

MALE STERILITY (Kaul 1988)

A. INFERTILITY VS. INCOMPATIBILITY

1. Sterility systems: spores and gametes are absent or abnormal. Individual failed to produce functional gametes to produce offspring.
2. Incompatibility systems: male and female gametes are normal and functional, but can only produce offspring when correct parents are mated; etc. self-incompatibility, cross-incompatibility.
3. Male sterility is defined as the failure of plants to produce functional anthers, pollen, or male gametes.
 - a. 1st documentation: 1763--Kölreuter observed anther abortion within species and species hybrids.
 - b. More prevalent than female sterility:
 - i. Male sporophyte and gametophyte less protected from environment than ovule and embryo sac.
 - ii. Easy to detect male sterility, because a large number of pollen for study available.
 - iii. Easy to assay male sterility: staining technique (caramine, lactophenol or iodine); female sterility (fst) requires crossing.
 - iii. Male sterility has propagation potential in nature (can still set seed) and is important for crop breeding, fst does not.
 - c. Arises spontaneously via mutations in nuclear and/or cytoplasmic genes

B. MANIFESTATIONS OF MALE STERILITY

- a. Absence or malformation of male organs (stamens) in bisexual plants or no male flowers in dioecious plants.
- b. Failure to develop normal microsporogenous tissue- anther.
- c. Abnormal microsporogenesis--deformed or inviable pollen.
- d. Abnormal pollen maturation; inability to germinate on compatible stigmata.0
- e. Nondehiscent anthers but viable pollen,- sporophytic control.
- f. Barriers other than incompatibility preventing pollen from reaching ovule.

C. CLASSIFICATION OF MALE STERILITY

1. Genetic male sterility:
 - a. Phenotypic (table 1.2)
 - i. Structural male sterility: anomalies in male sex organs (or missing all together)
 - ii. Sporogenous male sterility: stamens form, but pollen absent or rare due to microsporogenous cell abortion before, during, or after meiosis
 - iii. Functional male sterility: viable pollen form, but barrier prevents fertilization (anther indehiscence, no exine formation, inability of pollen to migrate to stigma or

other factors that affect fertilization --e.g. extended style, pollen is glued together so can't be released-soybean, peas)

b. Genotypic (fig 1.1)

- i. Genic male sterility: Mendelian inheritance due to nuclear not cytoplasmic genes
 - 1) Arisen as spontaneous mutants in most cases:- high frequency of mutation.
 - 2) Identified in 175 species.
 - 3) Few reciprocal differences observed.
 - 3) *ms* (recessive--most) or *Ms* (dominant--few)
 - 4) Many nonallelic genes are known in some species (e.g. 60+ in maize, tomato 55, etc.)
 - 4) Recessive: single genes ~70%; multigenes~15 (monocot)-23 (dicot)%: rest is dominant mutants- 7 (dicot) 15 (monocot)%.

- ii. Cytoplasmic male sterility: Non-Mendelian inheritance - cytoplasmic
 - 1) Reciprocal differences observed.
 - 2) N (normal) and S (sterile) cytoplasms.
 - 3) Male sterility inherited maternally.
 - 4) Not very common.

- iii. Gene-cytoplasmic male sterility: Both nuclear and cytoplasmic genes are involved
 - 1) N and S cytoplasm.
 - 2) Restorer of fertility (*Rf*) genes--distinct from genic male sterility genes.
 - 3) *Rf* requires to restore fertility in S cytoplasm. N *rf/rf* = fertiles; *SRf*- = fertiles; S *rf/rf* = male sterile.

D. GENE-CYTOPLASMIC MALE STERILITY

Reference: Schnable and Wise (1998), Levings (1993), and Wise et al. (1999).
Mackenzie and McIntosh (1999).

General features of CMS

1. Need both sterile cytoplasm and *rf* nuclear gene(s) to get male sterility expression--that is, S + *Rf* or N + *rf* will be fertile
2. *Rf* mutations (i.e., mutations to *rf* or no fertility restoration) are frequent, so N-*RfRf* is best for stable fertility
3. Maize: perhaps most well studied system:
 - a. Maize has a number of male sterile cytoplasms--T, S, and C are the most widespread and well characterized; see handout from Kaul, 1988, p. 561
 - b. T and other sterile cytoplasms differ from fertile or normal (N) cytoplasms in
 - i. mitochondrial RFLP
 - ii. mitochondrial RNA
 - iii. mitochondrial translation products

