Mitochondrial Transcript Processing and Restoration of Male Fertility in T-Cytoplasm Maize

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Cytoplasmic male sterility (CMS) systems have been useful in the production of hybrid seed in a number of crops. The Texas or T-cytoplasmic male-sterile (*cms-T*) system was used extensively in the 1960s to eliminate the need for hand detasseling in hybrid maize production. As a consequence of the 1970 epidemic of southern corn leaf blight, *cms-T* is no longer widely used commercially. However, it has been developed as a model system to study the genetic and molecular mechanisms underlying male sterility and fertility restoration. Male sterility in T-cytoplasm maize results from the action of a T-cytoplasm-specific mitochondrial gene, T-*urf13.* Full (or partial) fertility restoration of T-cytoplasm maize is mediated by the *Rf2* nuclear restorer in combination with one of three other restorers: *Rf1, Rf8,* or *Rf*. Rf2* encodes a protein highly similar to mitochondrial aldehyde dehydrogenases; *Rf1, Rf8,* and *Rf each mediate discrete T-*urf13* mitochondrial transcript processing events. To test the functionality of *Rf1, Rf8,* or *Rf**, a T-cytoplasm transformation system is under development. AFLP bulk-segregant analysis has been used to identify DNA markers closely linked to the *Rf8* locus. These tools will provide a foundation for determining mechanisms of nuclear-directed mitochondrial RNA processing and fertility restoration.**

Cytoplasmic male sterility (CMS) is a maternally inherited inability to produce viable pollen. The CMS trait is useful in the production of hybrid seed because it greatly reduces the cost of hand emasculation. CMS systems are found in more than 150 plant species and are often attributed to chimeric open reading frames in the mitochondrial genome. These open-reading frames encode unique proteins that can interfere with mitochondrial function and pollen development. Nuclear restorer (*Rf*) genes function to suppress the effects of CMS-associated mitochondrial abnormalities on male fertility. In many instances this suppression occurs via *Rf*-gene-dependent differential RNA processing activities. Although the specific molecular mechanisms involved are unknown, this process has been observed in maize, sorghum, oilseed rape, common bean, rice, and petunia (Schnable and Wise 1998).

One of our long-range goals is to characterize the mechanisms by which mitochondrial gene expression is modified by nuclear-encoded factors, specifically how the processing of mitochondrial transcripts can mediate the restoration of male fertility in CMS systems. An ideal model system for these studies is the T cytoplasm of maize (Wise et al. 1998). Male sterility in T-cytoplasm maize is attributed to the presence of the unique mitochondrial gene, T-*urf13* (Levings 1993). The 13 kD URF13 monomers encoded by T-*urf13* are assembled as tetrameric pore-forming structures spanning the inner mitochondrial membrane (Korth et al. 1991; Rhoades et al. 1994). Full suppression of the sterility-inducing effects of T-*urf13* requires the action of two dominant nuclear restorers, *Rf1* and *Rf2.* T cytoplasm is restored sporophytically; the genotype of the diploid, sporophytic anther tissue, rather than the haploid, gametophytic pollen, determines pollen development. Therefore a T-cytoplasm plant that is heterozygous for both restorer loci (*Rf1/rf1, Rf2/rf2*) will produce all fertile pollen even though only one-fourth of the pollen grains carries both *Rf1* and *Rf2* (Laughnan and Gabay-Laughnan 1983).

Although both *Rf1* and *Rf2* are necessary to restore fertility in T cytoplasm, they have very different functions. The recent cloning and sequencing of *Rf2* has revealed that the RF2 amino acid sequence is 75% similar and 60% identical to mammalian aldehyde dehydrogenases (ALDH),

