onto plants carrying T cytoplasm and homozygous for the stable recessive null allele, *rf2-R213*, to expose new mutations at the *rf2* locus (details are provided in the MATERIALS AND METHODS section, crosses 1–3). The progeny from this cross were then screened for the male-sterile phenotype conditioned by *rf2/rf2* (Figure 3A). Rare progeny that exhibited male sterility often carried a newly mutated *rf2* allele (designated *rf2-m*) contributed by the transposon donor parent and the stable recessive null allele from the female parent (*rf2-R213*). However, the male-sterile phenotypes from these screenings can arise via mutation at either *rf1* or *rf2*. These two classes of events were distinguished as described in the MATERIALS AND METHODS.

During the summer of 1990, 20,000 colored kernels (revertants from *c1-m5*) and a control population of 80,000 spotted kernels from the *Spm* population were screened for *rf-m* mutations; seven fully male-sterile plants were identified. During the summer of 1991

approximately 28,000 and 50,000 plants from the *Cy* and *Mutator* populations (respectively) were screened; 36 putative *rf-m* alleles were isolated. The phenotypes of these 36 plants ranged from fully male-sterile plants in which tassel glumes opened but anthers did not exert (open glume phenotype, Figure 3C) to plants that exerted apparently functional anthers at the base of otherwise fully sterile tassels (basal anther phenotype, Figure 3D).

Inheritance of male-sterile phenotypes isolated from the *Cy* and *Mutator* populations: Each of the 36 male-sterile plants isolated from crosses 1 and 2 represented a putative *Mutator*-induced *rf2-m* allele. These putative mutant alleles are designated *"rf2-m"* as opposed to confirmed mutants which are designated *rf2-m*. The male-sterile plants from crosses 1 and 2 were crossed (Cross 5) by stocks homozygous for *Rf1* and *Rf2* (Ky21, WF9-BG or the F_1 hybrid of these inbreds). In crosses involving Ky21 or its F_1 hybrid, *Wc1* served as a contamination marker. *Wc1* is a dominant marker that conditions a white cap on otherwise yellow kernels. In those instances where the *Wc1* contamination marker was not available (*i.e.*, when the male parent was WF9-BG), RFLP markers were used to rule out contamination.

Cross 5: **T** *Rf1-R213/rf1 "rf2-m" wc1/rf2-R213 wc1* × **N** *Rf1/Rf1 Rf2 Wc1/Rf2 Wc1* (inbred Ky21, inbred WF9-BG or their F_1 hybrid).

When available, white capped kernels from cross 5 were sent to our 91/92 winter nursery; otherwise, yellow kernels were sent. As expected, the progeny of cross 5 were male fertile. However, because the female parents in cross 5 carried two *rf1* alleles (*Rf1-R213* and *rf1*) and two *rf2* alleles (*"rf2-m"* and *rf2-R213*), the plants within a single family from cross 5 were of four possible genotypes. It was often possible to distinguish these genotypes by the use of linked RFLPs. The *rf2* locus is flanked by RFLP markers *umc153* and *sus1* (Figure 1). Based upon the crossing strategy, the *"rf2-m"* allele is "marked" by being in repulsion to RFLPs at *wx1*, *bnl5.10*, *umc153*, *sus1* and *umc95* that correspond to the inbred line R213. This use of RFLP markers that flank the *rf2* locus avoids the danger of genetic recombination confusing the *rf2-R213* with the *"rf2-m"* alleles in subsequent analyses. Each family from cross 5 was subjected to RFLP analyses using combinations of the five RFLPs linked to *rf2*. Unfortunately, polymorphisms could not be detected between the R213 and *"rf2-m"*-containing chromosomes at all the RFLP loci. For nine of the *"rf2-m"* alleles no polymorphisms could be detected relative to R213 at any of the five RFLP loci even after using at least four restriction enzymes. These mutants were not analyzed further because it was impossible to distinguish the *"rf2-m"* alleles from *rf2-R213* for the inheritance test (see below). RFLP polymorphisms were detected on only one side of *rf2* for seven *"rf2-m"* alleles (*e.g.*, *rf2-m8080* in Table 2). Polymorphisms that flanked *rf2* were detected for the remaining twenty *"rf2-m"* alleles (*e.g.*, *rf2-m8122* in Table 2).

Using R213 and the appropriate male-sterile parent of cross 5 as controls, it was often possible to identify those progeny of cross 5 that did not carry the R213 chromosome *9*, but instead carried the chromosome *9* derived from the appropriate *Mutator* or *Cy* parent of crosses 1 and 2. These plants were crossed by R213 (crosses 6A or 6B). Crosses 6A and 6B differ only in the *rf1* genotype of the female parent and reflect the two possible *rf1* genotypes expected from cross 5.

Cross 6A: **T** *Rf1/Rf1-R213 "rf2-m" wc1R/f2 Wc1* X **N** *Rf1/Rf1 rf2 wc1/rf2 wc1* (inbred line R213).
Cross 6B: **T** *Rf1/rf1 "rf2-m" wc1/Rf2 Wc1* X **N** *Rf1/Rf1 rf2 wc1/rf2 wc1* (inbred line R213).

The progeny from crosses 6A and 6B would be expected to segregate for male-sterile and male-fertile plants in a ratio of 1:1 if an *"rf2-m"* allele were heritable. These inheritance tests were grown during the summer of 1992 in Ames. Families carrying 18 of the 27 tested putative mutants segregated male-sterile plants in one or two independent inheritance tests (Table 2), suggesting that these 18 mutants were heritable. "Independent inheritance tests" refer to crosses (crosses 6A and 6B) involving different sibling progeny plants from cross 5, but that carry the same *"rf2-m"* allele.

For those putative mutants for which only one *rf2*-linked RFLP marker was available, two independent inheritance tests were performed. Such mutants were deemed heritable only if both inheritance tests were positive. A false-positive will therefore result only from two independent crossover events at a rate of the square of the map distance between *rf2* and the RFLP used to track the putative *rf2-m* allele. For example, if the single RFLP marker were *sus1*, which is approximately 6 cM from *Rf2* (Figure 1) the chance of a false-positive in a double inheritance test is only 0.36%. Four mutants (*rf2-m8040*, *rf2-m8049*, *rf2-m8128* and *rf2-m8164*) gave inconsistent inheritance tests (Table 2). The most likely explanation for these inconsistencies is that these four mutants were false-positives. The appearance of male-sterile plants in some families derived from these false-positives could reflect the segregation of an *rf1* allele contributed by *rf1*-containing R213 sublines used as male parents in the corresponding crosses (cross 6B). Alternatively, the positive tests for each allele may have resulted from a crossover that brought *rf2-R213* into coupling with the non-R213 marker. Because of these inconsistencies these four mutants were excluded from further analysis, even though other events consistent with their being valid *rf2-m* alleles (*e.g.*, reversion or methylation) could explain the lack of concordance between the two tests.

