Genetic characterization of the partial restorer of fertility gene, *Rf8*, in T cytoplasm maize

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics

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2010

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ABSTRACT

Texas (T) cytoplasm maize (*Zea mays* L.) is a model system for studying cytoplasmic male sterility and fertility restoration. The mitochondrial gene responsible for male sterility is T-*urf13*. Fertility is restored to T cytoplasm maize by the combined action of two nuclear genes, *Rf1* and *Rf2a*. *Rf1* is associated with the accumulation of additional 1.6/0.6 kb T-*urf13* transcripts while *Rf2a* is an aldehyde dehydrogenase and not involved in transcript accumulation. There are also partial restorers of fertility for T cytoplasm. *Rf8* and *Rf**, with the combined action of *Rf2a*, restore partial fertility and are associated with the accumulation of additional 1.42/0.42- and 1.4/0.4-kb T-*urf13* transcripts, respectively. *Rf8* was positioned on the long arm of chromosome 2 near *white pollen* (*whp1*) using a segregating backcross population. PCR markers were developed to map *Rf8*. Flanking PCR markers that span a physical distance of 4.56 Mb in the B73 maize genome sequence correspond to a genetic distance of 8.28 ± 3.25 cM. This region includes seven pentatricopeptide repeat genes. Partially male-fertile plants segregated for the presence or absence of the *Rf8*-associated transcripts, indicating that presence of the 1.42/0.42-kb transcript is not necessary for anther exertion. Fertility phenotypes decreased with successive generations of backcrossing to (N) W64A, where as re-introducing the (N) *wx1-m8* background increased fertility. The day after planting to the first flowering was observed for partially fertile and mostly fertile plants. Mostly male-fertile plants flowered 2.7 to 3.4 days earlier than partially male-fertile plants. These results demonstrate the flanking region *rf8* is located in and the possibility that male fertility is under the control of more than one nuclear locus.

CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

The main emphasis of this thesis is the genetic mapping of a partial restorer of fertility, *Rf8*, for Texas (T) cytoplasm maize. A review of T cytoplasm is presented in the first chapter. Topics included are the historical significance of T cytoplasm, the cytoplasmic male sterility (CMS) phenotype, sensitivity to host-selective toxins produced by *Cochliobolus heterostrophus* and *Mycosphaerella zeae-maydis*, the characteristics of T-*urf13* and its encoded URF13 protein, fertility restoration, and proposed mechanisms for CMS. Chapter 2 is a part of a paper to be submitted to The Plant Genome later this year, in combination with an *rf8/rf** linkage test performed previously by Deqing Pei. This paper describes the fine mapping of *Rf8* as well as fertility observations for *Rf8* plants. Last is a general conclusions chapter that describes the implications of this work and future experiments.

Literature Review

Introduction

Cytoplasmic male sterility (CMS) is the maternally inherited inability to produce functional pollen. Female fertility is unaffected. CMS is found in over 150 species (Laser and Lersten 1972; Wise and Pring 2002). There are three different major types of CMS in maize (Zea mays L.): Texas (T), USDA (S) and Charrua (C) (Beckett 1971). Nuclear genes can suppress the CMS phenotype and restore male fertility. T and C cytoplasms are characterized by sporophytic restoration, while S cytoplasm is characterized by gametophytic restoration. Sporophytic restoration means that all the male gametes (pollen) produced by a heterozygous individual for the restorer gene, regardless of the allele they carry, will be able to successfully fertilize the female gamete (ear). The restoration occurs at the diploid (sporophyte) level.

Gametophytic restoration means only the male gametes that carry the restorer allele will be able to successfully fertilize the female gamete because restoration is occurring at the haploid (gametophyte) level. CMS was recognized for its potential in producing hybrid seed in the 1950s (Duvick 1965). T cytoplasm became a successful way to produce hybrid seed in the 1950s and 1960s. However, T cytoplasm fell completely out of use because of significant crop loss during the southern corn leaf blight epidemic of 1970 (Pring and Lonsdale 1989; Ullstrup 1972).

T cytoplasm maize is highly susceptible to the host-selective T toxin of *Cochliobolus heterostrophus* (asexual stage *Bipolaris maydis*), the causal agent of southern corn leaf blight (Mercado and Lantican 1961). The mitochondrial gene, T-*urf13*, is responsible for male sterility and sensitivity to T toxin (Dewey et al. 1988; Wise et al. 1987). T cytoplasm maize is also highly susceptible to the PM toxin of *Mycosphaerella zeae-maydis* (asexual stage *Phyllosticta maydis*), the causal agent of yellow corn leaf blight (Scheifele and Nelson 1969; Scheifele et al. 1969). T cytoplasm plants restored to fertility are just as susceptible as nonrestored plants (Villareal and Lantican 1965). Losses from the 1970 epidemic were estimated at 700 million bushels (Ullstrup 1972). Since this epidemic, T cytoplasm has not been used for hybrid seed production.

Phenotype of CMS

The phenotype of T cytoplasm maize is dramatic. In normal cytoplasm, plump yellow anthers exert from the florets and pollen is shed. In T cytoplasm, the florets stay tightly closed and few anthers exert, if any. If exertion does occur, the anther is almost always brown and shriveled. There is no pollen shed in T cytoplasm plants and female fertility is not affected.

The tapetum is the inner most layer of cells around the locule which houses the developing microspores. This layer has more than one function. It provides the microspores with nutrients, it releases the microspores from the callose wall, and it produces the precursors for the development of the outer pollen wall (exine) (Bedinger 1992). As the exine develops, the tapetum undergoes programmed cell death that leads to the release of nutrients necessary for pollen development. Timing of the degradation is critical. Disruption usually leads to sterility.

In a normal cytoplasm, the microsporocytes are enclosed in a callose wall in the developing anther. During the meiocyte stage, the tapetal cells are undergoing mitosis without cytokinesis to become binucleate (Horner et al. 1993). The microsporocytes undergo two meiotic divisions to produce a tetrad of four microspores. The callose wall then degrades and the exine begins to develop. During the growth of the microspores, small vacuoles are present which merge into a single large vacuole. Asymmetrical division occurs producing a generative cell and a tube cell. The generative cell divides again producing two sperm cells. The tapetum begins to degrade giving off nutrients the pollen grains stores. The pollen then begins to dehydrate to prepare for dehiscence.

In T cytoplasm, the tapetum begins to enlarge and develop vacuoles as early as the tetrad stage (Warmke and Lee 1977; Yang 1989). The tapetum degradation is preceded by mitochondrial dysfunction. The mitochondria swell and become disorganized (Gengenbach et al. 1973; Warmke and Lee 1977; Watrud et al. 1975). Tapetal swelling causes the microspores to abort and the pollen stage is never reached.

CMS-associated tapetal degradation is not unique to maize. Tapetum degradation has been observed in other species including CMS radish (*Raphanus sativus*) (Liu et al. 2009), pigeonpea (*Cajanus cajan* L. Millspaugh) (Dalvi et al. 2008), pepper (*Capsicum annuum* L.)

(Luo et al. 2006), and sunflower (*Helianthus annuus*) (Zhang et al. 2010). In these species, the tapetum degenerates prematurely causing the death of the microspores. The tapetal degradation of CMS radish is characterized by numerous vacuoles in the tapetum similar to maize (Liu et al. 2009). CMS pigeonpea and sunflower are characterized by degeneration of the tapetum at the tetrad stage (Dalvi et al. 2008; Zhang et al. 2010). CMS pepper is slightly different than maize, radish, and pigeonpea. The tapetum degrades later at the uninuclete pollen stage instead of the tetrad stage (Luo et al. 2006). Because the tapetal layer is critical to pollen development, any disruption will cause sterility in a variety of species.

Restorer of fertility genes for T cytoplasm

CMS was first observed by Rhoades (1931). Restoration of fertility in T cytoplasm was initially thought to be controlled by one dominant gene (Edwardson 1955). However after crossing five fertility restoring lines (Ky21, K55, BH2, F5DD1 and WG3) to (T) Wf9, it was discovered that complete fertility is restored by the combined action of two dominant genes, *Rf1* and *Rf2* (Duvick 1956). *Rf2* is present in many inbred lines (Dill et al. 1997). *Rf1*and *Rf2* are located on chromosomes *3* and *9*, respectively (Duvick et al. 1961; Synder and Duvick 1969). *Rf2a* was the first restorer of fertility gene cloned from any species (Cui et al. 1996). It encodes an aldehyde dehydrogenase (Liu et al. 2001).

Dewey et al. (1986) first found two unique 1.6/0.6 kb transcripts in restored (T) B37 (*Rf1/Rf1, Rf2/Rf2*) as compared to sterile (T) B37 (*rf1/rf1, Rf2/Rf2*) when using portions of T-*urf13* (historically designated ORF13) sequence as probes for RNA gel blot analysis. Kennell et al. (1987) also observed the 1.6 kb transcript in five different restored inbred lines. Dewey et al. (1987) showed that *Rf1* alone is responsible for the accumulation of the 1.6 kb transcript by showing only plants *Rf1/--*, *Rf2/--* and *Rf1/--*, *rf2/rf2* displayed the transcript while *rf1/rf1, Rf2/--*

and *rf1/rf1, rf2/rf2* did not. Four *rf1-m* alleles were identified which showed reduced or no accumulation of the 1.6/0.6 kb transcripts (Wise et al. 1996). Taken together, these experiments show *Rf1* is associated with the accumulation of additional 1.6/0.6 kb T-*urf13* transcripts while *Rf2* is not involved in transcript processing.

