The *rf2* Nuclear Restorer Gene of Male-Sterile T-Cytoplasm Maize

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The T cytoplasm of maize serves as a model for the nuclear restoration of cytoplasmic male sterility. The *rf2* gene, one of two nuclear genes required for fertility restoration in male-sterile T-cytoplasm (cmsT) maize, was cloned. The protein predicted by the *rf2* sequence is a putative aldehyde dehydrogenase, which suggests several mechanisms that might explain *Rf2*-mediated fertility restoration in cmsT maize. Aldehyde dehydrogenase may be involved in the detoxification of acetaldehyde produced by ethanolic fermentation during pollen development, may play a role in energy metabolism, or may interact with URF13, the mitochondrial protein associated with male sterility in cmsT maize.

Cytoplasmic male sterility is a maternally inherited inability to produce functional pollen. It has been observed in more than 150 plant species (*1*) and is often associated with the expression of novel mitochondrial open reading frames (*2*). In many cytoplasmic male-sterile systems, male fertility can be restored in the presence of specific nuclear genes (*3*). These fertility-restorer genes are thought to block or compensate for cytoplasmic dysfunctions that are phenotypically expressed during pollen development. Efforts to characterize the molecular mechanisms by which these events occur have been hampered by the absence of cloned nuclear restorer genes.

The T cytoplasm of maize serves as a model for cytoplasmic male sterility and fertility restoration. Restoration of fertility to cmsT maize requires the combined action of the dominant alleles of two nuclear restorer genes, *rf1* and *rf2* (*4*). Until the 1970 epidemic of Southern corn leaf blight, cmsT maize was used to produce about 85% of the hybrid seed in the United States (*5*). T-cytoplasm maize is highly sensitive to the host-selective pathotoxin HmT, produced by *Cochliobolus heterostrophus* Drechsler race T (*6*). The mitochondrial gene T-*urf13*, which is present in T cytoplasm but absent in N cytoplasm, is responsible for male sterility and toxin sensitivity (*7*). T-*urf13* encodes a 13-kD mitochondrial polypeptide, URF13 (*8*, *9*).

Even in the absence of pathogens, URF13 appears to have a slight deleterious effect on maize cells (*10*), presumably as a consequence of subtle perturbations in mitochondrial function. However, under these conditions, the only striking phenotype conditioned by URF13 is male sterility, which occurs as a consequence of a degeneration of the tapetal layer of anthers (*11*). The selective nature of this degeneration is paradoxical because URF13 is expressed in many, if not all, maize tissues (*12*). It is possible that there is a tapetum-specific compound (factor X) that is a prerequisite for URF13-induced toxicity (*13*). Alternatively, tissue-specific degeneration could occur if tissues differ in their requirements for mitochondrial function (*14*). The accumulation of toxic URF13 is reduced about 80% in plants carrying *Rf1* (*8*, *15*), presumably as a consequence of this allele's ability to alter the accumulation and nature of T-*urf13* transcripts (*16*). In contrast, *Rf2* does not affect URF13 accumulation (*8*), and nothing is known about the essential role of *Rf2* in fertility restoration.

To clone this nuclear fertility-restorer gene, we used a transposon-tagging strategy to isolate *rf2* mutant (*rf2-m*) alleles (*17*). Genetic crosses were performed such that rare progeny plants carrying newly generated transposon-tagged *rf2* alleles were male sterile. Seven *rf2-m* alleles were isolated through a screen of about 178,300 plants. Six of these mutant alleles were derived from *Mutator* transposon stocks and may therefore have arisen by the insertion of *Mu* transposons. In an effort to identify such a transposon inserted within the *rf2* gene, we subjected DNA samples from test-cross progeny families (each of which involved a different *rf2-m* allele) to DNA gel-blot analysis with probes specific to individual classes of *Mu* transposons. In one such family derived from the cross T cytoplasm *rf2-m8122/Rf2-Ky21* × *rf2-ref/rf2-ref*, all 56 male-sterile progeny carried a 3.4-kb *Mu1*-containing Eco RI–Hind III DNA fragment that was absent in all 49 male-fertile siblings (Fig. 1). This result demonstrated that this DNA fragment was closely linked to or part of the *rf2* locus. The 3.4-kb DNA fragment was cloned as the plasmid pF#9 (Fig. 1C). The identity of this DNA fragment was established by allelic cross-referencing experiments. In such experiments (Fig. 2), the putative *rf2* clone revealed DNA polymorphisms between each of four progenitor alleles (*18*) and their corresponding *rf2-m* derivatives (Fig. 2). Because mutations at the *rf2* locus coincided with