TABLE 2

Summary of the analysis of "*rf2-m*" alleles derived from the *Mutator* and *Cy* populations

	Origin		RFLP tracking marker(s)[d]				No. of plants with the indicated male fertility status[g]				χ^2	
"*rf2-m*" allele[a]	Original phenotype[b]	Source subpopulation[c]	Proximal	Distal	1991g Plant[e]	1992 Row[f]	F	"S"	S	Total no. of plants	1:1[h]	1:3[h]
8032	S	YA	*wx1*	*umc95*	6162-7	2104	16	0	9	25	1.96 ns	20.3**
					6162-9	2105	9	2	7	18	0.00 ns	6.00*
8036	S	P	—	*umc95*	6163-1	2106	9	0	18	27	3.00 ns	1.00 ns
					6163-9	2107	15	0	12	27	0.333 ns	1.34 ns
8040	OG	P	*wx1*	*umc95*	6164-2	2108	6	1[i]	0	7	3.57 ns	13.8**
					6164-4	2109	21	0	8	29	5.83*	34.8**
8049	BA	P	—	*umc95*	6165-1	2110	15	0	15	30	0.00 ns	10.0**
					6165-5	2111	27	0	0	27	27.0**	81.0**
8080	BA	P	*wx1*	—	6169-1	2117	19	0	8	27	4.48*	29.6**
					6169-7	2118	11	2	17	30	2.13 ns	2.18 ns
8110	BA	P	—	*umc95*	6173-1	2123	19	3	4	26	5.53*	32.1**
					6173-2	2124	12	0	14	28	0.154 ns	6.21*
8122	S	YA	*umc153*	*sus1*	6174-5	2125	10	0	13	23	0.391 ns	4.19*
					6174-9	2126	5	1	13	19	4.26*	0.018 ns
8128	S	OA	*umc153*	—	6175-1	2127	17	0	10	27	1.82 ns	20.8**
					6175-2	2128	16	0	8	24	2.67 ns	22.2**
					6175-8	2129	24	0	0	24	24.0**	72.0**
8135	S	P	*wx1*	*sus1*	6176-3	2130	22	2	2	26	12.4**	49.3**
					6176-6	2131	18	0	10	28	2.29 ns	23.1**
8164	BA	P	*wx1*	*sus1*	6178-1	2134	25	0	0	25	25.0**	75.0**
					6178-5	2135	16	5	7	28	0.571 ns	15.4**
8181	OG	P	*wx1*	*umc95*	6179-9	2136	19	0	7	26	5.54*	32.0**
9323	S	G	*wx1*	*sus1*	6180-1	2137	9	2	13	25	1.50 ns	2.00 ns
					6180-3	2138	13	0	15	28	0.143 ns	6.85**
9352	S	OB	—	*sus1*	6183-1	2139	10	0	7	17	0.529 ns	10.4**
					6183-5	2140	21	0	9	30	4.80*	32.4**
9358	S	OB	*wx1*	*sus1*	6185-3	2143	19	0	10	29	2.79 ns	25.4**
9363	S	OB	*wx1*	*sus1*	6186-6	2144	19	0	8	27	4.48*	29.6**
9385	S	BB	*wx1*	*umc95*	6188-7	2146	24	1	5	30	10.8**	48.4**
					6188-10	2147	10	0	20	30	3.33 ns	1.11 ns
9390	S	G	*wx 1*	*sus1*	6189-4	2148	18	8	3	29	1.69 ns	21.3**
9437	S	G	*umc153*	*sus1*	6193-8	2152	14	4	9	27	0.037 ns	10.4**
					6193-10	2153	15	1	12	28	0.143 ns	12.2**

[a] "*rf2-m*" alleles that are not indicated in boldface exhibited disconcordant inheritance test results.
[b] S = fully male sterile; OG = open glume phenotype (male sterile with open anther, see Figure 3); BA = basal glume phenotype (male sterile tassel, but with fertile anthers exerted at the tassel base, see Figure 3).
[c] See Table 1 for a description of the subpopulations.
[d] The indicated RFLP markers were used to distinguish among progeny of cross 5 (see text) that carried "*rf2-m*" alleles from those that carried *rf2-R213*. — indicates no test.
[e] Progeny of cross 5 (see text).
[f] Derived from the indicated 1991 g plant via crosses 6A or 6B (see text).
[g] S, "S" and F indicate fully male sterile, partially male sterile and fully male fertile, respectively.
[h] ns, * and ** indicate not significantly, significantly (0.05 level) and highly significantly (0.01 level) different than the indicated ratio of male fertile to male sterile plants, respectively. Fully and partially male-sterile plants were pooled for these analyses.
[i] An "F" plant termed F for the purpose of the inheritance test.

Progeny of some of the plants that expressed novel male-sterile phenotypes, *i.e.*, the open glume (*rf2-m8040*) and basal anther (*rf2-m8080* and *rf2-m8110*, Figure 3) phenotypes, segregated male-sterile plants in the inheritance test. However, in none of these cases was the open-glume or basal anther phenotype observed in the inheritance test families, *i.e.*, these families segregated fully sterile tassels. The basal anther phenotype is likely the result of genetic modifiers because all plants observed with this phenotype arose in a single *Mutator* subpopulation (P). This phenotype may also be influenced by environmental conditions, *e.g.*, the basal portion of the tassel may experience higher levels of humidity at critical developmental stages because of its sheltered position within the uppermost leaves during the late stages of tassel emergence.

Analysis of *Mutator*- and *Cy*- derived male-sterile phenotypes not associated with *rf2*: The accepted model for the restoration of fertility to male-sterile T-cytoplasm maize (LAUGHNAN and GABAY-LAUGHNAN 1983) would predict that the female parents of crosses 6A and 6B whose progeny included male-sterile plants must have carried *rf2-m* alleles. However, because we have found that some R213 sublines carry *rf1*, RFLP markers linked to *rf2* were used to confirm that the male-sterile phenotypes segregating in the families derived from crosses 6A

TABLE 3

Correlation between male sterility and six *Mutator*-derived *rf2-m* alleles

rf2-m allele	1992 progeny row(s)[a]	Fertility status	No. of plants in the indicated progeny row with the indicated genotypes[b] *rf2-m/rf2-R213*	*Rf2/rf2-R213*
8110	2123	S	3	0
		"S"	3	0
		F	0	5
8122	2126	S	13	0
		"S"	1	0
		F	0	5
9323	2137, 2138	S	27	1
		"F"	1	1
		F	0	10
9385	2146	S	5	0
		"F"	1	0
		F	1	4
9385	2147	S	11	9
		F	1	4
9390	2148	S	3	0
		"S", "F"	6	2
		F	2	3
9437	2152, 2153	S	13	0
		"F"	3	1
		F	0	5

[a] Progeny of cross 6A or 6B (see text).
[b] All *rf2* genotypes were deduced using the *wx1* RFLP marker, with the exception of plants in progeny row 92 2126, where *umc153* was used.

and 6B were associated with *rf2-m* alleles. If the male sterility that segregated in a family was the result of the segregation of an *rf2-m* allele, almost all male-sterile plants derived from Cross 6A or 6B would be expected to carry the *rf2-m* allele. In a preliminary survey, three to nine male-sterile plants were analyzed for each mutant. Male sterility did not exhibit straightforward association with "*rf2-m*" alleles in families derived from eight (8032, 8036, 8080, 8135, 8181, 9352, 9358 and 9363) of the 14 "*rf2-m*" mutants (data not shown). In each instance, at least two male-sterile plants did not carry the *rf2-m* allele. Therefore, sterility in these families was not a consequence of the segregation of an *rf2-m* allele and probably reflects the segregation of an *rf1* allele contributed by an *rf1*-bearing R213 subline in cross 6B.

Analysis of *Mutator*- and *Cy*-derived *rf2-m* alleles: In all but one family from crosses 6A and 6B that carried six mutants (8110, 8122, 9323, 9385, 9390 and 9437), a plant's fertility status was highly correlated with its *rf2* genotype (Table 3, Figure 4); in most instances fully and partially male-sterile plants carried the *rf2-m* allele, while fertile siblings carried the *Rf2* allele. Rare exceptions to this rule be explained by crossovers between the RFLP markers and *rf2*. These six mutants therefore represent heritable *rf2-m* alleles.