Partial fertility is restored by the combined action of *Rf2a* and one of two nuclear genes, *Rf8* or *Rf**. *Rf8* and *Rf** are associated with the accumulation of additional 1.42/0.42 kb and 1.4/0.4 kb T-*urf13* transcripts, respectively (Dill et al. 1997). Dill et al. (1997) also reported that *Rf8* is environmentally sensitive and incompletely penetrant. *Rf8* is rare in maize germplasm. In a survey of 10 inbred lines, Dill et al. (1997) only found *Rf8* in the inbred line (N) *wx1-m8*. Pei (2000) showed *Rf8* is linked to *Rf**, another uncharacterized partial restorer allele.

Rf8 and *Rf** map near the *white pollen* (*whp1*) gene on chromosome *2L* (Pei 2000). *whp1* is a visual marker historically used because of its easily identifiable phenotype. S cytoplasm fertility restorer *Rf3* also maps to *2L* (Laughnan and Gabay-Laughnan 1983). Fine mapping positioned *Rf3* 4.3 cM distal to *whp1* (Kamps and Chase 1997). In addition, five *Rf* alleles from Mexican maize capable of restoring S cytoplasm were linked to *whp1* (Gabay-Laughnan et al. 2004). Clearly this complex locus is a hotspot for fertility restoration.

T-*urf13* and URF13

The T-*urf13* sequence is a unique rearrangement of mitochondrial DNA. Upstream of T-*urf13*, there is a 5 kb repeat similar to the ATPase subunit 6 gene (*atp6*). The *atp6* gene is found in normal and T cytoplasms while the duplication of this 5 kb segment is only found in T cytoplasm (Dale et al. 1984). This segment contains promoter sequences 5' to *atp6*. The 345 bp T-*urf13* coding sequence is similar to the 3' region of the 26S rRNA mitochondrial gene (*rrn26*) (Dale et al. 1984; Dewey et al. 1986). The 58 bp at the 3' terminus of T-*urf13* is 100% similar to

1,055-1,110 bp of *rrn26* which encode ribosomal RNA helices (Dale et al. 1984). Co-transcribed with T-*urf13* is the 663 bp *orf221*. At the 3' terminus of *orf221* is sequence similar to $tRNA^{Arg}$ that appears to be of chloroplast origin (Dewey et al. 1986). Multiple recombinations are needed to produce this unique mitochondrial sequence.

T-*urf13* encodes a 13 kD protein designated URF13 (Wise et al. 1987a). This is a ligand-gated, pore-forming protein in the inner mitochondrial membrane (Dewey et al. 1987; Rhoads et al. 1995). The T toxin binds to the protein causing a conformational change and ion leakage leaving the mitochondria incapable of oxidative phosphorylation (Braun et al. 1989; Matthews et al. 1979). Wise et al. (1987b) found a frameshift T-*urf13* mutant in maize, designated T-4, to be toxin insensitive. The T-4 T-*urf13* sequence is characterized by a guanine to adenine single nucleotide polymorphism at 213 bp and a five bp insertion at 214 bp (Wise et al. 1987b). These changes make the T-4 sequence homologous to 86 bp 3' of *rrn26* (Wise et al. 1987b). T-4 URF13 protein was truncated from 113 amino acids to 74 due to a frameshift that created a premature stop codon. Similarly, Braun et al. (1989) showed that URF13 must contain at least the first 83 amino acids to be toxin sensitive when expressed in *E. coli*. These studies show the carboxy terminal end of the protein is not essential for toxin sensitivity. Site directed mutagenesis of the aspartate at position 39 revealed this amino acid is required for the URF13/T toxin interaction (Braun et al. 1989).

There are seven major transcripts of T-*urf13*: 3.9, 2.0, 1.8, 1.5, 1.1, 1.0, and 0.8 kb (Dewey et al. 1986; Dill et al. 1997; Kennell and Pring 1989; Kennell et al. 1987; Wise et al. 1996). Many of these transcripts are thought to be processing events of the 3.9 kb transcript (Kennell and Pring 1989). Restored T cytoplasm plants carrying the *Rf1* allele are characterized by the presence of the 1.6/ 0.6 kb transcripts. The 1.6 kb transcript is a derivative of the 2.0 or

1.8 kb transcripts while the 0.6 kb transcript is a derivative of the 1.0 kb transcript (Kennell and Pring 1989; Kennell et al. 1987). Similarly, the 1.42 kb *Rf8*-mediated transcript is a derivative of the 2.0 or 1.8 kb transcript while the 0.42 kb transcript is a derivative of the 1.0 kb transcript (Dill et al. 1997).

A mechanism for T-*urf13*-mediated male sterility

The mechanism of T-*urf13*-mediated male sterility is poorly understood. It is unclear why T-*urf13* causes degradation of the tapetal layer in the absence of T toxin. One possibility suggests the mechanism for CMS and toxin sensitivity are the same (Flavell 1974). Although all T cytoplasm maize cells express the T-*urf13* gene (Hack et al. 1991), there many examples of tissue specific regulation of mitochondrial genes (Conley and Hanson 1994). The presence of a currently unidentified toxic "factor X" that is only active in the tapetal cell layer could induce tapetum degradation. Another possibility states that since different tissues have various metabolic activity levels, tissue specific degradation might occur if the metabolic activity is too high (Warmke and Lee 1978). Microsporogenesis requires a very high rate of metabolic activity. There is a 40 fold increase in mitochondria number in the tapetal layer while the nucellus and embryo sac have no significant increase (Lee and Warmke 1979). This could account for the male sterile yet female fertile nature of T cytoplasm.

Restorer of fertility genes in other species

To date, there are five species with cloned restorer of fertility genes—maize (Cui et al. 1996), petunia (*Petunia hybrida*) (Bentolila et al. 2002), radish (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003), rice (*Oryza sativa*) (Kazama and Toriyama 2003; Komori et al. 2003) and sorghum (*Sorghum bicolor*) (Klein et al. 2005). *Rf* genes seem to function by altering the expression of CMS-associated mitochondrial ORFs. With the exception of *Rf2a* of maize, all

other *Rf* genes contain PPR motifs (reviewed in Andrés et al. 2007; Saha et al. 2007; Schmitz-Linneweber and Small 2008). These motifs were first named by Small and Peeters (2000). PPR proteins are RNA-binding proteins specific to mitochondria and chloroplast with roles in RNA editing, stabilization and cleavage. This protein family is greatly expanded in terrestrial plants (O'Toole et al. 2008).

PPR proteins are divided into the P and PLS subfamilies (Lurin et al. 2004). PPR proteins are unique to eukaryotes while the PLS subfamily is unique to land plants (Lurin et al. 2004). The P subfamily includes proteins that have the 35 amino acid P motif tandemly repeated on average 12 times (Lurin et al. 2004). The PLS subfamily is characterized by two other plant specific motifs, PPR-like L (long) and the PPR-like S (short) (Lurin et al. 2004). Proteins from this subfamily have the P, L, and S motifs tandemly repeated, usually in this order.

There are three additional C terminal domains that are only found in some plant PPR-PLS proteins: E, E+, and DYW. These domains are almost never found in multiple copies. They are usually observed in the E - E+ - DYW order so that DYW is always C terminal. These domains are nested in an orderly fashion. If a protein has a DYW domain, it is almost always preceded by the E+ domain. If a protein has the E+ domain, it is almost always preceded by the E domain (Lurin et al. 2004). Cloned radish, rice, and petunia *Rf* genes are all members of the P subfamily of PPR proteins (Saha et al. 2007) while *Rf1* of sorghum is a PLS PPR containing an E terminal domain (Klein et al. 2005). How this E domain affects the mode of action for the sorghum *Rf1* gene is unknown.

CMS-associated PPR encoding genes are usually present in clusters typically with only one functional gene (Lurin et al. 2004). Each gene in the clusters shows high similarity suggesting recent gene duplication. The cloned *Rf* genes in petunia, radish, and rice are present

in clusters of PPR genes (Akagi et al. 2004; Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Komori et al. 2003). Interestingly, *Rf1* of sorghum is not located in a PPR cluster (Klein et al. 2005). A candidate gene has been identified for *Rf2* of sorghum. Similar to sorghum *Rf1*, the candidate gene is a lone PPR protein (Jordan et al. 2010). The differences between sorghum and the other species' *Rf* genes could be clues to the *Rf1* and *Rf8* loci since sorghum is a close relative of maize.

The recently sequenced B73 genome is a tremendous resource for the maize community. Integrated physical and genetic maps provide valuable resources for mapping. Faster and more efficient marker development allows for even finer resolution on genetic maps. Gene annotations and expressed sequence tag (EST) alignments provide essential information on features in a given region.

It is important to understand CMS/*Rf* systems because of their potential in creating hybrid seed. It is also important to learn about nuclear/plastid genome interactions as mitochondria significantly affect plant development. There are many unresolved questions about T cytoplasm maize. What are the molecular mechanisms underlying expression of *Rf1* and *Rf2a*? Why are restored T cytoplasm plants still highly susceptible to T toxin? Why do the truncated transcripts associated with *Rf1*, *Rf8*, and *Rf** correspond to the reduction of the URF13 protein? Do *Rf1*, *Rf8*, and *Rf** have functions in N cytoplasm? Are *Rf1*, *Rf8*, and *Rf** PPR proteins? What is the structure of the fertility locus on *2L*? Genetic mapping of the *rf8* locus is a first step in understanding T cytoplasm in greater detail.