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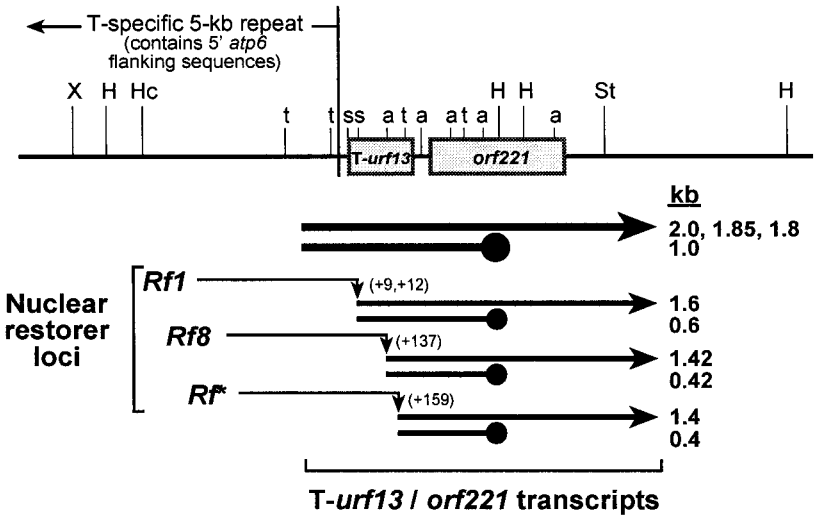

Figure 1. Effect of nuclear *Rf* genes on T-*urf13* mitochondrial transcript processing. The cotranscribed T-*urf13* and *orf221* reading frames most likely utilize a duplication of *atp6* promoter sequences within the 5 kb repeat region. Diagrammed below the chimeric T-*urf13/orf221* transcriptional unit, are novel T-*urf13* transcripts that accumulate in the presence of *Rf1, Rf8,* and *Rf**. Numbers in parentheses indicate 5′ termini (designated by the number of nucleotides 3′ of the start of the T-*urf13* reading frame) of each transcript as determined by primer extension experiments (Dill et al. 1997). To the right of each group of transcripts are their molecular sizes as determined by northern analyses. a, *Alu*I; H, *Hind*III; Hc, *Hinc*II; s, *Sau*3a; St, *Sst*II; t, *Taq*I; X, *Xho*I.

suggesting a possible mechanism for *Rf2*-mediated restoration (Cui et al. 1996). In contrast, the molecular phenotype mediated by *Rf1* and two recently described partial restorers, *Rf8* and *Rf**, are typified by the differential processing of T-*urf13* mitochondrial transcripts and the concurrent reduction of the URF13 CMS-associated protein (Dewey et al. 1986, 1987; Dill et al. 1997; Wise et al. 1996). In this review we describe our recent progress toward characterization of these three nuclear restorers that mediate the processing of T-*urf13* transcripts in maize mitochondria.

Nomenclature

According to the present maize nomenclature, loci and recessive alleles are designated by lowercase symbols, for example, the *rf1* allele of the *rf1* locus is a recessive mutant; dominant alleles are designated by uppercase symbols, for example, the *Rf1* allele of the *rf1* locus is wild type. Lines that carry T cytoplasm (sterile or fertile) are referred to as T-cytoplasm lines. Male-sterile lines that carry T cytoplasm are designated *cms-T.* Restored T-cytoplasm designates lines restored to fertility via the presence of nuclear restorer genes. Except in rare circumstances, N-cytoplasm lines are male fertile.

Overview of the T-*urf13*-Hybridizing Mitochondrial Transcripts

In *cms-T* maize, seven major transcripts of T-*urf13* and the cotranscribed *orf221* range in size between 1.0 and 3.9 kb (Dewey et al. 1986; Dill et al. 1997; Kennell et al. 1987; Kennell and Pring 1989; Wise et al. 1996). Many of these transcripts are products of a series of processing events stemming from the 3.9 kb transcript. Transcript capping experiments with guanylyl transferase identified the one known exception to this hypothesis; the 1.85 kb transcript is a primary (initiated) transcript and hence not the result of RNA processing (Kennell and Pring 1989).

Plants segregating for the *Rf1, Rf8,* or *Rf** restorers accumulate novel T-*urf13* transcripts. Genetic analyses of four *rf1-m* alleles established that *rf1-m* mutants that had lost their ability to restore fertility had also lost their ability to accumulate the *Rf1*-associated 1.6 and 0.6 kb T-*urf13* transcripts (Wise et al. 1996). These analyses provided critical genetic evidence of the role of *Rf1* in mitochondrial RNA processing.