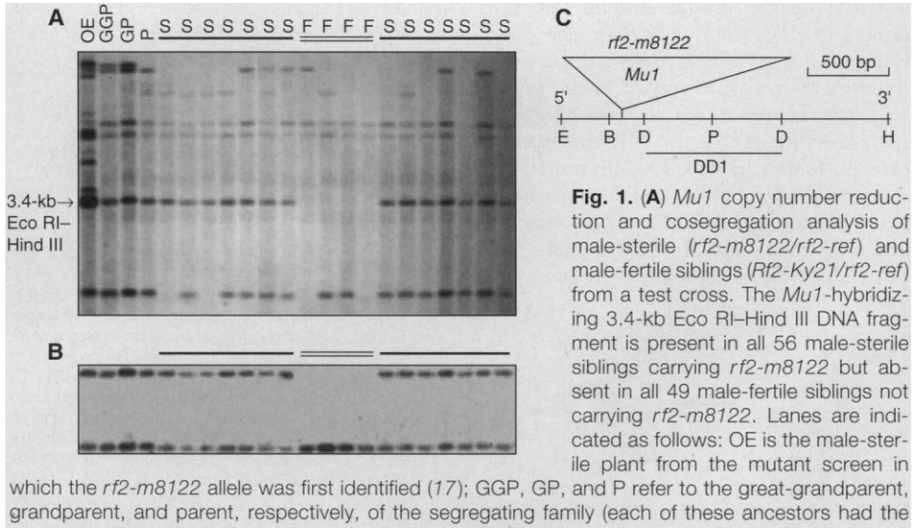

Fig. 1. (**A**) *Mu1* copy number reduction and cosegregation analysis of male-sterile (*rf2-m8122/rf2-ref*) and male-fertile siblings (*Rf2-Ky21/rf2-ref*) from a test cross. The *Mu1*-hybridizing 3.4-kb Eco RI–Hind III DNA fragment is present in all 56 male-sterile siblings carrying *rf2-m8122* but absent in all 49 male-fertile siblings not carrying *rf2-m8122*. Lanes are indicated as follows: OE is the male-sterile plant from the mutant screen in which the *rf2-m8122* allele was first identified (*17*); GGP, GP, and P refer to the great-grandparent, grandparent, and parent, respectively, of the segregating family (each of these ancestors had the genotype *rf2-m8122/Rf2*); and S and F refer to male-sterile and male-fertile siblings, respectively. The *rf2-ref* allele is the reference allele carried by the inbred line Wf9. (**B**) Cosegregation of sterility with an RFLP detected by DD1. The DNA blot from (A) was stripped and then reprobed with the 846–base pair Dra I fragment adjacent to the *Mu1* transposon in pF#9 [probe DD1 in (C)]. The resulting hybridization pattern established that DD1 is a low-copy sequence in the maize genome. Although not visible here, probe DD1 also hybridized weakly to a second genomic fragment. (**C**) Restriction map of the DNA insert of pF#9 (*24*). Abbreviations for restriction enzyme sites: E, Eco RI; B, Bgl II; D, Dra I; P, Pst I; and H, Hind III.

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DNA sequence rearrangements in the region detected by the probe DD1, we concluded that DD1 cross-hybridizes to DNA fragments containing the *rf2* locus.