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CHAPTER 2. RNA PROCESSING ACTIVITY ASSOCIATED WITH THE *Rf8* PARTIAL RESTORER OF FERTILITY COINCIDES WITH A PENTATRICOPEPTIDE REPEAT CLUSTER ON MAIZE CHROMOSOME *2L*

A paper to be submitted to The Plant Genome

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Abstract

Cytoplasmic male sterility (CMS) is often associated with chimeric mitochondrial open reading frames. In T cytoplasm maize, CMS results from the action of the unique T-*urf13* mitochondrial gene. Full (or partial) fertility restoration of T-cytoplasm maize is mediated by the *Rf2a* nuclear gene, in combination with one of three other genes: *Rf1*, *Rf8*, or *Rf**. *Rf2a* encodes a mitochondrial aldehyde dehydrogenase; *Rf1*, *Rf8*, and *Rf** are all associated with unique T-*urf13* mitochondrial transcript processing activity. Codominant and cleaved amplified polymorphic sequence (CAPS) markers were derived from the filtered gene set of Maizesequence.org release 4a.53, in order to amplify introns and 3' untranslated regions (UTRs), and used on a backcrossed population of 1,731 plants to genetically map *Rf8* to the *whp1*-umc36 interval on chromosome *2L*. RNA processing activity associated with *rf8* maps to a 4.56 Mb region on *2L* that contains seven pentatricopeptide repeat (PPR) encoding genes in B73. *Rf3,* which restores S cytoplasm, has also been mapped to this PPR cluster. Partially male-fertile plants segregated for the presence or absence of the *Rf8*-associated 1.42/0.42-kb transcripts,

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indicating that the presence of the transcript is not necessary for anther exertion. Plants with successive amounts of backcrossing and test-crossing were analyzed for their fertility phenotypes. Plants re-introduced with (N) *wx1-m8* (*rf1/rf1, Rf2/Rf2, Rf8/rf8*) background showed more fertility than plants backcrossed to (N) W64A (*rf1/rf1, Rf2/Rf2, rf8/rf8*). A statistically significant 2.7 to 3.4 day delay in flowering was observed between partially male-fertile and mostly male-fertile plants. Mostly sterile plants flowered at a significantly later day after planting than mostly fertile plants. Combined, these new results demonstrate the possibility that male fertility is under the control of more than one nuclear locus.

Abbreviations: cytoplasmic male sterility, CMS; pentatricopeptide repeat, PPR; sequence characterized amplified region, SCAR; amplified fragment length polymorphism, AFLP; bulk segregant analysis amplified fragment length polymorphism, BSA-AFLP; rapid amplified polymorphic DNA, RAPD; restriction fragment length polymorphism, RFLP; codominant amplified polymorphic sequence, CAPS; hexadecyltri-methylammonium bromide, CTAB; days after planting of the first flowering, DAPFF; restorer of fertility, *Rf*; *Rf*-like, RFL; untranslated region, UTR; tetratricopeptide repeat, TPR

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited inability to produce functional pollen. There are three types of CMS cytoplasm in maize (*Zea mays* L.): T, S, and C (Beckett, 1971). The mitochondrial gene responsible for male sterility in T cytoplasm is T-*urf13* (Dewey et al., 1987; Wise et al., 1987b). This gene encodes a 13 kDa protein designated URF13, which localizes to the inner mitochondrial membrane (Dewey et al., 1987; Wise et al., 1987a). T cytoplasm is also highly susceptible to T toxin produced by the ascomycete fungus, *Cochliobolus heterostrophus* (asexual stage *Bipolaris maydis*), the casual agent of southern corn leaf blight.

The presence of specific nuclear genes mediates the restoration of male fertility to CMS plants. In T cytoplasm, this is accomplished by the combined action of *Rf1* and *Rf2a* (Duvick, 1956; Wise et al., 1999a). *Rf1* mediates a series of mitochondrial transcript processing events, resulting in the accumulation of additional 1.6/0.6-kb T-*urf13* transcripts, whereas *Rf2a* is an aldehyde dehydrogenase and is not involved in transcript processing (Liu et al., 2001).

T cytoplasm maize also has nuclear genes that restore partial fertility. *Rf8* and *Rf** mediate partial restoration in combination with *Rf2a* (Dill et al., 1997). *Rf8* and *Rf** are associated with the accumulation of additional 1.42/0.42- and 1.4/0.4-kb T-*urf13* transcripts, respectively (Dill et al., 1997). While *Rf1* and *Rf2a* have been genetically mapped to chromosomes *3* and *9* respectively (Schnable and Wise, 1994), the position of *Rf8* has not yet been reported. Restriction fragment length polymorphism (RFLP) analyses of families segregating for *Rf8* showed that *Rf8* and *Rf1* are independent and unlinked (Dill et al., 1997). Because *Rf8*-associated flowering is environmentally sensitive, the most reliable way to assay the plants for *Rf8* is to determine via RNA gel blot analysis if the1.42/0.42-kb transcripts are present. *Rf8* and *Rf1* have similar molecular phenotypes. Both genes are associated with additional accumulation of T-*urf13* transcripts, and both are reported to restore at least some fertility to T cytoplasm plants (Dill et al., 1997; Wise et al., 1987a). Both genes are also associated with decreased accumulation of URF13 (Dewey et al., 1987; Dill et al., 1997). The *Rf8*-mediated URF13 reduction is less pronounced in ears than tassels, whereas the *Rf1*-mediated reduction occurs equally in ears and tassels (Dill et al., 1997; Wise et al., 1987a).

Of the five species with cloned restorer of fertility genes, four encode pentatricopeptide repeat proteins (PPR) (Akagi et al., 2004; Bentolila et al., 2002; Klein et al., 2005; Koizuka et al., 2003). PPR proteins are RNA binding proteins specific to mitochondria with functions in

editing, stabilization, and cleavage (Lurin et al., 2004; Small and Peeters, 2000). The cloned *Rf* genes of radish (*Raphanus sativus*), rice (*Oryza sativa*) and petunia (*Petunia hybrida)* are present in clusters of PPR encoding genes with one functional gene and multiple pseudo-genes suggesting recent gene duplication (Akagi et al., 2004; Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Komori et al., 2003). The *Rf1* gene of sorghum (*Sorghum bicolor*), interestingly, is not located in a PPR cluster (Klein et al., 2005). Because *Rf1*, *Rf8*, and *Rf** are associated with the additional accumulation of T-*urf13* transcripts, the possibility exists that they encode PPR proteins as well.

Analogous to T-*urf13, orf355-orf77* is the mitochondrial gene responsible for male sterility in S cytoplasm maize (Zabala et al., 1997). Likewise, S cytoplasm maize utilizes the nuclear gene *Rf3* to restore fertility to male sterile plants. *Rf3* cosegregates with a novel *orf355-orf77* transcript accumulation, suggesting an RNA editing function (Wen and Chase, 1999). The *rf3* locus was mapped to *2L* by Laughnan and Gabay (1978) by translocation and inversion heterozygotes. Kamps and Chase (1997) placed *rf3* 4.3 cM distal to RFLP whp1 and 6.4 cM proximal to RFLP bnl7.14. Shi et al. (1997) mapped *rf3* 4.8 cM distal to RFLP umc49 and 2.7 cM proximal to a rapid amplified polymorphic DNA (RAPD) marker, *E08-1.2*. Zhang et al. (2006) placed *rf3* 2.4 cM distal to a cleaved amplified polymorphic sequence (CAPS) marker and 1.8 cM proximal to a sequence characterized amplified region (SCAR) marker. Xu et al. (2009) observed cosegregation of *Rf3*-mediated fertility with three PPR encoding genes on *2L* in 900 segregating individuals.

In order to understand T cytoplasm in greater detail, the sequence and function of the *rf8* locus needs to be elucidated. The objective of this study was to identify PCR-based markers closely linked with *rf8*. Here we describe the mapping of *rf8* to a 4.56 Mb region on *2L* located

in contig 108 of Maizesequence.org release 4.53a. This region includes the *Rf3* candidate gene region including seven PPR genes. Plants restored to partial fertility segregated independently of the *Rf8*-associated 1.42/0.42-kb transcripts, suggesting the possibility of additional factors affecting pollen exertion in the genome.

Methods

Maize nomenclature

Loci and recessive alleles are designated by lowercase symbols, e.g., the *rf8* allele of the *rf8* locus is a recessive mutant. Dominant alleles are designated by uppercase symbols, e.g., the *Rf8* allele of the *rf8* 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.

Plant material

(N) W64A (*rf1/rf1, Rf2/Rf2, rf8/rf8*) and (N) *wx1-m8* (*rf1/rf1, Rf2/Rf2, Rf8/Rf8*) were the two primary inbred lines used in this study. As illustrated in Figure 1, our initial population consisted of progeny derived from a single cross, (T) *Rf8-8703*/*rf8-W64A* x (N) *rf8-W64A*/*rf8-W64A* BC_2, grown in the 1997 summer nursery at the Iowa State University Curtiss Research Farm in Ames, IA. One hundred seventeen segregating individuals were crossed by (N) *rf8-W64A/rf8-W64A* and second (unfertilized) ears were collected from each for DNA and RNA extractions. Ten plants from this 1997 population that possessed the T-*urf13*-derived 1.42/0.42-kb transcripts were interpreted as harboring the *Rf8* allele (genotype *Rf8-8703/rf8-W64A*), and thus, crosses derived from them were selected to create the 2008 high-resolution BC_3 population (see Table 2). This population was also grown at the Curtiss Research Farm in 2008. Young