Whereas additional 1.6 and 0.6 kb transcripts accumulate in plants segregating for *Rf1,* additional 1.42 and 0.42 kb transcripts accumulate in the plants segregating for the partial fertility restorer *Rf8,* and additional 1.4 and 0.4 kb transcripts accumulate in the presence of *Rf** (Figure 1; Dill et al. 1997). Physical mapping of these transcripts via northern blot analyses indicates that the *Rf1*-dependent 1.6 kb, the *Rf8*-dependent 1.42 kb, and the *Rf**-dependent 1.4 kb transcripts are all derivatives of the 1.8, 1.85, or 2.0 kb transcripts. Likewise, the smaller 0.6 , 0.42, and 0.4 kb transcripts likely originate from the 1.0 kb T-*urf13* transcript (Figure 1; Dill et al. 1997; Kennell et al. 1987; Kennell and Pring 1989; Rocheford et al. 1992; Wise et al. 1996). A series of primer extension experiments were used to determine the precise 5′ termini of the *Rf1*-, *Rf8*-, and *Rf**-dependent T-*urf13* transcripts. In these experiments, labeled extension products corresponding to nucleotides +9 or +12 of the T-*urf13* reading frame are uniquely associated with the 5′ termini of the 1.6 and 0.6 kb *Rf1*-associated transcripts. In contrast, extension products that correspond to nucleotides +137 and +159 are uniquely associated with the 5′ termini of the 1.42 and 0.42 kb *Rf8*-associated transcripts and the 1.4 and 0.4 kb *Rf**-associated transcripts, respectively (Dill et al. 1997).

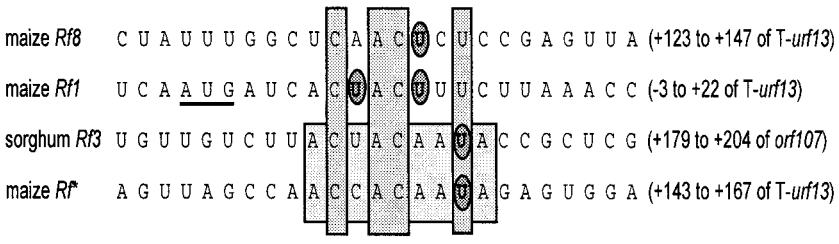

Figure 2. Sequence conservation among restorer-dependent RNA processing sites. The sequence 5′-CNACNNU-3′ overlaps the 5′ termini of the 1.6 and 0.6 kb (*Rf1*-associated), 1.42 and 0.42 kb (*Rf8*-associated), 1.4 and 0.4 kb (*Rf**-associated), and 380-nucleotide (*Rf3*-associated) transcripts from maize and sorghum. Letters highlighted by dark-gray-filled circles represent the 5′ termini of mtRNA transcripts associated with fertility restoration as determined by primer extension experiments (shown in Figure 1). Conservation between the *Rf3*- and *Rf**-associated processing sites [AC(U/C)ACAAUA] is highlighted in the light-gray horizontal boxed area. The translation initiation codon for T-*urf13* is underlined.

Rf-Mediated T-*urf13* Transcript-Processing Sites Exhibit Sequence Similarity

Sequences encompassing the 5′ termini of the *Rf1*-, *Rf8*-, and *Rf**-associated, T-*urf13* mitochondrial transcripts were examined for patterns that might indicate splice-site recognition. As shown in Figure 2, these inspections revealed a small conserved sequence that overlaps a 5′ terminal U of

each of these T-*urf13* transcripts (Dill et al. 1997). Based on this finding, we hypothesize that the *Rf1, Rf8,* and *Rf** restorers encode functionally similar gene products that mediate the specific modification of T-*urf13* transcripts (Figure 1).

Of interest, the highly conserved sequence, 5′-AC(C/U)ACAAUA-3′, overlaps the 5′ terminal U of the *Rf**-associated T-*urf13* transcripts and the *Rf3*-associated *orf107* transcript (Tang et al. 1996), revealing remarkable similarities among these restorer-associated processing sites in maize and sorghum. It is possible that this mitochondrial-sequence represents a recognition site for snRNAs or proteins regulated by the nuclear restorer genes.