T cytoplasm in maize as model system for cms (cms-T)

General information

1. Texas (T) cytoplasmic male sterility discovered in 1940s; used extensively throughout the 1960s.
2. Highly stable under all environmental conditions.
3. Characterized by failure of anther exertion and pollen abortion.
4. Plants bearing the T cytoplasm, but not others (S, C, or N) are susceptible to race T of the southern corn leaf blight - (*Cochliobolus heterostrophus* = *Bipolaris maydis*); the only previously identified race was race O, which wasn't deemed a serious problem. Race T is exceptionally virulent. Also susceptible to *Mycosphaerella zea-maydis*. Cause diseases by producing a toxin (β -polyketol).
5. Widespread use of T-cytoplasm for hybrid corn production led to epidemic in 1970 with the widespread rise of Race T.
6. Toxin produced by *C. heterostrophus* = T-toxin.
7. Susceptibility arises in T cytoplasm due to mitochondrial sensitivity to T-toxin.
8. Male sterility and sensitivity to fungal toxins- both are mediated by the same gene product.

T-urf13 gene in T cytoplasm maize

Mitochondrial gene T-urf13 is a unique chimeric sequence

1. Early studies in Prof. Leving lab (Forde et al. 1978) established that a 13 kDa polypeptide is observed in T-mitochondria, but not in N-mitochondrial.
2. The gene encoding this polypeptide arose through mitochondrial recombination which produced a chimeric reading frame (Figure 6 of Dewey et al. 1987, PNAS 84:5574-5378) and named *urf13-T*.
3. The *pcf* gene in petunia and the *orf107* gene in sorghum conferring CMS are also chimeric and encode a nonessential polypeptide (Figure 2 of Schnable and Wise 1998).
4. *T-urf13* encodes a nonessential polypeptide.
5. Dewey et al. (1987) raised antibody and showed that amount of the polypeptide is reduced in presence of the *Rf1* gene.
6. Transcript of *T-urf13* also regulated by restorer gene *Rf1* and two others *Rf8* and *Rf** (Dill et al. 1997). Transcripts are truncated near the beginning of the ORF (e.g. start codon methionine is missing-presumably no translation = no *T-urf13* product) (Figure 14 of Wise et al. 1999).
7. Usual mitochondrial genes appear to be normal.

T-urf13 is probably cause of male sterility and disease susceptibility

1. Only present in T cytoplasm
2. Confers both male sterility and disease susceptibility
3. Encodes a 13 kDa polypeptide (URF13) associated with inner mitochondrial membrane
4. Plants expressing this gene are also sensitive to the insecticide methomyl. Toxin/methomyl interact with URF13 interaction to permeablize the plasma membrane (by creating pores).
5. Premature degeneration of tapetum cells- metabolically active.

E. coli transformation experiments

1. *E. coli* expressing T-*urf13* are sensitive to methomyl and T-toxin, showing massive ion leakage, inhibition of glucose-driven respiration, and growth suppression.
2. Phenotypic effects are similar to those seen in maize T mitochondria.
3. URF13 is associated with plasma membrane in *E. coli*, similar to location of activity in mitochondria.

cms-T revertants show T-*urf13* responsible for male sterility and T-toxin sensitivity

1. developed in tissue culture can survive on toxin containing media.
2. Regenerants (that are revertants) from toxin resistant calli were all male fertile.
3. Most revertants show that T-*urf13* is deleted from mitochondrial genome by recombination, resulting in a simultaneous loss of cms and disease susceptibility; some revertants due to mutations (frameshift mutation or premature stop codon) in T-*urf13* that make non-functional URF13 protein.

These data suggest the traits are inseparable.

How does URF13 cause cms?Effect of URF13 protein

1. Degeneration of the tapetum during microsporogenesis (see Fig. 3 in Wise et al., 1999)
2. Disruption of pollen development leading to male cell abortion

URF13 toxicity

1. URF13 only toxic to anther cells, even though it is expressed throughout the plant.
2. Some small effects on plant growth and development have been reported (Duvick 1965)—statistically significant 1-3% reductions in plant height, leaf number, and grain yield.
3. URF13 may negatively affect callus cultures as well (Levings 1993).
4. Transgenic experiments show URF13 toxicity—*E. coli*, insect larvae and cell culture, tobacco, and yeast mitochondria—sensitive to T-toxin or methomyl (active ingredient of the insecticide Lannate) when expressing URF13 in transgenic plants, but only if targeted to the mitochondria.