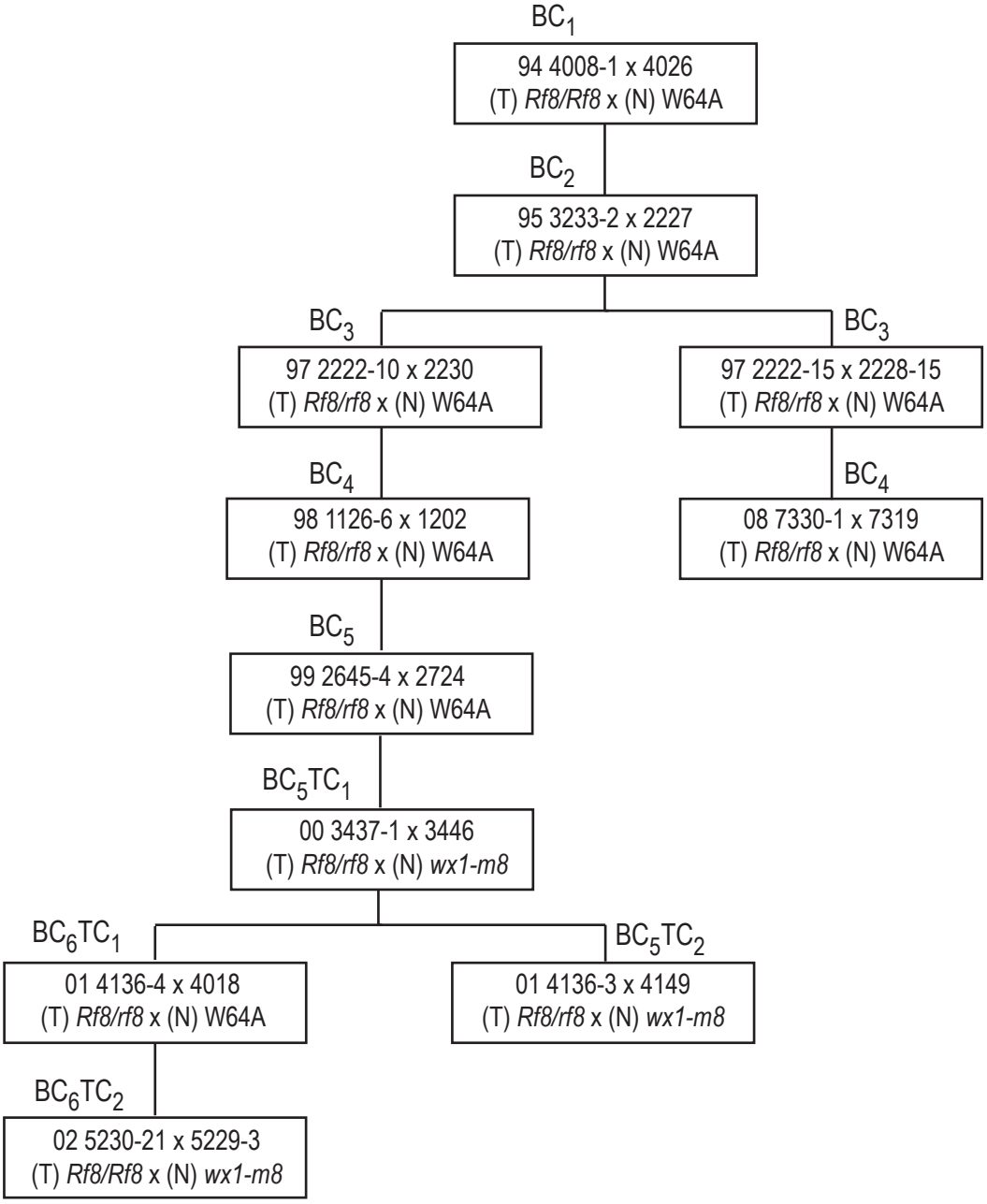

Figure 1. Pedigree of *Rf8*. Each box represents a cross. Plants were either backcrossed to (N) W64A (*rf1/rf1, Rf2/Rf2, rf8/rf8*) or test-crossed to (N) *wx1-m8* (*rf1/rf1, Rf2/Rf2, Rf8/Rf8*). Transcripts associated with *Rf8* were identified in the 1994 generation.The 1997 mapping population is a BC_3 generated from one cross. The 2008 mapping population is a BC_4 generation and is generated from ten 1997 individuals possessing the *Rf8*-associated 1.42 kb transcripts. Plants grown for fertility observation in 2009 are progeny from crosses BC_6, BC_5TC_1, BC_4 (2008 cross only), BC_6TC_1, BC_5TC_2, BC_7TC_1, and BC_6TC_2.

leaf tissue was collected from 1,731 individuals for DNA extractions and fertility phenotypes were recorded from adult plants. Tissue for total RNA extractions was collected from immature second ears from 952 individuals and 1,584 plants were crossed by (N) *rf8-W64A/rf8-W64A*.

Observation of fertility phenotypes

Male fertility was scored based on the four category system—S, sterile; "S", partially fertile; "F", mostly fertile; F, fertile—as described in Schnable and Wise (1994). Sterile indicates no anther exertion; partially fertile indicates >0 but <50% of the anthers on the tassel exerted; mostly fertile indicates >50% but <100% of the anthers exerted; fertile indicates 100% of the anthers exerted. The 2008 field was observed for fertility every day for 17 consecutive days, starting at the beginning of flowering (8/4/2008) and ending three days after anther exertion from the last plant (8/20/2008).

Plants containing an *Rf8* allele appear to lose fertility with increasing numbers of backcrosses to (N) W64A. To account for this observation, plants with successive amounts of backcrossing to (N) W64A and test-crossing to (N) *wx1-m8* were grown in 2009 at Curtiss Research Farm. The origin of the *wx1-m8* stock is described in detail in Wise and Schnable (1994) and is illustrated in Figure 1. Sixty progeny each from five crosses BC_5TC_1, BC_4 (2008 cross only), BC_6TC_1, BC_5TC_2, and BC_6TC_2 were grown. Fertility observations and leaf tissue for DNA analysis were collected from all plants. A subset of plants was tested for fertility by crossing as males onto (T) W64A or (N) *wx1-m8*. Pollen was tested from each genotype that flowered and all plants tested produced kernels.

DNA isolation and analysis

For the 1997 mapping population, DNA was extracted using a 1g Hexadecyltri-Methylammonium Bromide (CTAB) extraction (Wise et al., 1996). One hundred seventeen

individuals were subjected to Bulked Segregant Amplified Fragment Length Polymorphism (BSA-AFLP) analysis as described by Wei et al. (1999).

For the 2008 mapping population, isolation of DNA was performed using a modified 96-well CTAB extraction (Dietrich et al., 2002). PCR primers were designed from the filtered gene set on Maizesequence.org (Schnable et al., 2009) to amplify introns or 3' UTRs from linked genes (see Table 1). Primers were designed to be codominant markers, CAPS markers, or size polymorphic markers. PCR conditions were 3 min at 95°C, 30 sec at 95°C, 30 sec at Tm, 1.5 min at 72°C, 40 cycles, 10 min for 72°C, hold 4°C.

To efficiently screen the large 2008 mapping population, PCR primers were derived from the RFLP markers used in the 1997 mapping study. Overgo sequences were located at MaizeGDB.org (Lawrence et al., 2008) for the csu811 and umc36 RFLPs. These sequences were blasted against the maize genome using Maizesequence.org release 4a.53 (Schnable et al., 2009). These overgos aligned to two genes on *2L* (see Table 1). From these genes, PCR primers were designed to amplify interior portions of these genes.

RNA isolation and analysis

Total RNA was isolated from one gram of frozen second immature ear tissue via a Trizol-like reagent: 38% saturated phenol pH 4.3, 1 M guanidine thiocyanate, 1 M ammonium thiocyanate , 0.1 M sodium acetate pH 5.0, and 5% glycerol (Caldo et al., 2004). Eight µg of RNA were denatured with glyoxal (Ambion, Austin, TX) and size fractionated on a 1.8% SeaKem GTG agarose gel (FMC, Rockland, ME) with 0.01M iodoacetic acid (Sigma, St. Louis, MO) for 14 hours at 4°C. The gel and the circulating running buffer was 10mM Na_2HPO_4 pH 7.0. RNA was transferred to a Hybond XL membrane (GE Healthcare /Amersham Biosciences) for 4 hours using 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) as a transfer buffer, and

Table 1. List of *Rf8* mapping primers

Marker[†]	Located in Gene[‡]	Marker Type	Primer Sequence (5'-3')	Restriction Enzyme	Tm
76755p2	GRMZM2G076755	codominant	TGAAGAAATGGTGATGCGAGC ACAGATGGCACTCCTGATGTGTC	--	56
135087p1	GRMZM2G135087	size polymorphism	TGGAATACTTGCTTCTTGCTTGG CAATGGTTATGCGTGAACGGG	--	56
144635p4	GRMZM2G144635	codominant	TTGTGCTTGGGCTTTTCACG CCTGACTTCCTGCTTTTGTATCGC	--	54
66902p1	GRMZM2G066902	codominant	CGCTAACGCTTTCCTCTTGGAC CTGTTCCCCATCCTTTCTACATC	--	56
csu811_p9	AC217293.3_FGT007	codominant	CGAGGTCGAATCAAATTCTTCC GTACGGGCGGTTAAAGAAAC	--	56
J04p6	GRMZM2G108171	size polymorphism	CAAAGTCTCTGTCACTGTCACCTGG TCTTCTTCCTCCTCCCTTGGAC	--	60
J04p8	GRMZM2G108171	size polymorphism	TTAGTTGATTAGAGGAGGTTGCGG GTCATTTAGCGTTTAGCGTCCAAG	--	60
18p4	GRMZM2G092284	CAPS[§]	AAGATCATTCGGCGCGAGAA CGGAGCCAAAACATGTGAAA	*Mse*I	56
87p2	GRMZM2G035807	CAPS	ACATTGGTCTTTGTGGAGAC TTCACACCCAACAGGTTGAC	*Dra*I	56
10p2	GRMZM2G149935	CAPS	CGTAATGAAATGCGACGACG CGTAGCCAGGTCCATTAGCA	*Mse*I	56
26p3	GRMZM2G147819	CAPS	AGTTAAGGCTATCAGAATGA ACTGACGATCAAATCTGATC	*Apo*I	56
umc36_p10	GRMZM2G059033	codominant	CCTGGTGCACCATGTGATAGTTT TTACCATGCCAATGGAATTG	--	56

[†]Marker names are based on the predicted gene they are designed from on Maizesequence.org release 4a.53 (Schnable et al., 2009).