Rf-Mediated Processing of T-*urf13* Transcripts is Accompanied by a Reduction in URF13

Rf-mediated processing of CMS-associated mitochondrial transcripts is often concurrent with a reduction in the accumulation of the CMS-associated mitochondrial proteins. Although *Rf2* has no detectable effect on transcript processing or URF13 accumulation in maize (Dewey et al. 1987; Wise RP, Dill CL, and Schnable PS, unpublished data), the abundance of URF13 is reduced by approximately 80% in all tissues examined in plants that possess the *Rf1* restorer (Dewey et al. 1987; Forde and Leaver 1980). *Rf8* can also mediate a reduction in URF13; however, the effect is not as pronounced as that mediated by *Rf1* and may even be organ dependent. For example, although plants that carry *Rf1* exhibit a marked decrease of URF13 accumulation in both tassels and ears, the difference in URF13 accumulation among plants segregating for *Rf8* is much more apparent in young tassels than in ear shoots (Dill et al. 1997).

The molecular mechanism for *Rf*-mediated, URF13 reduction is as yet unresolved. An individual plant can accumulate an abundance of *Rf1*-, *Rf8*-, or *Rf**-associated transcripts, with no obvious decrease in the steady-state accumulation of the mature 2.0, 1.8, or 1.0 kb transcripts (Dill et al. 1997; Wise et al. 1996). This is in contrast with the effect of the sorghum *Rf3* on the abundance of *orf107* transcripts. In this case, a marked decrease in the 1.11, 0.87, and 0.81 kb unprocessed transcripts accompanies the accumulation of a novel 0.38 kb *orf107* transcript (Tang et al. 1996). Therefore, despite the similarities in processing sites as shown in Figure 2, there appear to be some distinct differences in the mechanism of these restorers.

Transposon-Tagged Mutants of *Rf1* and *Rf2* Provide Germplasm for the Molecular Isolation of Nuclear Restorers

Efforts are under way to clone nuclear restorer genes in a number of laboratories. Because known gene products are not associated with fertility restoration, restorer genes are most readily isolated via genetic methods that rely on a mutant phenotype, such as map-based methods (Bentolila et al. 1998; Delourme et al. 1994) or transposon tagging (Schnable and Wise 1994; Wise et al. 1996). In maize, the *Mutator* transposon system is an efficient tool for transposon tagging due to the high transposition rate of *Mu* elements. Utilizing the inserted *Mu* elements as molecular probes, this system has been used to clone a variety of genes (Walbot 1992). To facilitate the generation of stocks necessary to transposon tag restorer genes in T-cytoplasm maize, *Rf1* and *Rf2* were positioned in reference to closely linked RFLP and visible markers (Wise and Schnable 1994). Subsequently, seven *rf2-m* and four *rf1-m* alleles were isolated from populations of 178,300 and 123,500 transposon-bearing plants, respectively (Schnable and Wise 1994; Wise et al. 1996).

To identify DNA fragments containing *rf2* sequences, DNA gel-blot cosegregation analyses were performed. A 3.4 kb *Mu1*-hybridizing *Eco*RI-*Hind*III restriction fragment that cosegregated with the *rf2-m8122 Mutator*-induced allele in large testcross families was cloned. This fragment was shown to contain a portion of the *rf2* gene via allelic cross-referencing experiments. Allelic cross-referencing is the comparison of new DNA polymorphisms between a progenitor allele and its corresponding mutant derivatives. This is a particularly powerful method if a number of independent mutations are available. However, this method requires the use of a single- (or low-) copy sequence as a hybridization probe. Because independent mutations at the *rf2* locus coincided with DNA sequence rearrangements in the region detected by a single-copy probe derived from the *rf2-m8122* genomic clone, it could be concluded that this probe hybridized to a portion of the *rf2* gene.

Initially a similar approach was taken to identify *rf1* sequences. Male sterility mediated by the *rf1-m3207* allele cosegregated with a 5.5 kb *Mu1*-hybridizing *Eco*RI-restriction fragment, suggesting that it contained a *Mu* transposon insertion in the *rf1* gene (Wise et al. 1996). This candidate *rf1* DNA fragment was subsequently isolated from a subgenomic phage library constructed from a single plant containing the *rf1-m3207* allele. To identify candidate cDNAs representing *Rf1,* sequences flanking the *Mu1* insertion from p*rf1-m3207* were hybridized with 1×10^6 independent clones from a cDNA library constructed from RNA isolated from a plant with the genotype: *Rf1/Rf1, Rf2/Rf2.* The 3′ UTR (untranslated region) of the cDNA, p6140-1, cosegregated with the *rf1* locus in over 100 backcross progeny. In addition, part of the sequence of this candidate *Rf1* cDNA was consistent with the expected function of an *Rf1* allele (described below). However, this linkage analysis cannot rule out the possibility that this cloned sequence does not represent *Rf1,* even though it flanks a transposon and cosegregates with the *rf1* locus. Hence it is essential to distinguish between transposons inserted at the *rf1* locus from those that are only tightly linked to it. Because most of the candidate *rf1* sequences from the p6140-1 cDNA and the p*rf1-m3207* genomic clone are highly repetitive; they cannot be used for allelic cross-referencing experiments in the manner used for *rf2.* Instead, other approaches are being utilized to verify the identity of candidate *Rf1* clones.