Why would mitochondrial dysfunction specifically affect pollen development?

1. Mitochondrial gene functions are essential to all cells—electron transfer, ATP formation, and translation of mitochondrial mRNA.
2. Interruption of any of these functions would be expected to be lethal.

CMS plants have essentially normal phenotypes, except for pollen fertility

1. They must have few or no agronomic effects in order to be used in crop breeding! (1-3% reduction in crop yield, etc. Duvick 1965).
2. Therefore, CMS must not result from a lesion in a major mitochondrial gene.

Possible that a mitochondrial gene or its product can have an altered function in anther cells

If so, then the specific response seen with URF13 could occur.

Mode of action of URF13

Hypothesis 1:

1. Energy requirements are high in anthers at time of pollen development, resulting in a 40x increase in mitochondria/cell in tapetum and 20x increase in sporogenous cells of the maize anther; an increase that has not been observed in other cell types.
2. Mitochondrial mutants like *T-urf13* could interfere with mitochondrial replication, electron transport, or phosphorylation specifically in the tapetum.
3. URF13 is expressed in all cells (hence the susceptibility to methomyl or T-toxin), but its effect on general mitochondrial functions isn't severe enough to hurt growth and development—therefore, the tapetum may be more sensitive to subtle mitochondrial perturbations than the rest of the plant.

Hypothesis 2: (see Fig. 5 in Wise et al., 1999)

1. By the same mechanism that results disease sensitivity—that is, an *anther-specific* compound interacts with URF13 to permeabilize the inner mitochondrial membrane
2. This anther specific compound would have similar properties to T-toxins and methomyl, but must be limited to the anther so that other cells function normally.
3. However, the anther specific compound has not yet been isolated—though some evidence it exists (see Wise et al., p. 119).
4. Transgenic tobacco failed to show male sterility even when URF13 targeted to the mitochondrial membrane— the factor may not be present to result the pore formation (Chaumont et 1995).

Restoration of fertility through *Rf₁* and *Rf₂*

Restoration of fertility

1. T cytoplasm will produce URF13, but the plant will have normal male fertility if dominant alleles occur at **two** nuclear genes, *Rf1* and *Rf2* (need dominant alleles at both loci to restore fertility)
2. Restoration of fertility in T-cytoplasm is a sporophytic restorer system (acts premeiotically)
 - a. Pollen from plants heterozygous at both loci is only $\frac{1}{4}$ *Rf1*-/*Rf2*-
 - b. If plant carries male-sterile cytoplasm, fertility is completely restored even in heterozygotes (sporophytic determination)—i.e., all pollen will be functional, even if it does not have dominant *Rf* alleles in its haploid genome. Thus, if the plant is *Rf1rf1Rf2rf2*, $\frac{1}{4}$ of its pollen will have the genotype *rf1rf2* yet be fully functional.
 - c. Note that S cytoplasm requires one gene, *Rf3*, and is a gametophytic system—if the plant has a sterile cytoplasm and is heterozygous *Rf3rf3*, then $\frac{1}{2}$ of its pollen will be sterile.
3. Nuclear genes must interact in some manner with the URF13 protein, *T-urf13* transcription, or *T-urf13* transcripts in a manner to rescue the damage done potentially caused by the active protein.

Can *Rf1* and *Rf2* lines restore resistance to the pathogens?

- i) Restores fertility but show intermediate reaction to the pathogens.
- ii) N-cytoplasm is always immune to T-race.

Loci involved in fertility restoration

1. *Rf1*:
 - a. Maps to chromosome 2.
 - b. Not cloned.
 - c. Alters transcript profile of T-*urf13* and decreases URF13 expression by 80%.
 - d. The *Rf1* allele is rare among maize lines—indicating it only functions in male sterility restoration (i.e., it only arose in response to T-cytoplasm; otherwise, no selection was present to make it necessary).
2. *Rf2* (Cui et al., 1996; Levings, 1996)
 - a. Maps to chr. 9
 - b. Has been cloned (transposon-tagging approach).
 - c. Homology to mammalian aldehyde dehydrogenases.
 - d. The *Rf2* allele is common among many maize lines, including those that have never been exposed to sterile cytoplasms—therefore, probably has a function in the cell in addition to its role as a nuclear restorer.
3. Duplicate loci for fertility restoration (Dill et al. 1997)
 - a. *Rf8* and *Rf**
 - b. Can each partially restore fertility in absence of *Rf1*.
 - c. Edit *urf13* transcripts- truncated at the 5'-end. (Wise et al. 1997 Figure 14).
 - d. Also rare in maize populations.