[‡]Genes are from the filtered gene set from Maizesequence.org release 4a.53.

[§]Cleaved amplified polymorphic sequence marker.

crosslinked with 220 MJ of UV light emitted by 312 nm bulbs in a Stratalinker 2400 (Stratagene, La Jolla, CA), followed by baking at 80°C for 1 hour. The fixed RNA was de-glyoxylated by treating the membrane in 20 mM Tris-Cl pH 8 at 65°C for 30 minutes. The T-*urf13* derived T-st308 DNA probe (Wise et al., 1996) was used for hybridization. Probe DNA was random labeled with α-^{32}P dCTP (Perkin Elmer, Waltham, MA) (Feinberg and Vogelstein, 1983). Hybridization was carried out for 18 hours at 65°C in 7% SDS, 1% BSA, 1mM Na_2EDTA, 0.5 M $NaHPO_4$, pH 7.2 (Church and Gilbert, 1984). Membranes were incubated at 65°C in 1x SSPE 0.1x SDS (20x SSPE contains 0.2 M monobasic sodium phosphate, 3.6 M NaCl, 20 mM EDTA, pH 7.4) for two 30 minutes washes, followed by a one hour wash. A more stringent wash in 0.1x SSPE 0.1x SDS was done for 15-20 minutes and membranes were exposed to CL-XPosure film (Thermo Scientific, Rockford, IL) for 1-10 days at -80°C using two Dupont Cronex Lightning Plus intensifying screens (Sigma, St. Louis, MO).

Results

The *rf8* locus maps to maize chromosome *2L*

To position the gene encoding the *Rf8*-associated T-*urf13* processing activity on the maize genetic map, DNA from 117 individuals from the 1997 mapping population were tested for *Rf8*-associated transcripts via RNA gel blot analyses. This information was utilized in the design of a BSA-AFLP strategy (see Methods). Two-hundred fifty six pairwise combinations of *Eco*RI and *Mse*I primers were tested on the mapping parents, (T) *Rf8-8703*/*rf8-W64A* and (N) *rf8-W64A*/*rf8-W64A*, and two contrasting DNA pools; one representing 16 progeny displaying the T-*urf13*-derived 1.42/0.42-kb transcripts and the other representing 16 progeny without the 1.42/0.42-kb transcripts. Three-hundred twenty

five polymorphisms were found with twenty conserved between the mapping parents. Of these, three polymorphic AFLPs confirmed linkage to *Rf8*. Sequence tagged site markers were designed from these cloned AFLP fragments (Yu and Wise, 2000) and one, designated *ias21*, displayed a polymorphism between the T232 and CM37 parents of the Brookhaven mapping population (Burr et al., 1988). The *ias21* forward and reverse primers, 5'-TGCCACACTTTATCTAAGGTT-3' and 5'-TTGCTTTTGCGACAACGACGA-3', respectively, corresponding to *Arf8.3* (E-AGA/M-CTA) AFLP, were used to amplify a DNA fragment that cosegregated in the Brookhaven low-resolution mapping population with *whp1* on *2L*. RFLP markers linked to *whp1* were also tested and csu811 and umc36 cosegregated closely with *Rf8*.

Positioning *rf8* on the maize genome sequence

Results derived from the 1997 BC_2 mapping population indicated that the gene mediating the accumulation of the additional 1.42/0.42-kb T-*urf13* transcripts is closely linked to the csu811 and umc36 RFLPs near *whp1* on *2L* (Pei, 2000). In order to further characterize the *rf8* locus and take advantage of the newly sequenced maize genome (Schnable et al., 2009), progeny from ten 1997 BC_3 crosses were used to create a large 2008 BC_3 mapping population. RFLP markers csu811 and umc36 were converted into the PCR markers csu811_p9 and umc36_p10 by amplifying the gene associated with the RFLP (see Table 1).

Two hundred fifty-three primer pairs were designed to amplify 3'UTRs and introns of genes around the *whp1*-csu811-umc36 region. Amplicons derived from these primers were screened against (N) *wx1-m8* and (N) W64A for size polymorphisms. CAPS markers were developed from sequence of monomorphic amplicons of the parents and a small subset of

segregants. This allowed for the identification of informative SNPs that differentiate the *wx1-m8* and W64A parents present in the mapping population. As shown in Table 1, ten additional markers were linked to *Rf8*. These markers were tested on the population and, along with RNA blot analysis to determine accumulation of the 1.42/0.42-kb T-*urf13* transcripts, genetic distance was calculated (see Figure 2 and Table 2). Based on this analysis, *rf8* resides between polymorphic markers 76755p2 and 135087p1. Figure 3 displays the flanking markers on a subset of the segregating population. The flanking region is an 8.28 ± 3.25 cM region in the 2008 population, which corresponds to a 4.56-Mb region in B73 in contig 108.

Table 2. Comparison of genetic distances among PCR markers flanking the *rf8* locus

Interval	No. of Plants Tested	Minimum No. of Recombinant Plants	Maximum No. of Recombinant Plants	Genetic Distance[†]	B73 Physical Size[‡]	kb/cM Ratio
76755p2 to 135087p1	616	31	71	8.25 ± 3.25	4.56 Mb	552.73
135087p1 to 144635p4	701	1	54	3.92 ± 3.78	0.81 Mb	206.63
144635p4 to 66902p1	700	22	75	6.93 ± 3.79	2.01 Mb	290.04
66902p1 to csu811_p9	1141	22	30	2.28 ± 0.35	0.84 Mb	368.42
csu811_p9 to umc36_p10	1106	40	43	3.75 ± 0.14	1.00 Mb	266.67

[†]Genetic distances are averages of the maximum and minimum amount of recombination possible for a given region.

[‡]Corresponding B73 physical size taken from Maizesequence.org release 4a.53 (Schnable et al., 2009)

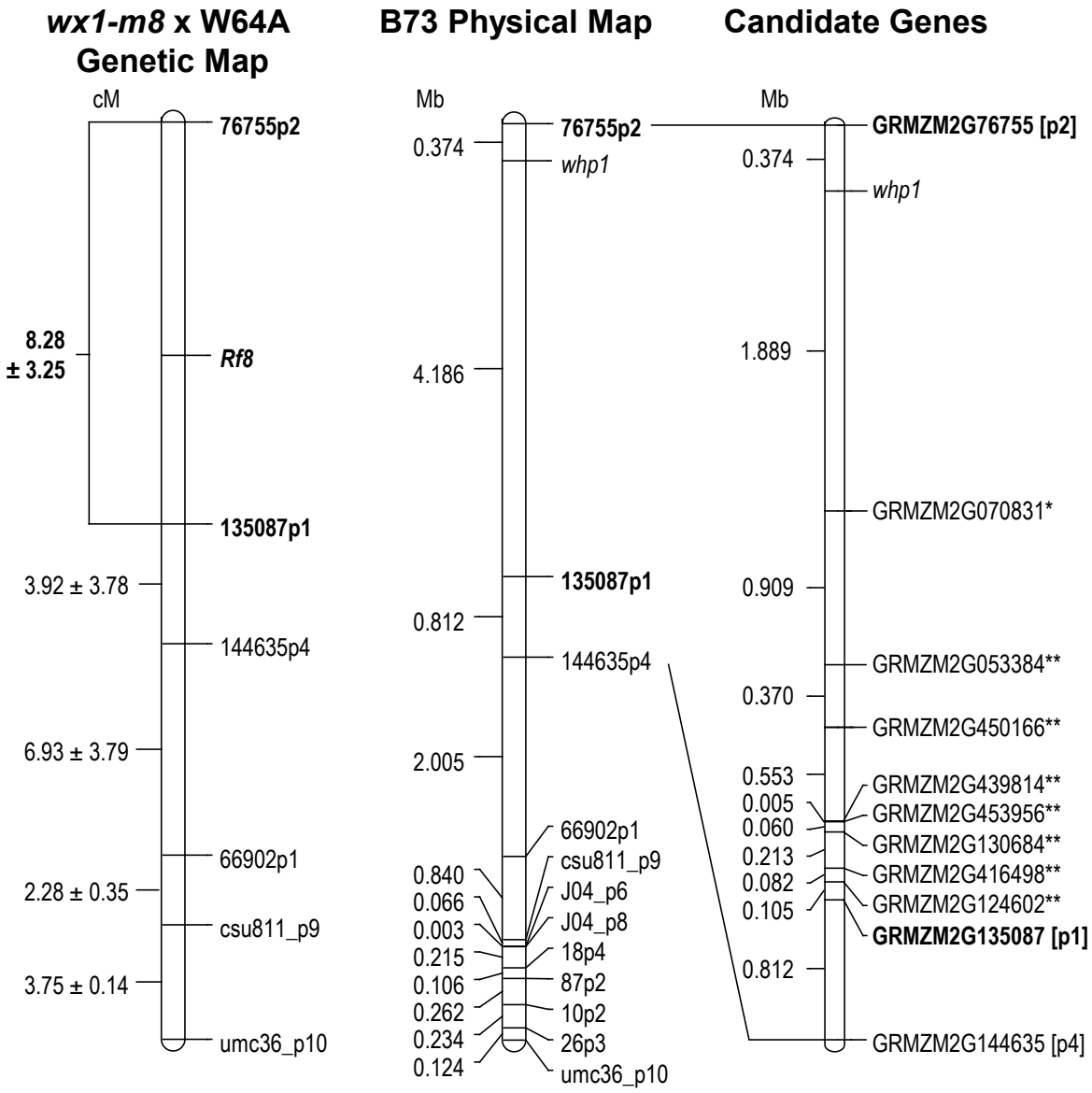

Figure 2. *wx1-m8* x W64A genetic map, B73 physical map, and candidate genes in the flanking region. *Rf8* is located is the 8.28 ± 3.25 cM flanking region between PCR markers 76755p2 and 135087p1 on 2L corresponding to contig 108. Interval distances in the genetic map are in centimorgans and interval distances in the physical maps are in megabases. Positions of primers and names of genes are taken from Maizesequence.org release 4a.53 (Schnable et. al. 2009). The flanking region contains seven PPR genes (denoted **) and one pre-mRNA processing gene (denoted *).