Even in the exceptional family (92 2147) in which male sterility was not highly correlated with *rf2* genotypes, only one *rf2-m/rf2-R213* plant was male-fertile. This is consis-

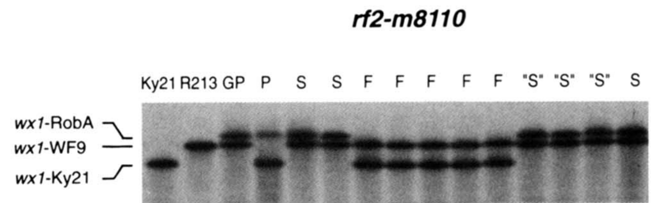

FIGURE 4.—In the progeny of the cross: *rf2-m8110/Rf2-Ky21* × *rf2-R213/rf2-R213* (R213) (cross 6B, see text), the *wx1* allele in coupling with *rf2-m8110* (*wx1-RobA*) co-segregated with full and partial male sterility, thereby confirming that this mutant is heritable. Ky21 and R213 represent inbred lines used in these analyses and are the sources of the *Rf2-Ky21* and *rf2-R213* alleles, respectively. GP designates the grandparent of the segregating family analyzed with this DNA gel blot. This grandparent was an exceptional male-sterile progeny of a cross between T cytoplasm R213 and a *Mutator* stock (cross 1, see text) and carried the *wx1-RobA* and *wx1-R213* alleles. P designates the parent of the segregating family under analysis. This plant was derived from the cross of the GP by Ky21 (cross 5, see text). As expected, it carried the *wx1-Rob* and *wx1-Ky21* alleles. S, F and "S" designate fully sterile, fully fertile and partially sterile progeny of cross 6B, respectively.

tent with the conclusion that the putative mutant carried in this family (*rf2-m9385*) is heritable. However, unexpectedly, many of the *Rf2/rf2-R213* plants in 92 2147 were male sterile. It is possible that the unexpected male sterility of *Rf2/rf2-R213* plants in this family is the result of the segregation of an *rf1* allele contributed by the particular R213 subline used in the corresponding cross 6A.

Three of the ten families resulting from cross 6A or 6B and carrying one of the six *rf2-m* alleles exhibited statistically significant deviations from the expected 1:1 ratio of male-sterile:male-fertile plants (Table 2). One of these deviations was the result of an excess of male-sterile plants. In this instance (family 91 2126 which carried *rf2-m8122*, Table 2), RFLP analyses have established that the excess of male-steriles can be explained by the enhanced transmission of the *rf2-m* chromosome relative to the *Rf2* chromosome (Table 3). In the other two instances (family 91 2123, which carried *rf2-m8110* and family 91 2146, which carried *rf2-m9385*), the deviations from 1:1 ratios were the result of a shortage of male-sterile plants (Table 2). However, in both cases, the paired inheritance test (cross 6A or 6B carrying the same mutant allele, but derived from a different female parent from cross 5) did not exhibit significant deviations from 1:1. This demonstrates that the reduced rates of transmission of the *rf2-m* bearing chromosomes are not characteristics of the *rf2-m8110* and *rf2-m9385* alleles, but are peculiar to the affected families.

Inheritance of male-sterile phenotypes isolated from the *Spm* population: All male-sterile plants from the *Spm* population (progeny of cross 3) were crossed as females as shown in cross 7.

Cross 7: **T** *Rf1-R213/rf1 Bz1 wx1 "rf2-m"/Bz1 Wx1 rf2-R213* × **N** *rf1/rf1 bz1 wx1 Rf2-McC/bz1 wx1-m8 Rf2-McC.*

The progeny from cross 7 were grown in our 1990/1991 winter nursery in Hawaii. Male-fertile progeny from cross 7 that did not carry the R213 alleles of the five RFLPs in the vicinity of *Rf2* (*wx1*, *bnl5.10*, *umc153*, *sus1* and *umc95*) were selected. As discussed above, this procedure avoids the danger of genetic recombination confusing the *rf2-R213* allele with the putative newly generated *rf2-m* alleles. Flanking polymorphisms were detected for each of the *Spm*-induced *rf2-m* alleles; however, in several instances it was necessary to use different restriction enzymes for each marker. Progeny that did not carry *rf2-R213* carried either *rf2-m* (if a putative mutant was heritable) or *Rf2* (if a putative mutant was not heritable).

As described in the section on the analysis of the *Mutator*-induced *rf2-m* alleles, the ideal test to distinguish between these possible genotypes (*rf2-m/Rf2 vs. Rf2/Rf2*) would involve testcrossing male-fertile progeny from cross 7 that carried the putative *rf2-m* allele (as indicated by RFLP markers) to R213 (*Rf1*, *rf2*). However, R213 did not flower in synchrony with the *rf2-m* lines in our 1990/1991 winter nursery. The male-fertile plants from cross 7 that carried *rf2-m* (as determined by RFLP analysis) were instead selfed (crosses 8A-D). In addition to segregating for *rf2*, the progeny of cross 7 also segregated for *rf1* (*rf1/Rf1 vs. rf1/rf1*). In the 1990/1991 winter nursery, some of the *rf1/rf1* plants (which were identified via RFLP analysis) were unexpectedly male fertile and could therefore be selfed.

Crosses 8A–D reflect the diversities of *rf1* and *rf2* genotypes present among the progeny of cross 7 that were selfed to test the inheritance of the "*rf2-m*" alleles. The plants involved in crosses 8A and 8C carried a mutant *rf2* allele (*i.e.*, *rf2-m/Rf2*), while plants involved in crosses 8B and 8D did not (*i.e.*, they are *Rf2/Rf2*). Hence, crosses 8B and 8D represent instances in which the exceptional male-sterile phenotypes observed among the progeny of cross 3 did not result from heritable mutations at *rf2*. Plants in Crosses 8A and 8B were *Rf1/rf1*, while those in crosses 8C and 8D were *rf1/rf1*.

If the *Rf1/rf1* male-fertile plants carried a genuine *rf2-m* allele, the resulting progenies of cross 8A would be expected to segregate 9:7 (male fertile:sterile). This ratio arises due to the independent segregation of the two restorer factors (*Rf1* and *Rf2*) in these crosses. A nonheritable event would be indicated if a 3:1 segregation were noted (segregation of only *rf1* in cross 8B). The existing genetic model for the restoration of cmsT would predict that all progeny from crosses 8C and 8D would be male sterile; this was not observed (see below). For each mutant we grew one to three selfed families. Each selfed family contained at least 52 and up to 135 plants.

Cross 8A: **T** *Rf1/rf1 Bz1 Wx1 rf2-mb/z1 wx1-m8 Rf2-McC* selfed.

Cross 8B: **T** *Rf1/rf1 Bz1 Wx1 Rf2/bz1 wx1-m8 Rf2-McC* selfed.

Cross 8C: **T** *rf1/rf1 Bz1 Wx1 rf2-m/bz1 wx1-m8 Rf2-McC* selfed.

Cross 8D: **T** *rf1/rf1 Bz1 Wx1 Rf2/bz1 wx1-m8 Rf2-McC* selfed.

The recovery of bronze (*bz1/bz1*) kernels from crosses 8A–D for six of the seven putative mutants ruled out contamination in cross 7 for those six putative mutants. No non-contaminant progeny of "*rf2-m*"*8974* were recovered. Because of the inherent difficulties in analyzing such contaminant progeny, this mutant was not analyzed further.