Cloning *Rf2* and its mechanism of action

1. Cui et al. (1996) used a transposon (Mu) tagging strategy
2. isolated a 2.2 kb cDNA containing a mitochondrial targeting sequence
3. 75% similarity to mammalian mitochondrial aldehyde dehydrogenases (ALDH)
4. Plant ALDH may be used to detoxify ethanol and acetaldehyde after brief periods of fermentation
5. Highest levels of accumulation of RF2 protein in tapetum
6. Numerous other plant ALDH have been cloned from maize and *Arabidopsis*; all have ALDH activity on acetaldehyde

What does *Rf2* do?

"Metabolic" hypothesis

1. In normal cells, RF2-ALDH produces energy by oxidizing fatty acids (this specifies a role for RF2 in normal cells)
2. This energy is essential when URF13 alters mitochondrial function.
3. Alternatively, ALDH scavenges and detoxifies acetaldehyde in normal plants, so that RF2 prevents tapetum poisoning and death by metabolizing acetaldehyde.
4. Elimination of acetaldehyde is essential if URF13 alters mitochondrial function toward more ethanolic fermentation, which produces lots of acetaldehyde and/or ethanol, both of which are toxic.

"Interaction" hypothesis

1. RF2 interacts directly or indirectly with URF13 to diminish its deleterious effects.
2. ALDHs have broad specificities and diverse metabolic functions; therefore, RF2 could modify component(s) of inner mitochondrial membrane or the tapetum specific factor (if it exists).
3. If the anther/tapetum specific compound is an aldehyde, RF2 would oxidize it to eliminate the problem.
4. If it is not an aldehyde, then the metabolic hypothesis is favored.

What do we now know about RF2 function?

- a. RF2 encodes mitochondrial aldehyde dehydrogenase (activity now measured) and involves in floret development (Liu et al. 2001).
- b. *Rf2* (nuclear gene) encodes a mitochondrial protein.
- c. An aldehyde dehydrogenase and accumulates in the tapetal cell layer (handout).
- d. Development of lower florets is retarded in the *rf2* mutant (handout).

Other CMS systems1. **Types of sterility genes:**

- a. Identified also in many other crop species including green beans, sorghum, beet, carrot, onion, petunia, *Brassica napus*, rye, sunflower, and wheat.
- b. Results following mutagenesis experiments (mutation of restorer genes), wide crosses, segregation of nuclear restorer gene through crossing, inter-specific crosses.
- c. Most CMS associated genes are chimeric mitochondrial sequences–, (see review by Schnable and Wise 1998) –but few similarities exist among them.
- b. Difference - In at least one well characterized system (*Nicotiana sylvestris*), CMS arises due to a deletion of an entire gene (Gutierrez et al., 1997).

2. **Mechanisms of the sterility development vary:**

- a. Maize and many others–need expression in the tapetum to get the sterility phenotype–i.e., tapetal dysfunction/breakdown causes CMS.
- b. *Phaseolus*–ORF239 protein appears to be toxic to microspores, whether or not it is mitochondrially targeted (He et al., 1996). It is associated with the cell wall of microspores.
- c. *Nicotiana sylvestris*–CMS mutants I and II show large deletions in the mitochondrion, with only the *nad7* gene, involved in mitochondrial respiration, is missing. This results in significant morphological deficiencies, including slower development, smaller plants, etc. Results indicate respiration dysfunction to cause male sterility under low light (partial otherwise) (Gutierrez et al., 1997).

3. **Mechanisms of fertility restoration vary:**

- a. Loss of the mitochondrial sequence causing CMS
 - i. *Phaseolus*–in the presence of the nuclear *Fr* gene (fertility restorer, the mitochondrial sequence *pvs* (which includes *orf239*) that is responsible for sterility is lost. (ORF239 protein appears to be toxic to microspores).