Additional Strategies to Verify the Identity of a Cloned *rf* Allele

PCR primers corresponding to sequences flanking a *Mu* transposon inserted at *rf1* might be expected to detect DNA rearrangements among members of our *rf1-m* allelic series in comparison to their wild-type progenitor alleles. Through sequence analyses of candidate *Rf1* cDNAs, we have developed PCR primers throughout the nucleotide sequence of p6140-1. Therefore, because much of the p6140-1-cDNA hybridizes to repetitive sequences, PCR amplifications with each of these p6140-derived primers paired with a *Mu* terminal-inverted repeat (TIR) primer may amplify a fragment that is positioned between a p6140-derived primer and the transposon TIR primer in the independent *rf1-m* alleles. Subsequent hybridization, cloning, and sequencing of these fragments would verify their identity. Thus it should be possible to perform allelic cross-referencing via PCR if the transposon insertion is in or near the region represented by the candidate cDNA (Gray et al. 1997).

Computational Analysis of the Candidate cDNA, 6140-1

Analysis of the DNA sequence of the candidate *Rf1* cDNA, p6140-1, was used to provide clues as to the possible function of this clone. Twenty-six residues of the predicted amino acid sequence of the p6140-1 cDNA revealed 96% similarity and 69% identity ($P = 3.1e^{-09}$) with mammalian small nuclear ribonucleoprotein E (snRNP E). The snRNP E protein is an 11 kD basic protein integral to RNA processing reactions. It is one of four "core" proteins associated with the snRNAs of the U (uridine rich) family (U1, U2, U4, U5, and U6) (Fautsch et al. 1992; Stanford et al. 1988a,b). The U1 snRNA is initially bound to the 5′ splice site and is released upon recruitment of U4/U5/U6. In mammalian systems, where RNA processing has been studied extensively, mutations in snRNP E abolish its ability to assemble into an snRNP complex. Hence the snRNP E protein is not thought to bind RNA directly, but may have a role in the assembly of the ribonucleoprotein particle, which consists of at least six elements (D1, D2, D3, E, F, and G). Therefore it has been hypothesized to be an essential scaffolding-like protein for the snRNP core (Weiben ED, personal communication).

As stated previously, the short conserved sequence 5′-CNACNNU-3′ overlaps the 5′ terminal U of each of the *Rf*-associated processing sites in the T-*urf13* transcripts (Figure 2; Dill et al. 1997). Although each of the *Rf* genes may encode proteins that mediate RNA processing activities, the precise molecular mechanism of specific site recognition is unknown. However, if a particular RF protein is part of an RNA processing complex, and the snRNP E domain is essential, the interaction among various snRNAs and unique domains of the different RF proteins may determine processing specificity.

Development of a Diagnostic Transformation System for T Cytoplasm—Testing *Rf1* Candidate Constructs in A188 (T) × Hi-II (N) Callus

If mtRNA processing involves a complex of RNAs and proteins, a system is needed that contains all the biological components necessary for the ultimate expression of the *Rf*-mediated phenotype. To decipher the function of *Rf*-encoded proteins (first by complementation and subsequently by in vitro mutagenesis), we have adapted a well-known system for maize transformation from which to base our experiments.