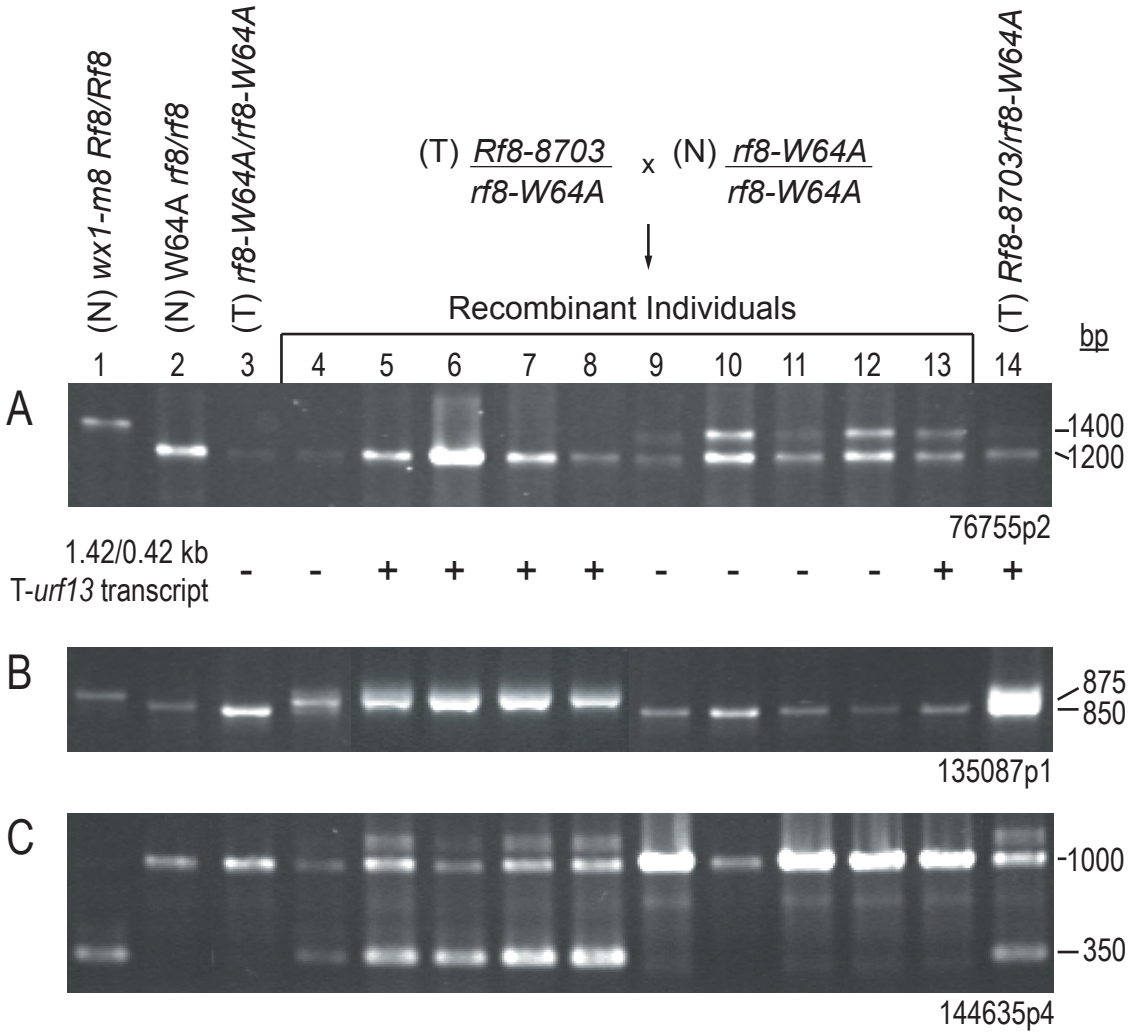

Figure 3. PCR markers illustrating the *Rf8* flanking region. Panel A contains marker 76755p2, panel B contains 135087p1, and panel C contains 144635p4. Lanes 1 and 2 display the polymorphism between the mapping parents of the 2008 mapping population. Lanes 3-14 are a subset of the segregating 2008 progeny in order of recombination breakpoint. Lanes 3, 4, and 9-12 demonstrate the absence of the *Rf8*-associated 1.42 kb transcript while lanes 5-8, 13, and 14 display the presence of the 1.42 kb transcript. Lanes 4 and 13 display a recombination breakpoint on the distal side of the 1.42 kb transcript while lanes 5-12 display a recombination breakpoint on the proximal side of the 1.42 kb transcript.

Candidate gene analysis

As shown in Table 3, the identified 76755p2 – 135087p1 region flanking *rf8* contains 4.56 Mb and 108 genes from the filtered gene set in the B73 genome. This region contains the markers located in pentatricopeptide repeat encoding genes that cosegregated perfectly with 900 segregating individuals in an *Rf3,* S-cytoplasm, mapping population (Xu et al., 2009). There are a total of seven PPR encoding genes in the 4.56-Mb *Rf8* flanking region. Two, GRMZM2G450166 and GRMZM2G053384, are lone PPR encoding genes separated by 369 kb. Approximately 554 kb distal to GRMZM2G053384 is a cluster of three PPR encoding genes, GRMZM2G439814, GRMZM2G453956, and GRMZM2G130684, spanning a 65 kb region. Two-hundred twelve kb distal to this cluster are two more PPR encoding genes, GRMZM2G416498 and GRMZM2G124602, 82 kb apart. GRMZM2G053384 is significantly different from the other six PPR encoding genes in this region in that it does not belong to the clade of PPRs that contain *Rf* genes. The other six PPRs belong to a clade of PPRs that encompass known restorer of fertility genes in plants (Akagi et al., 2004; Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Klein et al., 2005). GRMZM2G416498 and GRMZM2G124602 are similar to each other in that they have longer cDNAs than the other five PPR encoding genes. In addition to these PPR encoding genes, GRMZM2G070831 is a pre-mRNA processing factor positioned 909 kb proximal to the PPRs and GRMZM2G000936 is a tetratricopeptide repeat (TPR) encoding gene located in this PPR cluster. It would be very interesting to generate more markers closer around these genes to finer map this region. Because previously cloned *Rf* genes encode PPR proteins, with the exception of maize *Rf2a*, and are involved in RNA processing, this is

strong evidence *Rf8* might be a PPR protein as well. The genes in this region are ideal candidates for *Rf8*.

Table 3. Genes in the *rf8* flanking region

Gene[†]	Predicted Function	Sequence Coordinates[‡]
GRMZM2G076755[§]	Unknown/ Sodium symporter	220,473,907-220,484,805
GRMZM2G130379	Rubredoxin/ electron transfer	220,550,618-220,552,170
GRMZM2G410567	GH3 auxin-responsive promoter	220,594,675-220,601,030
GRMZM2G322844	Natural resistance-associated macrophage protein	220,683,705-220,690,184
GRMZM2G027130	Thiolase/ chalcone synthase	220,758,932-220,760,360
GRMZM2G056088	Unknown	220,762,697-220,767,088
GRMZM2G151227	*whp1* Thiolase/ chalcone synthase	220,851,374-220,855,359
GRMZM2G003043	Cyclin-like	220,938,266-220,945,517
GRMZM2G166776	Unknown	221,039,208-221,040,102
GRMZM2G166674	Unknown	221,110,152-221,111,536
GRMZM2G166661	Unknown	221,120,460-221,122,428
GRMZM2G173377	Unknown	221,130,826-221,131,827
GRMZM2G065144	Ferric reductase	221,211,336-221,217,052
GRMZM2G169095	Peptidase M24	221,307,146-221,319,083
GRMZM2G358619	Ferric reductase	221,359,430-221,365,687
GRMZM2G358633	Unknown	221,386,897-221,389,623
GRMZM2G037993	Ferric reductase	221,437,834-221,442,320
GRMZM2G038024	Unknown	221,443,416-221,444,381
GRMZM2G414114	TCP transcription factor	221,498,388-221,502,599
GRMZM2G114948	Unknown/ DUF247	221,510,904-221,512,518
GRMZM2G023328	Tropomyosin	221,521,249-221,522,336
GRMZM2G023585	Unknown	221,527,375-221,529,196
GRMZM2G105317	Histone fold	221,566,199-221,570,137
GRMZM2G455945	Unknown	221,633,859-221,647,475
GRMZM2G703399	Unknown	221,674,750-221,675,272

Table 3. (continued)