All but one of the 267 individuals in two families derived from selfs of *rf1/rf1* plants carrying "*rf2-m*"*8703* (cross 8C or 8D) were fully or partially sterile (Table 4), but the inheritance of this putative mutant was not further tested.

Crosses 8A–D and 9 and 10 established that four of the putative *rf2* mutants (8960, 8892, 8975 and 8713) were not heritable. One of the families carrying "*rf2-m*"*8960* and derived from the self of an *Rf1/rf1* plant (cross 8A or 8B; rows 91 2153–2154) displayed a segregation ratio not significantly different than 9:7 (Table 4). However, RFLP analyses conducted on this family failed to demonstrate co-segregation between male sterility and homozygosity of this putative *rf2-m* allele (Table 5). Hence, "*rf2-m*"*8960* is not a heritable *rf2-m* allele. The segregation ratios observed in some progeny tests of cross 8A or 8B that carried 8892 (rows 91 2178–2180) or 8975 (rows 91 2164–2166) were close to, or not significantly different from, 3:1 (Table 4). Therefore, the male-sterile phenotypes associated with these two putative mutants in the observation plot were probably not due to mutations at *rf2*. Based on the results from cross 8C or 8D, it was not possible to draw conclusions as to whether "*rf2-m*"*8713* was heritable, but testcrosses (crosses 9–10) and RFLP analyses (data not shown) established that this male-sterile phenotype was not heritable.

The observed ratios of male-fertile to male-sterile plants in both families derived from cross 8A that carried *rf2-m8904* were not significantly different from the expected 9:7 ratio (Table 4). If these ratios were the result of the independent segregation of *rf1* and a newly generated *rf2-m* allele, plants homozygous for either the *umc97* allele from the *wx1-m8* line in cross 7 (*i.e.*, in coupling with *rf1*) or the *umc153* allele derived from the *Spm* parent of cross 3 (*i.e.*, in coupling with *rf2-m*) would be expected to be male sterile. RFLP analyses of 97 plants from these two families established that sterility co-segregated with *rf1* and *rf2-m*, *i.e.*, plants that were homozygous for either recessive allele were generally male sterile. The observed fertility status of 86 of these 97 plants was consistent with their RFLP genotypes (Table 5). This is the expected result if the 9:7 ratios reflect the independent segregation of *rf1* and *rf2* in

TABLE 4

Summary of the analysis of "*rf2-m*" alleles from the *Spm* population

"*rf2-m*" alleles	1990 g plant[a]	Genotype of 1990 g plant[b]	1991 progeny rows[c]	No. of plants with the indicated male fertility status[d]			Total number of plants	χ^2	
				F	"S"	S		3:1[e]	9:7[e]
8703	1138-5	*rf1-LC/rf1-McC* "*rf2-m*"/*Rf2-McC*	2150–2152	0	5	108	113	—	—
	1138-6	*rf1-LC/rf1-McC* "*rf2-m*"/*Rf2-McC*	2167–2169	1	15	118	134	—	—
8713	1139-1	*rf1-LC/rf1-McC* "*rf2-m*"/*Rf2-McC*	2170–2171	16	24	50	90	165**	57**
	1139-4	*rf1-LC/rf1-McC* "*rf2-m*"/*Rf2-McC*	2172–2174	23	23	64	110	171**	55**
	1139-5	*rf1-LC/rf1-McC* "*rf2-m*"/*Rf2-McC*	2175–2177	9	14	106	129	318**	127**
8892	1132-1	*Rf1-R213/rf1-LC* "*rf2-m*"/*Rf2-McC*	2178–2180	95	0	17	112	5.8*	37.1**
8904	1263-4	*Rf1-R213/rf1-McC* "*rf2-m*"/*Rf2-McC*	2181–2183	61	3	51	115	29.5	0.48 ns
	1263-5	*Rf1-R213/rf1-McC* "*rf2-m*"/*Rf2-McC*	2184–2186	74	0	45	119	10.4	1.7 ns
8960	1137-2	*Rf1-R213/rf1-McC* "*rf2-m*"/*Rf2-McC*	2153–2154	41	1	28	70	10.1	0.153 n
	1137-4	*rf1-LC/rf1-McC* "*rf2-m*"/*Rf2-McC*	2155–2156	12	10	30	52	74.7	23
8975	1133-4	*rf1-LC/rf1-McC* "*rf2-m*"/*Rf2-McC*	2161–2163	30	28	72	135	186	58
	1259-7	*Rf1-R213/rf1-McC* "*rf2-m*"/*Rf2-McC*	2164–2166	80	1	22	103	0.39 ns	19

[a] Progeny of cross 7 (see text). All 1990 g plants were male fertile in Hawaii and carried the *bz1* contamination marker which was recovered via crosses 8A–D (see text).
[b] Genotypes were established using various RFLP markers flanking *rf1* and *rf2*.
[c] Progeny of crosses 8A–D (see text) involving the indicated 1990 g plant.
[d] S, "S" and F designate fully sterile, partially sterile and fully fertile plants, respectively.
[e] ns, * and ** indicate not significantly, significantly (0.05 level) and highly significantly (0.01 level) different than the indicated ratio of male fertile to male sterile plants, respectively. Fully and partially male-sterile plants were pooled for these analyses.

TABLE 5

Test of the correlation between male sterility and two *Spm*-derived *rf2-m* alleles

Genotype[a]	Predicted fertility status[b]	Observed fertility status			
		rf2-m8904 progeny rows 91 2181, 2182, 2184		*rf2-m8960* progeny rows 91 2153, 2154	
		F	S	F	S
Rf1/- Rf2/-	F	48[c]	5	20	3
Rf1/- rf2-m/rf2-m	S	5	15	9	2
rf1/rf1 Rf2/-	S	1	12	5	14
rf1/rf1 rf2-m/rf2-m	S	0	11	0	6

Analyses were performed on progenies from the cross *rf1/Rf1* "*rf2-m*"/*Rf2* selfed (crosses 8A or 8B, see text), which segregated 9:7 for male fertility *vs.* male sterility.
[a] *rf1* and *rf2* genotypes were established using RFLP probes *umc97* and *umc153*, respectively. *Rf1/-* and *Rf2/-* designate *Rf1/Rf1* or *Rf1/rf1* and *Rf2/Rf2* or *Rf2/rf2-m*, respectively.
[b] Based on genotypes.
[c] No. of plants with the indicated fertility status.

Cross 8A. Most significantly, 15 of the 20 plants that were deduced to carry *Rf1* and to be homozygous for *rf2-m*, were male sterile. Therefore this 9:7 ratio established that *rf2-m8904* is heritable. The five exceptional male-fertile, *rf2-m8904/rf2-m8904* plants in these families will be discussed below.

The inheritance of 8904 was further tested by crossing progeny from cross 8A that were homozygous for the *rf2-*

TABLE 6

Results from the second inheritance test of *rf2-m8904*

1992 rows[a]	No. of plants with the indicated male fertility status[b]		Total no. of plants	χ^2	
	No. F	No. S		1:1[c]	1:3[c]
2173	13	16	29	0.310 ns	6.08*
2174	10	13	23	0.391 ns	4.19*
2175	6	21	27	8.33**	0.111 ns
2176	10	16	26	1.39 ns	2.51 ns
2177	7	20	27	6.26**	0.012 ns
2178	7	19	26	5.54**	0.051 ns

[a] Progeny of cross 10 (see text).
[b] S and F designate fully male-sterile and male-fertile plants, respectively.
[c] ns, * and ** indicate not significantly, significantly (0.05 level) and highly significantly (0.01 level) different than the indicated ratio of male fertile to male-sterile plants, respectively. Fully and partially male-sterile plants were pooled for these analyses.

m8904 chromosome by the *wx1-m8* line (cross 9). Progeny from cross 9 were testcrossed by R213 (cross 10).