For the study of *Rf* function, it is essential to use T-cytoplasm lines, because the T-*urf13* gene upon which these corresponding gene products act is unique to T (and not N)-cytoplasm mitochondria. However, Hi-II, the standard germplasm for maize transformation (Armstrong and Green 1985; Armstrong et al. 1991) carries the N cytoplasm. Hence, to test the functionality of p6140-1 and other *Rf1*-candidate clones, we are developing a T-cytoplasm transformation system. This system is based on the cross A188 (T) × Hi-II (N). Of importance, the restorer genotypes of both the A188 and B73 parents of the Hi-II stock are *rf1/rf1, Rf2/Rf2, rf8/rf8, rf*/rf**. The dominant *Rf1, Rf8,* and *Rf** alleles independently mediate the processing of T-*urf13* transcripts in T-cytoplasm maize lines as described above. The dominant *Rf2* allele is necessary in combination with *Rf1, Rf8,* or *Rf** for expression of male fertility. Thus the A188 (T) × Hi-II (N) genotype will facilitate detection of transgenic *Rf1*-mediated, T-*urf13* transcript processing activity in addition to restoration of male fertility. In addition, this system will be valuable for future transgenic characterization of *Rf8* and *Rf**.

We have developed a PCR-based assay to ensure that our embryogenic callus lines contain an intact T-*urf13* sequence. Verification of the intactness of T-*urf13* is essential because it has been shown that the tissue-culture environment can yield mtDNA rearrangements and subsequent changes in T-*urf13* expression (Fauron et al. 1990; Gengenbach et al. 1981; Kemble et al. 1982; Rottmann et al. 1987; Wise et al. 1987a,b). In addition, we have shown that the A188 (T) × Hi II (N) callus lines display the T-*urf13* transcript patterns appropriate for an *rf1/rf1, Rf2/Rf2, rf8/rf8, rf*/rf** plant and that this callus is readily regenerable. Thus biolistic (or *Agrobacterium*-mediated) transformation of candidate *Rf1* constructs should complement the A188 (T) × Hi-II (N) recipient embryogenic callus, and T-*urf13* transcript processing will be discernible via northern blot analyses. Callus lines that are positive for the transgene and T-*urf13* can be bulked up for mtRNA isolations and T-*urf13* transcript analysis. The mtRNA can be assayed for the levels of the *Rf1*-dependent, 1.6 and 0.6 kb T-*urf13* transcripts, which are easily visualized via autoradiography subsequent to hybridization with diagnostic T-*urf13* probes (Wise et al. 1996). In this way we can test many different constructs for RNA processing activity at the callus stage before proceeding further with the more expensive whole-plant regenerations.

Identification of the *Rf8* and *Rf** Regions of the Maize Genome

The availability of a quick DNA-based assay (PCR or hybridization) for newly described restorer loci is essential to follow them (alone or in combination) in genetic crosses designed to study RNA processing activities. Although excellent molecular markers exist for *Rf1* and *Rf2* (Wise and Schnable 1994), *Rf8* and *Rf** are not yet genetically mapped.

To begin marker analysis for *Rf8,* segregating progeny from the cross (T) *Rf8-8703/rf8-W64A* × (N) *rf8-W64A/rf8-W64A* were grown in the summer of 1997. One hundred twenty individual plants were observed for fertility and backcrossed by W64A. However, since *Rf8* is a weak fertility restorer, the most robust method to identify plants that carry it is to assay (via mitochondrial northern hybridization) for the accumulation of the 1.42 kb T-*urf13* transcript (Dill et al. 1997). Hence immature second ear shoots from each plant were also harvested for DNA and mtRNA isolations. Individual mtRNAs were assayed via northern hybridizations for the presence of the 1.42 and 0.42 kb, *Rf8*-dependent T-*urf13* transcripts. This analysis identified the *Rf8/rf8* versus *rf8/rf8* plants. Pools of 16 DNAs each from *Rf8/rf8* and *rf8/rf8* plants were used in bulk-segregant AFLP analyses (Cnops et al. 1996; Michelmore 1991) to identify polymorphic amplified DNA fragments linked to *Rf8.*

Out of 256 ^{33}P-labeled *Eco*RI-*Mse*I AFLP primer pairs tested on the parents and the bulks, flanking AFLP markers have been observed at 4.5 and 15.6 cM from the *Rf8* locus (Figure 3). Subsequent sequence analyses of the cloned AFLP fragment will facilitate the design of PCR primers that specifically amplify the polymorphic bands. These primers will be used to follow *Rf8* alleles as well as position *Rf8* on the maize genetic map. Transposon tagging of *Rf8* is precluded because of its environmentally sensitive, partial fertility restoration. Hence as we proceed through the genomic analyses, we expect to identify markers with tighter linkage until we are able to use the markers to identify a maize bacterial artificial chromosome (BAC) clone. Sequence and functional analysis of these