Using DD1 as a probe, we isolated a 1.2-kb partial *Rf2* cDNA clone from a cDNA library prepared from RNA isolated from immature tassels of the inbred line W22. Subsequently, we isolated a 2.2-kb *Rf2* cDNA clone from a cDNA library prepared from RNA isolated from seedlings of the inbred line B73 (*19*). The latter *Rf2* cDNA detected a 2.3-kb transcript in RNA isolated from immature tassels (*20*) of the *Rf2*-containing inbred line Ky21 (Fig. 3). Although this transcript also accumulated to at least normal amounts in the tassels of plants homozygous for the *rf2-ref* allele, it was not detectable in total RNA isolated from tassels homozygous for *rf2-m8904*, *rf2-m9323*, and *rf2-m9437* and was barely detectable in RNA isolated from tassels homozygous for *rf2-m8110* and *rf2-m8122* (Fig. 3).

This 2.2-kb *Rf2* cDNA appears to be near full length because its size corresponds well with that of the *Rf2* transcript (Fig. 3). Additionally, the sequence (*21*) contains in-frame stop codons 5′ of two methionine residues and the cDNA encodes a putative mitochondrial targeting signal (Fig. 4). Computer-based homology searches of GenBank and other databases with various derivatives of the Blast algorithm (*22*) revealed substantial similarity (about 60% identity and 75% similarity) between the predicted *Rf2*-encoded protein and mammalian mitochondrial aldehyde dehydrogenases (ALDHs), which catalyze the oxidation of a broad range of aldehydes to acids (Fig. 4). The two catalytic domains present in ALDHs are conserved in the RF2 protein. Although several ALDHs have been partially purified from plants (*23*), their role in plant metabolism has not been clarified. In yeast, *Drosophila*, and mammals, the enzymes alcohol dehydrogenase (ADH) and ALDH function together to oxidize ethanol through acetaldehyde to acetate. It is this NADH (reduced form of nicotinamide adenine dinucleotide)–producing pathway that allows these organisms to survive under aerobic conditions on diets in which ethanol constitutes the sole carbon and energy source. Hence, one possible role for ALDH in plants is the detoxification of ethanol and acetaldehyde after brief periods of fermentation.

Our working hypothesis is that the RF2 protein has a function independent of its role as a nuclear restorer in cmsT maize (*17*). According to this hypothesis, the RF2 protein has been recruited in T-cytoplasm maize to ameliorate the mitochondrial lesion associated with T-*urf13* expression. Assuming the *rf2* gene encodes an ALDH, we have developed two hypotheses for the mechanism by which this could occur.

According to the "metabolic" hypothesis, the normal metabolic role of ALDH becomes essential in T-cytoplasm cells as a result of URF13-mediated alterations of mitochondrial function. For example, the energy produced by ALDH-catalyzed α-oxidation of fatty acids could become essential if T-cytoplasm tapetum cells have an energy deficit. Alternatively, the potential role of ALDH in scavenging and detoxifying acetaldehyde could become essential if URF13 alters mitochondrial function such that additional pyruvate is shunted into aerobic fermentation (*24*).

The "interaction" hypothesis suggests that the RF2 protein interacts directly or indirectly with URF13 and thereby diminishes its deleterious effects. Because of the broad specificities of ALDHs (*25*) and their diverse metabolic functions (*26*), the RF2 protein could act by modifying (i) some component or components of the inner mitochondrial membrane or (ii) factor X. For example, an ALDH-catalyzed oxidation of an aldehyde component of the mitochondrial membrane could alter the binding of

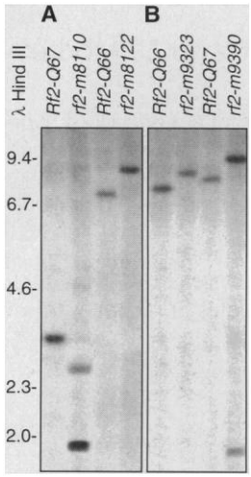

Fig. 2. Allelic cross-referencing experiments. The DD1 fragment of pF#9 (Fig. 1C) was hybridized to gel blots containing DNA isolated from plants carrying four independent *rf2* mutants and their respective wild-type progenitor alleles. *rf2-m8110* and *rf2-m9390* are derived from the wild-type allele *Rf2-Q67*; *rf2-m8122* and *rf2-m9323* are derived from *Rf2-Q66* (*18*). (**A**) Eco RI; (**B**) Hind III.