Cross 9: **T** *"rf2-m"/"rf2-m"* × **N** *rf1/rf1 wx1-m8 Rf2/wx1-m8 Rf2.*

The *Rf2* genotypes assigned to the female parents of cross 9 were determined based upon their RFLP configurations using *umc153*. The *rf1* genotypes of the female parents of cross 9 were established using the RFLP probe *umc97*. However, because the apparent *rf1* genotypes varied, they are not presented here for simplicity's sake.

Cross 10: **T** *"rf2-m"/Rf2* × **N** *Rf1/Rf1 rf2/rf2* (inbred R213).

The female parents in cross 10 were either *rf1/rf1* or *rf1/Rf1*.

All families derived from Cross 10 and carrying a heritable *rf2-m* allele would be expected to segregate 1:1 for male sterility *vs.* male fertility in each independent inheritance test. A non-heritable mutant would be expected to yield cross 10 families that were all male fertile. The segregation of male sterility in all six progeny tests from cross 10 confirmed that 8904 is heritable (Table 6). However, the segregation ratios of male-fertile and male-sterile plants in three of these progeny tests (92 2175, 92 2177, 92 2178) were significantly different from 1:1, but not significantly different from 1:3. This result could reflect the inadvertent use of an R213 subline that carried *rf1* as the male parent in the corresponding cross 10.

Test for the presence of an *Spm* insertion in *rf2-m8904*: RFLP analyses were used to identify plants from segregating families (derived from cross 8A) that carried *rf2-m8904* (91 2181–2184). Such plants were crossed by a *wx1-m8* line as shown in cross 4. As described in the MATERIALS AND METHODS section, this cross assays for the presence of genetically active *Spm* elements. None of the nine tested plants that carried *rf2-m8904* contained *Spm* elements (data not shown). It can therefore be concluded that this *Spm*-derived *rf2-m* allele does not contain an *Spm* insert. Instead, it contains a *dSpm* element, contains some other insertion, or arose via a mechanism other than transposon insertion.

Reversion of *rf2-m8904*: As discussed above, five plants from cross 8 and homozygous for *rf2–8904* (as deduced from RFLP analysis) were unexpectedly male fertile in cross 8A. This lack of correlation between the observed fertility status and the deduced *rf1* and *rf2* genotypes could have been the result of either crossovers between the *umc153* (the RFLP marker used to deduce *rf2* genotypes) and *rf2* or alternatively could have been the result of reversion of *rf2-m* to an *Rf2'* allele. To distinguish between these two possibilities, the genotypes at *umc95* (which lies on the opposite side of *rf2* as does *umc153*) of three of the five male-fertile plants that were assumed to carry *Rf1* and to be homozygous for *rf2-m* based on their *umc153* genotypes were determined. If these discordant plants carried recombinant chromosomes they would be expected to carry the *umc153* allele derived from the *Spm* population (the male parent of cross 3), *Rf2*, and the *umc95* allele derived from *wx1-m8*. In fact, one of the three plants (91 2182–24) had this genotype. Therefore, the unexpected male fertility of this plant was probably the result of a crossover between *umc153* and *rf2*. However, the two remaining plants (91 2181–37 and 91 2184–33) were homozygous for the *umc153* and *umc95* alleles derived from the *Spm* population. Although these chromosomes could have arisen via double crossovers between *umc153* and *rf2-m* and between *rf2-m* and *umc95*, such double crossovers are expected to occur very rarely based on the close linkage between *umc153* and *umc95* (Figure 1B). Hence, it is likely that plants 91 2181–37 and 91 2184–33 carried revertant alleles (*Rf2'*) that arose from *rf2-m*, perhaps via excision of a transposon inserted in *rf2-m8904*. Because the parents of 91 2181–37 and 91 2184–33 did not carry *Spm* (see above), if these reversions were caused by excision of a transposon, this transposon is not an *Spm* or *dSpm* element.

Test for independent origins of *rf2-m* alleles: In developing our transposon populations (crosses 1–3) the transposon donor was invariably the pollen parent. If a transposon insertion at *Rf2* occurred early enough in tassel development in one of these transposon donor pollen parents, multiple pollen grains could be produced that carried the identical transposon-induced allele (a tassel sector). The potential therefore exists that not all recovered male-sterile plants were of independent origin. Of course, male-sterile plants recovered from different transposon populations (or subpopulations) must be of independent origin. To establish the independent origins of the *rf2-m* alleles within each subpopulation an extensive RFLP analysis was conducted

TABLE 7

RFLP fingerprinting of *rf2-m* chromosomes

rf2-m allele	Subpopulation	RFLP alleles at specified loci[a] *bnl5.10*	*umc153*	*sus1*	*umc95*
9385	OA (*Cy*)	A	A	C	E
8122	YA (*Mutator*)	A	D	B	B
8110	P (*Mutator*)	B	B	D	C
9323	G (*Mutator*)	A	D	B	B
9390	G (*Mutator*)	B	B	D	C
9437	G (*Mutator*)	A	F	B	F

[a] Alleles were distinguished by analysis with four restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Hin*dIII). Letters are arbitrary codes to represent distinctive alleles on the *rf2-m* chromosomes.

in the vicinity of each *rf2-m* allele. In the absence of genetic recombination, two *rf2-m* alleles that reside on chromosomes with different RFLP alleles must be of independent origin. To increase the sensitivity of the analysis, total genomic DNA preparations carrying each of the *rf2-m* chromosomes from the *Mutator* and *Cy* populations were subjected to digestion with four restriction enzymes and hybridization with four probes that reveal RFLPs in the vicinity of the *rf2* locus (Table 7). This level of analysis is appropriate because two alleles that are indistinguishable with a single enzyme can often be differentiated using a battery of enzymes. In addition, chromosomes that are indistinguishable at one or two loci can often be differentiated when more loci are examined.

Three of the six *Mutator/Cy*-induced *rf2-m* alleles (9385, 8122 and 8110) arose in separate subpopulations (Table 7). These mutants are therefore each of independent origin. The remaining three *Mutator*-induced *rf2-m* alleles (9323, 9390 and 9437) all arose in subpopulation G. However, each of the three corresponding *rf2-m*-containing chromosomes is distinguishable from the others (Table 7). This result demonstrates that in the absence of recombination in the transposon donor parents of cross 1, each of these three alleles is also of independent origin.

Comparison of tagging protocols: The mutation rates observed in each of the transposon tagging populations are shown in Table 1. It should be noted that the mutation rates in the transposon populations may be somewhat underestimated because nine putative mutants from the *Mutator/Cy* population and two from the *Spm* population were not tested for their inheritance. However, given this small potential bias, the *Mutator* population had approximately double the mutation rate of the *Cy* population (3.6 *vs.* 9.9 mutants per 100,000 gametes, Table 1), this difference is not statistically significant; the nonsignificant homogeneity Chi-square value for the two mutation rates, with one degree of freedom, is 0.52. The two were therefore pooled to yield a mutation rate of 7.7 per 100,000.