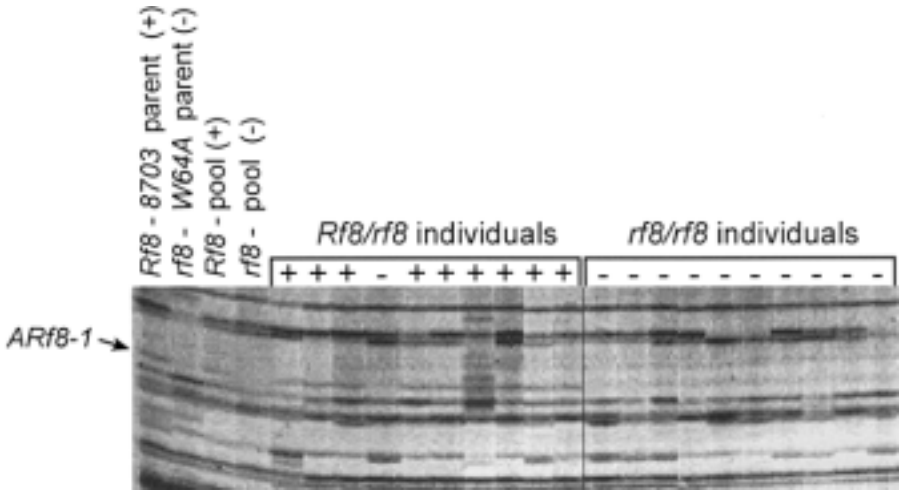

Figure 3. Bulk-segregant AFLP analysis of DNAs from maize parents, pools, and individual progeny using the *Eco*RI-AAA and *Mse*I-CAT primers. DNAs from plants segregating for the 1.42 and 0.42 kb T-*urf13* transcripts were pooled together to make up the *Rf8* bulk, whereas DNAs from plants lacking these transcripts were used to construct the *rf8* bulk. This subset of amplifications from *Rf8/rf8* (+1.42 and 0.42 kb transcripts) and *rf/rf8* (−1.42 and 0.42 kb transcripts) individuals demonstrates the linkage of AFLP marker *ARf8-1,* which is positioned 4.5 cM from the *rf8* locus.

large-insert clones should provide clues as to the identification of *Rf8.*

Epilogue

CMS systems are models for studying nuclear-cytoplasmic interactions because fertility restoration relies upon nuclear-encoded gene products that suppress cytoplasmic dysfunction. *Rf1* and two similar restorers, *Rf8* and *Rf**, mediate the differential processing of T-*urf13* transcripts and the concurrent reduction of the URF13 protein. These processing events result in unique T-*urf13* transcripts, but do not significantly decrease the levels of the progenitor transcripts. The *Rf*/T-*urf13* interactions can serve as a model for restorer-mediated processing of mitochondrial transcripts because this process occurs in many CMS systems, such as sorghum, rice, petunia, and oilseed rape. Investigations of these interactions should therefore contribute to a general understanding of mechanisms of mtRNA processing in plants.

Secondary structure predictions of T-*urf13* transcripts suggest that each of the 5′ processing sites mediated by the *Rf1, Rf8,* and *Rf** all occur in open loops that overlap the 5′-CNACNNU-3′ conserved sequence outlined in Figure 2 (Dill et al. 1997). Yet how different nuclear (*Rf*) genes evolved to mediate recognition of specific sequences within mitochondrial transcripts is an unresolved question. Our goal is to determine the molecular mechanism of *Rf*-mediated processing activity and specifically define what determines splice-site recognition. The *Rf1, Rf8,* and *Rf** DNA markers will facilitate the tracking of progeny in families segregating for multiple restorers. This in turn will simplify cDNA expression and microarray experiments to identify the proteins (or protein complexes) that bind to the 5′-CNACNNU-3′ site of the T-*urf13* RNA in different *Rf* backgrounds. Experiments to test the functional domains of candidate *Rf* genes can then be designed based on our T-cytoplasm transformation system. Because CMS remains a widely used tool for the production of hybrid seed in a number of economically important crops such as *Brassica,* sorghum, and rice, we anticipate that a common mechanism among *Rf1*-like restorer genes will be applicable to these crops as well.

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