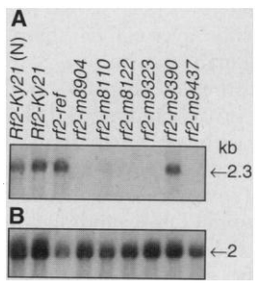

Fig. 3. Gel blot containing RNA prepared from immature tassels homozygous for the indicated *rf2* alleles (*20*). All plants were T-cytoplasm maize and were derived from the Ky21 inbred background (after four to five backcrosses) except the one used in the first lane, which was an N-cytoplasm Ky21 plant (N). RNAs were hybridized with either the 2.2-kb *rf2* cDNA (**A**) or the maize tubulin cDNA *tub1* (**B**).

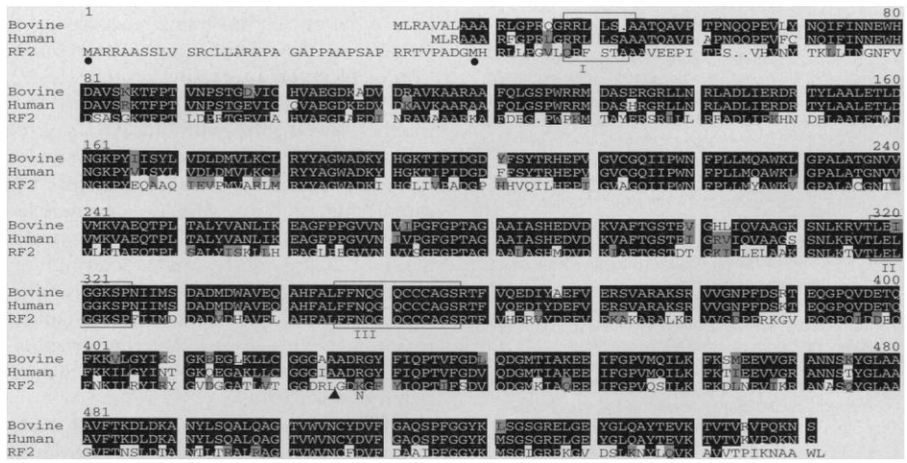

Fig. 4. Sequence alignment of the predicted RF2 protein (GenBank accession number U43082) with bovine (GenBank accession number P20000) and human (GenBank accession number P05091) mitochondrial ALDH (*27*). Solid circles indicate positions of the two possible translation-initiation codons of *rf2*. Mitochondrial targeting signals (box I) predicted by the PSORT algorithm (*28*) and the Glu (box II) and Cys (box III) catalytic domains of ALDH identified by the Motifs algorithm of the GCG sequence analysis software package are indicated. The insertion site of the *Mu* transposon that generated the *rf2-m8122* allele is indicated by a solid triangle. Identical and similar amino acid residues are indicated by black and gray backgrounds, respectively. There is a single nucleotide polymorphism between the *Rf2-B73* and *Rf2-W22* alleles; this G to C transversion results in the substitution of an asparagine for a lysine residue at position 427.

URF13 to that membrane. Alternatively, if factor X is an aldehyde, it could be inactivated through oxidation. Either of these events could alter the toxicity associated with the accumulation of URF13 in the mitochondria without affecting URF13 accumulation (consistent with the behavior of *Rf2*).