In the *Spm* population, the preselection of *Spm* excision events from *c1-m5* (revertants) did not significantly increase the mutation rate over the control *Spm* population (0 *vs.* 1.25 per 100,000 gametes, Table 1). In addition, it is unlikely that the use of an *Spm* element (at *c1-m5*) linked to *Rf2* had any positive effect on the efficiency of the tagging effort, because none of the recovered mutants resulted from the insertion of an *Spm* element (see above).

The pooled mutation rate at *rf2* in the *Mutator/Cy* population was significantly higher than that in the *Spm* population; the significant homogeneity Chi-square value, with one degree of freedom, is 5.2.

Evidence for a third restorer locus: Families derived from 8713 unexpectedly segregated for male fertility. For example, all three inheritance tests of "*rf2-m*"*8713* involved selfs of plants with the genotype *rf1-LC/rf1-McC Rf2/Rf2* as determined by RFLP analyses (cross 8D) and yet each segregated for at least some male-fertile plants. As discussed previously, these *rf1/rf1 Rf2/Rf2* plants were unexpectedly male fertile in the 1990/1991 winter nursery. According to the accepted model for cmsT restoration, all of the resulting selfed progeny from such "escapes" would have been expected to have been male sterile under the hotter drier conditions prevalent in Ames during the summer of 1991, because all the progeny were *rf1/rf1*. However, as noted above, variable proportions of the progeny from these selfs were unexpectedly male fertile or partially male fertile (Table 4). Combining the partially male-fertile plants with the fertile class did not result in either a 3:1 or a 9:7 ratio. RFLP analyses of 38 and 33 individuals from two of these families (91 2170 and 91 2175, respectively) were conducted to determine the association of particular *rf1* and *Rf2* alleles with the unexpected male fertility in these pedigrees. The ability of either *rf1-LC* or *rf1-McC* to function as a weak *Rf1* allele would explain the unexpected male fertility in this pedigree. However, because neither *rf1-LC* nor *rf1-McC* is uniquely required for the unexpected male fertility (*i.e.*, both *rf1-LC* and *rf1-McC* homozygotes exhibited male fertility, Table 8), it appears that the unexpected male fertility is not associated with the *rf1* locus. We therefore hypothesize that a third, previously undescribed, cmsT restorer gene that can substitute for *Rf1*, is segregating in this pedigree. To fit the observed segregation ratios, this third restorer must be variably and weakly penetrant. This model would also explain the aberrant segregation patterns observed in some families carrying 8960 (91 2155–2156, Tables 4 and 6) and 8975 (91 2161–2163, Table 4). The third restorer hypothesis would also be an appropriate explanation for the appearance of the fully or partially male-fertile plants in two families derived from selfs of *rf1/rf1* plants carrying "*rf2-m*"*8703* (cross 8D and Table 4). However, because other models could also explain these results (*e.g.*, both *rf1-McC* and *rf1-LC* could be weak *Rf1* alleles), the existence of this third restorer is currently being confirmed.

TABLE 8

RFLP analysis of families derived from 8713 and 8960 and displaying unexpected male-fertile plants

Genotype[a]		No. of plants with the indicated fertility status[b]								
		rf2-m8713 progeny row 91 2170			*rf2-m8713* progeny rows 91 2175			*rf2-m8960* progeny rows 91 2155		
rf1	rf2	F	"S"	S	F	"S"	S	F	"S"	S
rf1-LC/rf1-LC	*Rf2-McC/Rf2-McC*	0	1	3	0	0	2	0	0	0
	Rf2-McC/"Rf2"[c]	1	1	1	0	0	3	1	0	0
	"Rf2"/"Rf2"	0	0	4	0	0	2	0	1	2
rf1-LC/rf1-McC	*Rf2-McC/Rf2-McC*	2	1	0	0	0	1	2	1	7
	Rf2-McC/"Rf2"	5	3	5	1	2	6	1	1	7
	"Rf2"/"Rf2"	2	2	1	0	0	6	2	0	0
rf1-McC/rf1-McC	*Rf2-McC/Rf2-McC*	0	0	2	0	0	0	1	0	2
	Rf2-McC/"Rf2"	2	1	0	2	3	2	0	1	1
	"Rf2"/"Rf2"	0	2	1	0	1	2	0	2	1

Analyses were performed on progenies from selfed plants (cross 8C or 8D) with the genotype *rf1/rf1 "rf2-m"/Rf2* and which segregated more than 7/16 male-sterile plants.

[a] *rf1* and *rf2* genotypes were established using RFLP probes *umc97* and *umc153*, respectively.

[b] S, "S" and F designate fully male sterile, partially male sterile and fully male fertile plants, respectively.

[c] *"Rf2"* represents the false-positive 8713 and 8960 alleles.

DISCUSSION

Because there is no molecular phenotype associated with the *rf2* locus, it has not been possible to determine whether *Rf2* or *rf2* is the "functional" allele. For example, if the *rf2* locus were haplo-insufficient, *rf2* alleles could actively condition male sterility in T cytoplasm. Under this model, the *Rf2* allele would represent an amorph or hypomorph. However, our success in obtaining recessive mutant derivatives from the *Rf2* allele at normal rates for transposon mutagenesis experiments (1–10 mutants/100,000 gametes), strongly suggests that the *Rf2* allele produces a gene product necessary for fertility restoration in cmsT. This conclusion, in combination with the low frequency with which T cytoplasm is found in maize populations, suggests that the *Rf2* allele produces a gene product that by chance has the ability to interact with the T cytoplasm. Hence, *Rf2*'s functionality could either arise from a novel gene product or via ectopic expression of a "normal" gene product. However, because *Rf2* has no known effect on N-cytoplasm maize, its prevalence in Corn Belt populations remains unexplained.

As a first step toward answering these and other questions related to *Rf2*'s role in restoration of cmsT, we have generated a collection of seven *rf2-m* alleles from two different transposon sources (six mutants from *Mutator/Cy* and one from *Spm*). We are fortunate to have a collection derived from different transposon sources because this diversity is often useful in gene isolation efforts (O'Reilly *et al.* 1985; Menssen *et al.* 1990).

In the process of isolating these *rf2* mutants, we identified a number of factors in the experimental design that may ensure success of similar tagging efforts. Specifically, this report details a mutant screening protocol that would be applicable to isolating mutants at any gene expressed at the time of flowering. By ensuring efficient field layout and record keeping, this protocol makes it possible for a small crew to screen very large populations for putative mutants. The protocol emphasizes the importance of using inbred lines that contain contamination markers as parents in crosses to the putative mutants identified in the screen. The protocol includes the DNA fingerprinting of putative mutants, and thereby facilitates the tracking of mutant alleles through subsequent generations. These fingerprints also make it possible to determine whether two mutants isolated from the same subpopulation represent independent mutational events. However, this approach is not suited to the analysis of mutants derived from inbred transposon donor subpopulations. For example, the *Cy* population was generated by backcrossing a *Cy* line to the inbred line Vebz (or derivatives of this inbred) for over five generations. One would therefore expect most plants in this population to carry the Vebz alleles of most RFLPs. Indeed, four of five chromosome 9s that were analyzed from this subpopulation (OA) were indistinguishable (data not shown).

Thirty-six male-sterile plants were isolated from the *Mutator/Cy* populations. Of these, nine were not analyzed because it was not possible to identify RFLPs flanking the *"rf2"* allele. Of the remaining 27 putative *rf2* mutants, six have been shown to represent heritable *rf2-m* alleles. Two of the seven *"rf2-m"* alleles derived from the *Spm* population were not tested to determine if they were heritable, four were not heritable, and one was heritable.