Gene[†]	Predicted Function	Sequence Coordinates[‡]
GRMZM2G097896	Patatin/ storage protein	221,708,947-221,716,048
GRMZM2G064475	Unknown	221,782,209-221,795,694
GRMZM2G064366	Unknown	221,805,987-221,814,288
GRMZM2G301399	Unknown	221,826,298-221,827,593
GRMZM2G107922	Unknown	221,869,911-221,870,634
GRMZM2G107931	Unknown	221,886,689-221,887,451
GRMZM2G107945	Galactose oxidase-like	221,889,489-221,893,852
GRMZM2G408768	14-3-3 protein binding domain	221,894,448-221,897,770
GRMZM2G408809	Unknown	221,896,924-221,898,014
GRMZM2G367348	Unknown/ Ca++ chelating serine protease	221,957,410-221,957,916
GRMZM2G066546	Unknown	221,962,968-221,963,545
GRMZM2G056977	Unknown	221,993,469-221,994,155
GRMZM2G355940	Peptide chain release factors	221,997,252-222,006,779
GRMZM2G057056	MAF/ putative inhibitor of septum formation	222,010,013-222,011,627
GRMZM2G090559	Unknown/ ankyrin repeat	222,053,912-222,058,724
GRMZM2G099765	Peptidase C1A	222,131,459-222,133,577
GRMZM2G099862	Unknown/ DNA binding	222,134,172-222,137,583
GRMZM2G022863	Knottin	222,148,257-222,148,880
GRMZM2G074496	Unknown/ defense	222,202,183-222,205,027
GRMZM2G460429	Unknown	222,235,488-222,241,159
GRMZM2G139813	Aminotransferase	222,274,112-222,282,345
GRMZM2G448692	Unknown/ DUF724	222,293,549-222,296,161
GRMZM2G040932	Knottin	222,299,265-222,299,873
GRMZM2G426158	unknown	222,383,480-222,384,604
GRMZM2G055594	unknown	222,418,832-222,419,648
GRMZM2G355760	unknown	222,423,800-222,426,506
GRMZM2G458077	Protein kinase-like	222,473,523-222,475,729
GRMZM2G068011	unknown	222,480,641-222,481,878

Table 3. (continued)

Gene[†]	Predicted Function	Sequence Coordinates[‡]
GRMZM2G159756	Protein kinase-like	222,622,444-222,626,799
GRMZM2G001405	decarboxylase	222,675,628-222,676,953
GRMZM2G170161	BSD domain	222,698,315-222,702,905
GRMZM2G470984	Phytosulfokine	222,702,423-222,702,779
GRMZM2G070831	Pre-mRNA processing	222,740,921-222,743,823
GRMZM2G372058	Unknown/ leucine rich repeat	222,756,468-222,759,377
GRMZM2G372068	UDP glycosyltransferases	222,781,284-222,783,413
GRMZM2G135354	Prefoldin	222,825,277-222,827,621
GRMZM2G433731	Unknown	222,831,054-222,835,549
GRMZM2G703402	Unknown	222,998,264-222,999,255
GRMZM2G067713	Unknown	223,024,157-223,026,712
GRMZM2G313110	Unknown	223,152,021-223,153,218
GRMZM2G023652	Unknown	223,175,575-223,180,049
GRMZM2G457381	DNA binding	223,296,617-223,297,569
GRMZM2G106531	Carotene isomerase	223,340,203-223,345,192
GRMZM2G106604	Unknown/ DUF593	223,347,756-223,351,926
GRMZM2G451729	Unknown	223,384,093-223,386,877
GRMZM2G150813	Unknown	223,396,530-223,400,729
GRMZM2G083095	Chaperone/ tailless complex polypeptide	223,420,920-223,425,765
GRMZM2G083599	Glycoside hydrolase	223,426,404-223,428,432
GRMZM2G066394	Pseudouridine synthase	223,496,051-223,496,754
GRMZM2G345622	Peptidase S8	223,604,047-223,607,752
GRMZM2G053384	Pentatricopeptide repeat protein	223,650,313-223,652,692
GRMZM2G353343	Unknown/ lipid transfer protein	223,652,940-223,654,236
GRMZM2G000936	Tetratricopeptide repeat/ protein binding	223,673,828-223,679,284
GRMZM2G177008	Unknown	223,965,177-223,967,729
GRMZM2G475554	Unknown	223,980,488-223,982,402
GRMZM2G406131	Unknown	223,992,476-223,992,865

Table 3. (continued)

Gene[†]	Predicted Function	Sequence Coordinates[‡]
GRMZM2G318393	Unknown	223,993,853-224,001,784
GRMZM2G318412	Unknown/ Homeodomain-like	224,003,547-224,005,842
GRMZM2G450166	Pentatricopeptide repeat protein	224,019,777-224,020,722
AC196106.2_FG001	Unknown	224,147,225-224,149,150
GRMZM2G303463	Unknown/ DNA binding domain	224,238,351-224,242,411
GRMZM2G081377	Unknown	224,276,996-224,278,364
GRMZM2G158298	Histone H2A	224,518,641-224,520,201
GRMZM2G158288	Nucleic acid-binding proteins/ OB fold	224,520,402-224,525,465
GRMZM2G158279	Unknown	224,526,051-224,526,826
GRMZM2G158175	Unknown	224,568,325-224,572,222
GRMZM2G439814	Pentatricopeptide repeat protein	224,573,474-224,574,223
GRMZM2G439788	Unknown	224,574,326-224,575,450
GRMZM2G453956	Pentatricopeptide repeat protein	224,577,905-224,579,311
GRMZM2G408232	Unknown	224,585,779-224,591,928
GRMZM2G008865	Histone H2A	224,631,239-224,632,618
GRMZM2G130684	Pentatricopeptide repeat protein	224,638,493-224,639,728
GRMZM2G416498	Pentatricopeptide repeat protein	224,850,717-224,854,307
GRMZM2G116461	Unknown/ antifreeze	224,856,514-224,860,414
GRMZM2G416541	Unknown	224,903,535-224,905,474
GRMZM2G416544	Histone H2A	224,906,096-224,920,835
GRMZM2G124602	Pentatricopeptide repeat protein	224,932,695-224,936,524
GRMZM2G124616	Unknown/ peptidase-like	224,938,225-224,945,742
GRMZM2G097511	Histone H2A	224,977,472-224,979,165
GRMZM2G135195	Glycotransferase	225,008,737-225,011,848
GRMZM2G436001	MiaB methiolase	225,020,198-225,024,319
GRMZM2G436000	Unknown	225,023,999-225,024,577
GRMZM2G135087[§]	Unknown/ DUF295 domain	225,038,370-225,040,487

[†]Genes taken from the filtered gene set and coordinates are from Maizesequence.org release 4a.53 (Schnable et al., 2009). Shaded are the predicted PPR encoding genes, a TPR encoding gene, and an RNA editing gene.

‡Sequence coordinates corresponding to Maizesequence.org release 4a.53 (Schnable et al. 2009)

§ These genes contain the flanking markers for *Rf8*. Gene GRMZM2G76755 contains the proximal flanking marker while gene GRMZM2G135087 contains the distal flanking marker.

Additional *Rf* loci in the *whp1*-umc36 interval

As described above, T-cytoplasm plants segregating for *Rf8* accumulate additional 1.42/0.42-kb T-*urf13* transcripts. Likewise, plants segregating for *Rf1* accumulate additional 1.6/0.6-kb transcripts (Wise et al., 1996), and those segregating for an additional *Rf* locus, *Rf**, accumulate additional 1.40/0.40-kb T-*urf13* transcripts (Dill et al., 1997; Wise et al., 1999b). All three of these T-cytoplasm restorers share a small, conserved target sequence in the T-*urf13* ORF, yet independently control the modification of T-*urf13,* CMS-associated, transcripts (Dill et al., 1997). Interestingly, this same target sequence is also highly conserved among sites for *Rf**-mediated T-*urf13* processing and the CMS-associated *orf107* processing regulated by sorghum *Rf3* (Tang et al., 1996; Wise et al., 1999a).

Since we already identified *Rf8*-linked DNA markers, we further tested the relationship between *Rf8* and the additional partial restorer, *Rf**, by testing *Rf8*-linked markers on segregating progeny of an *Rf** mapping population. A population of 88 progeny segregating for *Rf** was established using the same procedure used to generate the *Rf8* mapping population (see Methods) and the *Rf8*-linked 144635p4 PCR-based marker was found to be linked to the *Rf** locus (Figure 4).

Having established that *Rf** was in the *whp1*-umc36 interval on maize chromosome *2L*, we performed an additional test to see whether we could directly identify recombinants between *Rf8* and *Rf**. The experiment was based on RNA blot analysis using two different

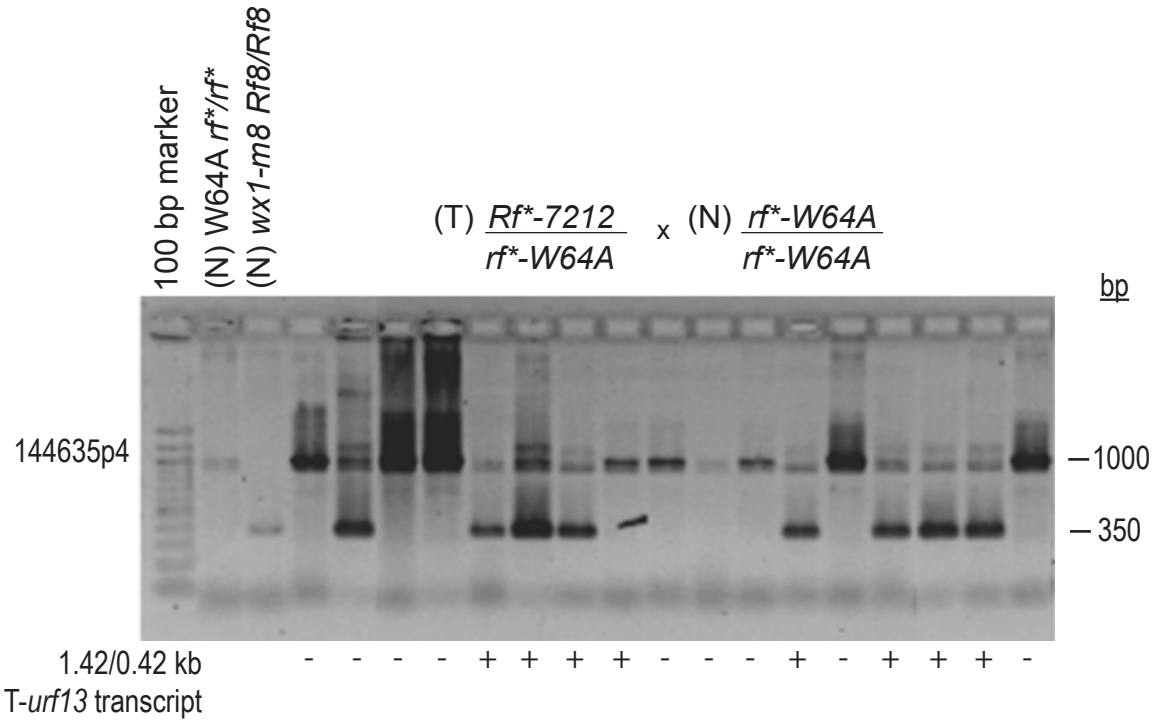

Figure 4. PCR marker 144635p2 illustrating linkage to the *Rf** locus. Inbred lines (N) *wx1-m8* and (N) W64A and 17 progeny from the *Rf** mapping population were tested for PCR marker 144635p4. The presence or absense of the 1.42/0.42-kb *Rf**-associated T-*urf13* transcript as detected by probe T-st308 is given.

probes that can differentiate the *Rf8* and *Rf** specific T-*urf13* transcripts. Probe T-st308 can hybridize to the *Rf8* specific transcripts (1.42/0.42 kb) as well as the *Rf**-specific transcripts (1.40/0.40 kb). The oligo probe CD1721 can only hybridize to the *Rf8*-specific transcripts (Dill et al., 1997).