- b. Alteration of transcript profiles of CMS inducing genes
 - i. *Rf1* in maize with T-cytoplasm
 - ii. Restoration of *Brassica napus pol* and *nap* sterile cytoplasms is conditioned by different alleles of the *Rfp* (*Rfn*) locus, which reduce the transcript of the chimeric genes *orf224* (for the *pol* cytoplasm) and *orf222* (for the *nap* cytoplasm) (Li et al., 1998). The *Rfn* allele was not found in any of 51 varieties examined except those with fertility-restored *nap* cytoplasms, suggests that *Rfn* evolved with the *nap* cytoplasm. Similar to *Rf1*'s evolution-only with its specific sterile cytoplasm- for restoration of fertility.
 - iii. Restoration of sorghum fertility in the IS1112C cytoplasm requires the two locus gametophytic system (itself a rarity) of *Rf3* and *Rf4*. Fertile progeny exhibit increased transcript processing activity, in which full length transcripts of the sterility chimeric mitochondrial gene *orf107*, specific to IS1112C cytoplasm, are not produced (Tang et al., 1998). Similar mechanism observed for *Rf1* in maize.
 - iv. Restoration of fertility in maize S-cytoplasm requires the *Rf3* nuclear allele (gametophytic determination); when present, it reduces the transcript lengths of two chimeric genes present in S-cytoplasm, *orf335* and *orf77*, resulting in restored fertility (Wen and Chase, 1999).
- c. *Rf2* story described earlier.
- d. Post-transcriptional editing (RNA editing):
 - i. In plant mitochondria changes C changed to U most commonly perhaps to create new start/stop codons (UAA, UAG or UGA) by RNA editing.
 - ii. It is possible that tissue-specific editing targeting only microsporocytes can result in male sterility. For example: *atp6* editing (but not editing of other mitochondrial transcripts) in sterile sorghum is reduced relative to fertile plants in anthers but not in etiolated seedling shoots; progeny that have been restored to fertility have an increase in editing of *atp6* (Howard and Kempken, 1997). Loss of RNA editing is associated with CMS.

E. GENIC MALE STERILITY (GMS)

(Chaudhury, 1993; Horner and Palmer, 1995)

1. Nuclear genes causing male sterility

- a. *ms* genes in nucleus are the main class of male sterile genes
 - b. Genes such as *apetela-3 (ap3)*, *pistillata (pi)*, and *antherless (at)* in *Arabidopsis* which affect floral morphology produce structurally male sterile plants.
 - c. Genes which affect other characteristics in plants may have pleiotropic effects on male fertility—e.g., mutants of *CUT1*, an *Arabidopsis* gene involved in cuticular wax synthesis, cause male sterility because waxless pollen has problems interacting with the stigma—although this problem can be overcome if the pollen germinates in very humid conditions (Millar et al., 1999).
 - d. The flavonoid pathway is apparently important for normal pollen maturation. An antisense chalcone synthase gene was targeted to anthers in petunia, turning off pigment synthesis. Plants with white anthers were male sterile because male gametophyte development was arrested (van der Meer et al., 1992).
- 2. Most GMS systems are sporogenous**—i.e., they affect microsporogenesis or microgametogenesis—rather than structural.
- ### **3. Timing of GMS systems**
- a. Most *ms* genes act early in pollen development
 - b. Tapetum is primary tissue where many abnormalities occur
 - c. Tapetum is innermost wall of microsporangium—provides enzymes, hormones, food for developing PMCs and microspores

F. ENGINEERING MALE STERILITY

(Goldberg et al. 1993)

- a. Anther development (Fig. 1)
 - i. Tapetum–stomium/circular cell cluster–microspores are the major targeting sites for manipulation
 - ii. Tapetum involved in microspore maturation.
 - iii. Stomium/ccc involved in dehiscence of pollen grains.
- b. Two phases of development
 - i. phase 1: Histodifferentiation of various anther cell types (Fig. 3).
 - ii. phase 2: Cell degeneration and dehiscence (programmed destruction of CCC/connective and stomium leading to pollen release).
- c. Temporal and spatial regulation of gene expression (Figure 5 and Table 2) and identification of suitable promoters for engineering male sterility.
- d. Barnase/barstar system for engineered male sterility
(p. 1226-1227 in Goldberg et al., 1993)]
 - i. Barnase is extracellular RNase; barstar is inhibitor of barnase (both from bacterium *Bacillus amyloliquefaciens* which uses barnase for protection from microbial predators and barstar to protect itself from barnase.
 - ii. Fuse the barnase and barstar genes to TA29 promoter–TA29 is a plant gene that has tapetum specific expression–good for a wide range of dicot and monocot crop plants.
 - iii. Plants containing the TA29–barnase construct are male sterile; those with TA29–barstar are not affected by the transgene.
 - iv. Cross male sterile (barnase) with male fertile (barstar) to get hybrid seed, which now has both barnase and barstar expressed in tapetum and, hence, is fully fertile. Barstar is dominant over barnase.