The mutation rates at *rf2* in the *Mutator/Cy* and *Spm* populations were significantly different (6/78,300 *vs.* 1/100,000, respectively). Because this study included two modifications to the standard *Spm* tagging approach, this finding extends those of earlier studies

which showed that mutation rates are typically substantially higher in *Mutator vs. Spm* populations (ROBERTSON and MASCIA 1981). The modifications used in this study were based on the observations by the MCCLINTOCK and PETERSON laboratories (MCCLINTOCK 1962; PETERSON 1970; NOWICK and PETERSON 1981) that *Spm* preferentially engages in intra-chromosomal transpositions. We therefore reasoned, based on the success of this approach with *Ac*, which also exhibits preferential intra-chromosomal transposition (GREENBLATT and BRINK 1992; VAN SCHAIK and BRINK 1959), that placement of an *Spm* in the vicinity of *Rf2* and selection for *Spm* excisions might increase the rate of *Spm* insertion into *Rf2*. This approach was previously exploited by NELSON and KLEIN (1984) to transposon tag *bz1*. Although the mutation rates in our *c1-m5* revertant and *c1-m5* control populations were not significantly different, the statistical precision of this experiment was such that only relatively large differences in mutation rates would have been observed. Even so, there is no evidence to suggest that the considerable effort involved in preselecting excision events can be justified. Further, the finding that the single *rf2-m* allele from this population (8904) did not have an *Spm* insertion suggests that the use of a linked *Spm* element did not increase the mutation rate at *rf2*. Although *Spm* typically transposes farther from the excision site than does *Ac*, one explanation for the failure of these tagging modifications to improve the mutation rate may be that the *c* locus is too far away from *rf2* (*ca.* 50 cM).

The RFLP markers that have recently been mapped in the vicinity of *rf1* and *rf2* (WISE and SCHNABLE 1994). proved invaluable in deciphering segregation patterns in progenies derived from exceptional male-sterile plants isolated in the tagging screen. The use of these markers allowed us to track distinct alleles of *rf1* and *rf2* from generation to generation. In our previous mapping efforts, we utilized these markers to confirm the complementary roles of *rf1* and *rf2* in restoring male fertility to T-cytoplasm maize. In this study, we extended these analyses by characterizing the behavior *rf1* and *rf2* alleles in lines segregating for putative *Mutator*-, *Cy*-, and *Spm*-derived *rf2-m* alleles. By providing a means to track alleles of *rf1* and *rf2*, the RFLP markers enabled us to determine the *rf1* and *rf2* genotypes of individual progenies of a particular cross. We were thereby able to establish unambiguously in seven instances that male sterility co-segregated with newly generated *rf2-m* alleles. These results confirmed that these seven *rf2-m* alleles were heritable. These analyses also established that the partially male-sterile plants segregating in these families carried the *rf2-m* alleles (Table 4 and Figure 4).

In addition, by using RFLP markers linked to *rf1* and *rf2* to analyze segregation patterns in segregating families, we have obtained preliminary evidence for a weak third restorer gene of T cytoplasm. Under the models developed here to explain the aberrant segregation ratios that appeared in inheritance tests of several of the *Spm*-induced male steriles, this third restorer can partially substitute for *Rf1* in *rf1/rf1* plants. Although previous reports have described weak alleles of *rf1* that can restorer T cytoplasm imperfectly (D. N. DUVICK, 1966 *Maize Genetics Coop. Newsl.* **40:** 122–123), this putative weak restorer is not allelic to *rf1*. Hence, this weak restorer may represent one of the "modifiers" of fertility restoration that has previously been postulated to explain the partial restoration that occurs in some genetic backgrounds (DUVICK 1956).

The authors wish to recognize the efforts of our 1990 and 1991 summer crews who helped screen the transposon populations for male-sterile plants: BERNADETTE HACKBART, SHANE HEINEN, ANTONY HISZCZYNSKYJ, JODI JANS, UTE RODE and KARIN GOBELMAN-WERNER. KARIN GOBELMAN-WERNER (assisted by DARREN GRUIS) also provided technical assistance with the RFLP-based allele tracking. ANDY SMITH and TSUI-JUNG WEN provided technical assistance in the genetics nursery and seed laboratory. We appreciate the generosity of the maize research community for sharing genetic stocks and thank DON DUVICK for helpful discussions. We wish to thank JOHN IMSANDE and KEITH SCHERTZ for their comments on the manuscript. ROBBIE KERKOVE provided secretarial assistance. This research was supported by U.S. Department of Agriculture (USDA)-National Research Initiative/Competitive Grants Program grants GAM 9001136 and AMD 9201761 and a competitive research grant from Pioneer Hi-Bred International. This manuscript is a joint contribution from the Iowa Agriculture and Home Economics Experiment Station and the Field Crops Research Unit, USDA-Agricultural Research Service and represents Journal Paper No. J-15465 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa (Project Nos. 2447 and 3152).

LITERATURE CITED

BARKER, R. F., D. V. TALBOT, D. R. SWANSON and J. L. BENNETZEN, 1984 Nucleotide sequence of the maize transposable element *Mu1*. Nucleic Acids Res. **12:** 5955–5967.

BECKETT, J. B., 1971 Classification of male-sterile cytoplasms in maize (*Zea mays* L.). Crop Sci. **11:** 721–726

BENNETZEN, J., 1984 Transposable element *Mu1* is found in multiple copies in Robertson's *Mutator* maize lines. J. Mol. Appl. Genet. **2:** 519–524.

BENNETZEN, J. L., J. SWANSON, W. C. TAYOR and M. FREELING, 1984 DNA insertion in the first intron of maize *Adh1* affects message levels: Cloning of progenitor and mutant *Adh1* alleles. Proc. Natl. Acad. Sci. USA **81:** 4125–4128.

BENSEN, G. JOHAL, P. CLOSE, P. SCHNABLE and S. BRIGGS, 1993 Cloning gibberellic acid biosynthetic genes from maize. Plant Physiol. **102** (Suppl.): 8.

BROWN, W. E., D. S. ROBERTSON and J. L. BENNETZEN, 1989 Molecular analysis of multiple *Mutator*-derived alleles of the *Bronze* locus of maize. Genetics **122:** 439–445.

BUCKNER, B., T. L. KELSON and D. S. ROBERTSON, 1990 Cloning of the *y1* locus of maize, a gene involved in the biosynthesis of carotenoids. Plant Cell **2:** 867–876.

CONE, K. C., F. A. BURR and B. BURR, 1986 Molecular analysis of the maize anthocyanin regulatory locus *C1*. Proc. Natl. Acad. Sci. USA **83:** 9631–9636.

CONE, K. C., R. J. SCHMIDT, B. BURR and F. A. BURR, 1988 Advantages and limitations of using *Spm* as a transposon tag, pp. 149–160 in *Plant Transposable Elements*, edited by O. NELSON. Plenum Press, New York.

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:** 292–295.

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:** 439–449.

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.

DUVICK, D. N., 1956 Allelism and comparative genetics of fertility restoration of cytoplasmically pollen sterile maize. Genetics **41:** 544–565.

DUVICK, D. N., 1965 Cytoplasmic pollen sterility in corn. Adv. Genet. **13:** 1–56.

FAURON, C. M.-R., M. HAVLIK and R. I. S. BRETTEL, 1990 The mitochondrial genome organization of a maize fertile cmsT revertant line is generated through recombination between two sets of repeats. Genetics **124:** 423–428.

FLEENOR, D., M. SPEEL, D. ROBERTSON and S. WESSLER, 1990 Nucleic acid sequence of the maize *Mutator* element, *Mu8*. Nucl. Acid. Res. **18:** 6725.