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18. On the basis of their pedigrees, five of the *rf2-m* alleles isolated in the *Mutator* transposon-tagging experiment (*rf2-m8110, rf2-m8122, rf2-m9323, rf2-m9390*, and *rf2-m9437*) would be expected to have been derived from *Rf2-Q66, Rf2-Q67, Rf2-B77*, or *Rf2-B79*. Their wild-type progenitor alleles were determined by comparing the restriction fragment length polymorphism (RFLP) fingerprints through the use of 3′ *sus1* and *umc153* markers that flank the *rf2* locus [R. P. Wise and P. S. Schnable, *Theor. Appl. Genet.* **88**, 785 (1994)] and *rf2* intron sequences of DNA samples carrying mutant alleles with those carrying the potential progenitor alleles.
19. The inbred lines W22 and B73 carry *Rf2* alleles. The 1.2- and 2.2-kb *Rf2* cDNAs were isolated from the 2ts library prepared by the Dellaporta laboratory [A. Delong, A. Calderon-Urrea, S. L. Dellaporta, *Cell* **74**, 757 (1993)] and from a library prepared by A. Barkan, respectively (A. Barkan, personal communication).
20. Total RNA was isolated from immature tassels according to the method of Dean *et al.* [*EMBO J.* **4**, 3055 (1985)]. About 10 μg of total RNA was loaded in each lane of the RNA gel [H. Lehrach *et al.*, *Biochemistry* **16**, 4743 (1977)]. RNAs were transferred onto a Magna Charge nylon membrane (Micron Separations) and hybridized with an *rf2* cDNA probe at 68°C. RNA loading was quantified by stripping the membrane and then rehybridizing it to a tubulin cDNA probe (*tub1*) [R. Villemur *et al.*, *J. Mol. Biol.* **227**, 81 (1992)].
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27. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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An Orphan Nuclear Hormone Receptor That Lacks a DNA Binding Domain and Heterodimerizes with Other Receptors

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SHP is an orphan member of the nuclear hormone receptor superfamily that contains the dimerization and ligand-binding domain found in other family members but lacks the conserved DNA binding domain. In the yeast two-hybrid system, SHP interacted with several conventional and orphan members of the receptor superfamily, including retinoid receptors, the thyroid hormone receptor, and the orphan receptor MB67. SHP also interacted directly with these receptors in vitro. In mammalian cells, SHP specifically inhibited transactivation by the superfamily members with which it interacted. These results suggest that SHP functions as a negative regulator of receptor-dependent signaling pathways.

The yeast two-hybrid system (*1*, *2*) has proved useful in both the characterization of the heterodimeric interactions of members of the nuclear hormone receptor superfamily and the isolation and characterization of receptor-interacting proteins (*3*–*9*). We used this system to isolate proteins that interact specifically with mCAR, a close murine relative of the human orphan receptor MB67 (*10*). Among several mCAR-interacting proteins, one isolate included the ligand-binding and dimerization domain of a previously uncharacterized orphan member of the receptor superfamily. On the basis of its small size and its ability to interact with several superfamily members, we called this protein SHP (small heterodimer partner).

SHP is encoded by an ~1.3-kb transcript expressed in liver and at lower levels in heart and pancreas (Fig. 1A). Extensive screening of two murine liver cDNA libraries produced a number of cDNA clones (*11*). Two lines of evidence suggested that these clones were full length. The first was simply that the 5′ ends of 10 of 12 independent clones fell within a short 32-nucleotide (nt) segment located upstream of the initiator methionine. More importantly, their length [~1120 base pairs (bp)] corresponded precisely to that of the SHP transcript without its presumably 100- to 300-nt polyA tract. A similar clone was isolated from a human liver cDNA library.

The human and murine SHP proteins are well conserved (Fig. 1B). Both include complete ligand-binding and dimerization domains, with sequences similar to those associated with heterodimerization in other superfamily members, but neither contains a conventional DNA binding domain. Addition of the at least 200 nt necessary to encode the DNA binding domain would increase the predicted size of the mRNA to between 1.4 and 1.6 kb, substantially greater than that observed.

The closest relative of SHP in the receptor superfamily is DAX-1 (Fig. 1C), the only other family member that lacks a conventional DNA binding domain (*12*). These two proteins are approximately as

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