GLAB, N., R. P. WISE, D. R. PRING, C. JACQ and P. SLONIMSKI, 1990 Expression in *S. cerevisiae* of a gene associated with cytoplasmic male sterility from maize: respiratory dysfunction and uncoupling of yeast mitochondria. Mol. Gen. Genet. **223:** 24–32.

GRACEN, V. E., and C. O. GROGAN, 1974 Diversity and suitability for hybrid production of different sources of cytoplasmic male sterility in maize. Agron. J. **65:** 417–421.

GREENBLATT, I. M., and R. A. BRINK, 1962 Twin mutations in medium variegated maize. Genetics **47:** 489–501.

HUANG, J., S-H LEE, C. LIN, R. MEDICE, E. HACK *et al.*, 1990 Expression in yeast of the T-URF13 protein from Texas male-sterile maize mitochondria confers sensitivity to methomyl and to Texas-cytoplasm-specific fungal toxins. EMBO J. **9:** 339–247.

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.

KORTH, K. L., C. I. KASPI, J. N. SIEDOW and C. S. LEVINGS III, 1991 URF13, a maize mitochondrial pore-forming protein, is oligomeric and has a mixed orientation in *Escherichia coli* plasma membranes. Proc. Natl. Acad. Sci. USA **88:** 10865–10869.

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

MCCLINTOCK, B., 1961 Further studies of the *Suppressor-Mutator* system of control of gene action of maize. Carnegie Inst. Washington Year Book **60:** 469–476.

MCCLINTOCK, B., 1962 Topographical relations between elements of control systems in maize. Carnegie Inst. Washington Year Book **61:** 448–461.

MCCLINTOCK, B, 1963 Further studies of gene-control systems in maize. Carnegie Inst. Washington Year Book **62:** 486–493.

MENSSEN, A., S. HÖHMANN, W. MARTIN, P. S. SCHNABLE, P. A. PETERSON *et al.*, 1990 The *En/Spm* transposable element of *Zea mays* contains splice sites at the termini generating a novel intron from a *dSpm* element in the *A2* gene. EMBO J. **9:** 3051–3057.

NELSON, O. E., JR., and A. S. KLEIN, 1984 Characterization of an *Spm*-controlled *bronze*-mutable allele in maize. Genetics **106:** 769–779.

NEUFFER, M. G., L. JONES and M. S. ZUBER, 1968 *The Mutants of Maize.* Crop Science Society of America, Madison, Wisc.

NOWICK, E. M., and P. A. PETERSON, 1981 Transposition of the *Enhancer* controlling element system in maize. Mol. Gen. Genet. **183:** 440–448.

OISHI, K., and M. FREELING, 1988 A new *Mu* element from a Robertson's *Mutator* line, pp. 289–292 in *Plant Transposable Elements*, edited by O. NELSON. Plenum Press, New York.

O'REILLY, C. SHEPHERD, N. S. PERIERA, ZS. SCHWARZ-SOMMER, I. BERTRAM *et al.*, 1985 Molecular cloning of the *a1* locus of *Zea mays* using the transposable elements *En* and *Mu1*. EMBO J. **4:** 591–597.

PAZ-ARES, J., U. WIENAND, P. A. PETERSON and H. SAEDLER, 1986 Molecular cloning of the *c* locus of *Zea mays*: a locus regulating the anthocyanin pathway. EMBO J. **5:** 829–833.

PETERSON, P. A., 1965 A relation between the *Spm* and *En* control systems in maize. Am. Nat. **99:** 391–398.

PETERSON, P. A., 1970 The *En* mutable system in maize. III. Transposition associated with mutational events. Theor. Appl. Genet. **40:** 367–377.

PETERSON, P. A., 1988 The mobile element systems in maize, pp. 43–64 in *Plant Transposable Elements,* edited by O. NELSON. Plenum Press, New York.

ROBERTSON, D. S., 1978 Characterization of a mutator system in maize. Mutat. Res. **51:** 21–28.

ROBERTSON, D. S., 1983 A possible does-dependent inactivation of Mutator (*Mu*) in maize. Mol. Gen. Genet. **191:** 86–90.

ROBERTSON, D. S., 1985 Differential activity of the maize mutator *Mu* at different loci and in different cell lineages. Mol. Gen. Genet. **200:** 9–13.

ROBERTSON, D. S., and P. N. MASCIA, 1981 Test of 4 controlling-element systems of maize for mutator activity with *Mu* mutator. Mutat. Res. **84:** 283–289.

ROTTMANN, W. H., T. BREARS, T. P. HODGE 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.

SAGHAI-MAROOF, M. A., K. M. SOLIMAN, R. A. JORGENSEN and R. W. ALLARD, 1984 Ribosomal DNA spacer-length polymorphisms in barley. Proc. Natl. Acad. Sci. USA **81:** 8014–8018.

SCHMIDT, R. J., F. A. BURR and B. BURR, 1987 Transposon tagging and molecular analysis of the maize regulatory locus *opaque2*. Science **238:** 960–963.

SCHNABLE, P. S., and P. A. PETERSON, 1989 Genetic evidence of a relationship between two maize transposable element systems: *Cy* and *Mutator.* Mol. Gen. Genet. **215:** 317–321.

SCHNABLE, P. S., P. A. PETERSON and H. SAEDLER, 1989 The *bz-rcy* allele of the *Cy* transposable element system of *Zea mays* contains a *Mu*-like element insertion. Mol. Gen. Genet. **217:** 459–463.

SCHWARZ-SOMMER, ZS., A. GIERL, R. B. KLOESGEN, U. WIENAND, P. A. PETERSON *et al.*, 1984 The *Spm* (*En*) transposable element controls the excision of a 2-kb insert at the *wx-m8* allele of *Zea mays.* EMBO J. **3:** 1021–1028.

SULLIVAN, T. D., L. I. STRELOW, C. A. ILLINGWORTH, R. L. PHILLIPS and O. E. NELSON, JR., 1991 Analysis of maize *brittle-1* alleles and a defective *Suppressor-Mutator*-induced mutable allele. Plant Cell **3:** 1337–1348.

VAN SCHAIK, T., and R. A. BRINK, 1959 Transpositions of modulator, a component of the variegated pericarp allele in maize. Genetics **44:** 725–738.

VON ALLMEN, J. M., W. H. ROTTMANN, B. G. GENGENBACH, A. J. HARVEY and D. M. LONSDALE, 1991 Transfer of methomyl and *HmT*-toxin sensitivity from *T*-cytoplasm maize to tobacco. Mol. Gen. Genet. **229:** 405–412.

WALBOT, V., 1992 Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertion mutagenesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43:** 49–82.

WIENAND, U., U. WEYDEMANN, U. NIESBACK-KLÖSGEN, P. A. PETERSON and H. SAEDLER, 1986 Molecular cloning of the *c2* locus of *Zea mays*, the gene coding for chalcone synthase. Mol. Gen. Genet. **203:** 202–207.

WISE, R. P., and P. S. SCHNABLE, 1994 Mapping complementary genes in maize: Positioning the *rf1* and *rf2* nuclear-fertility restorer loci of the Texas (T) cytoplasm relative to RFLP and visible markers. Theor. Appl. Genet. (in press).

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

WISE, R. P., A. E. FLISS, D. R. PRING and B. G. GENGENBACH, 1987b *urf13*-T of T-cytoplasm maize mitochondria encodes a 13 kD polypeptide. Plant Mol. Biol. **9:** 121–126.

Communicating editor: M. R. HANSON