An individual heterozygous for *Rf** (*Rf*-7212/rf*-W64A*) was used to pollinate a plant heterozygous for *Rf8* (*Rf8-8703/rf8-W64A*) and a BC_1 population was generated by pollination with (N) W64A (*rf8/rf8, rf*/rf**). Two BC_1 families derived from individuals carrying both *Rf8* and *Rf** (98 1233 and 98 1236) were identified from twenty-four planted families via RNA blot analysis in the 1998 summer nursery. A total of fifty-three individual plants from the two families (12 from 98 1233 and 41 from 98 1236) were analyzed via RNA blot analysis using probes T-st308 and CD1721. Results are summarized in Table 4.

If *Rf8* and *Rf** are encoded by separate, but linked open reading frames, then the BC_1 families would be derived from an individual with the genotype *Rf8-8703, Rf*-7212 / rf8-W64A, rf*-W64A* and any of four genotypes could arise (*Rf8-8703, Rf*-7212 / rf8-W64A, rf*-W64A*; *rf8-W64A, rf*-W64A / rf8-W64A, rf*-W64A*; *Rf8-8703, rf*-W64A / rf8-W64A, rf*-W64A*; and *rf8-W64A, Rf*-7212 / rf8-W64A, rf*-W64A*). If *Rf8* and *Rf** are alleles of one locus, the two BC_1 families would be derived from an individual with the genotype *Rf8-8703/Rf*-7212* and the expected segregation pattern would consist of two genotypes (*Rf8-8703/rf-W64A* and *Rf*-7212/rf-W64A*. As described above, plants carrying *Rf8* and *Rf** can be distinguished by hybridization of RNA gel blots with the T-st308 and CD1721 probes. When hybridized with T-st308, all fifty-three individuals displayed either the 1.42-kb or the 1.40-kb transcripts ($\chi^2_{1:0}=0 < \chi^2_{1,\,0.05}=3.84$, p-value > 0.05). Twenty-five of these individuals hybridized to the 1.42-kb transcript when hybridized with the probe CD1721,

Table 4. Chi-square test of the expected and observed outcome of linkage test of *Rf8* and *Rf.**

Assumptions[†]	T-st308[†]				CD1721[†]			
		No. of progeny accumulating *Rf8*- and/or *Rf**- mediated T-*urf13* transcripts				No. of progeny accumulating *Rf8*- or *Rf**- mediated T-*urf13* transcripts		
		Rf8 or *Rf**	*rf8* or *rf**[‡]	χ^2		*Rf8*	*Rf**[§]	χ^2
Independent	Expected (3:1)	39.7	13.3	17.6	Expected (1:1)	26.5	26.5	0.17
Allelic	Expected (1:0)	53	0	0	Expected (1:1)	26.5	26.5	0.17
	Observed	53	0		Observed	25	28	

[†]Under the assumption of independence, probe T-st308 would detect a 3:1 segregation for the presence of *Rf8*- and/or *Rf**-mediated T-*urf13* transcripts (*Rf8-8703, Rf*-7212 / rf8-W64A, rf*-W64A*; *Rf8-8703, rf*-W64A / rf8-W64A, rf*-W64A*; and *rf8-W64A, Rf*-7212 / rf8-W64A, rf*-W64A*) to the absence of *Rf8*- and/or *Rf**-mediated T-*urf13* transcripts (*rf8-W64A, rf*-W64A / rf8-W64A, rf*-W64A*). Probe CD1721 would detect a 1:1 segregation for the presence of *Rf8*- mediated T-*urf13* transcripts and the presence of *Rf**- mediated T-*urf13* transcripts (*Rf8-8703/rf-W64A* or *Rf*-7212/rf-W64A*). Under the assumption of allelism, probe T-st308 would detect presence *Rf8*- or *Rf**- mediated T-*urf13* transcripts to the inability to detect the *Rf**- mediated T-*urf13* transcripts.

[‡]Indicates these individuals do not accumulate any *Rf*-mediated novel T-*urf13* transcripts.

[§]Indicates these individuals accumulate the *Rf**-mediated T-*urf13* transcripts, but cannot be detected by CD1721 probe.

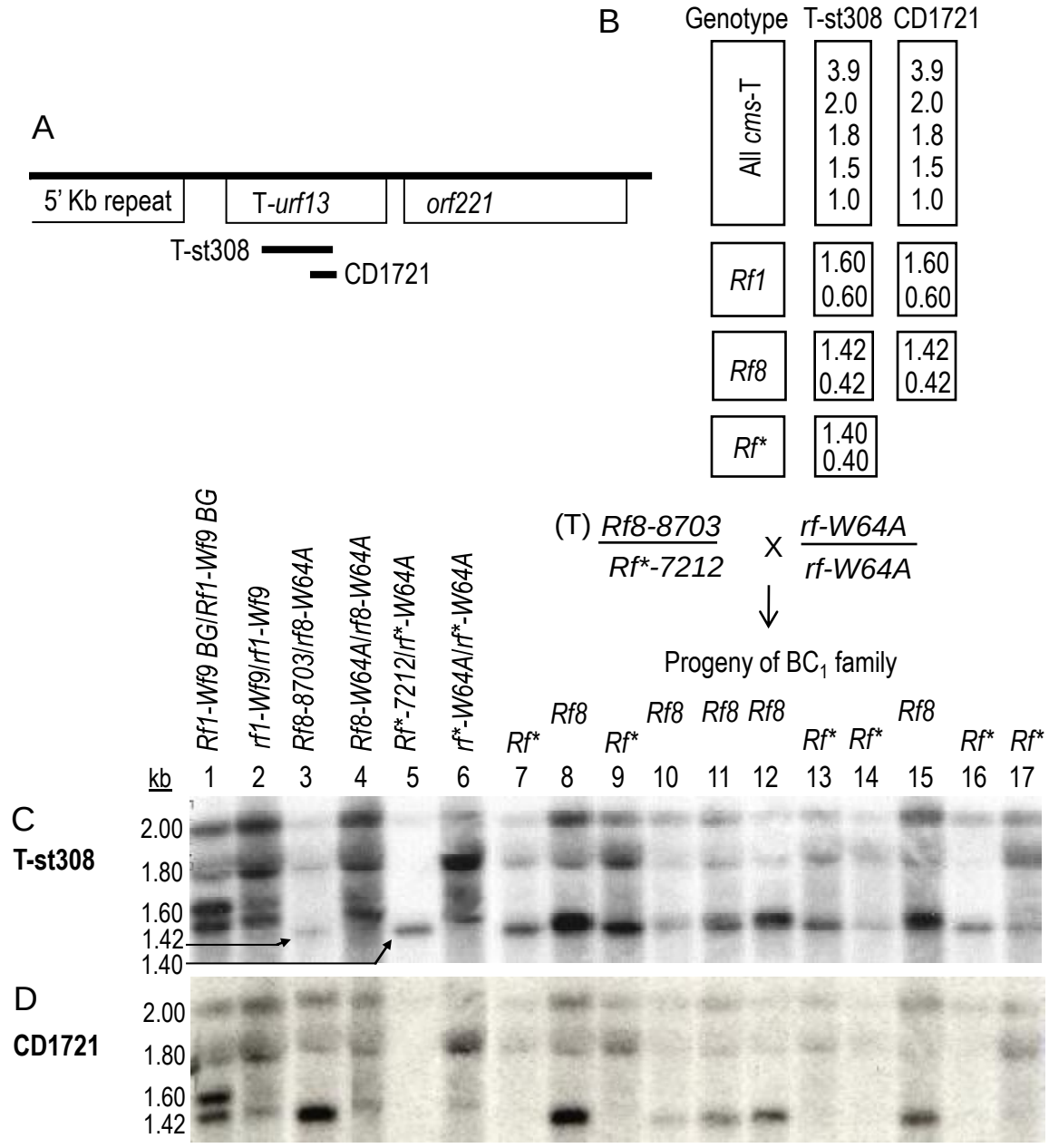

Figure 5. Analysis of progeny from BC_1 families segregating for *Rf8* and *Rf**. Panel A shows the position of the probes on the T-*urf13* sequence. Panel B shows the expected transcript accumulation when using the T-st308 probe and the oligo CD1721 probe. Panel C illustrates the T-st308 probe hybridizes to all of progeny that carry either *Rf8* or *Rf** genes in the subset progeny of BC_1 segregating family. Panel D shows the CD1721 probe only hybridizes to progeny that carry the *Rf8* gene.

Table 5. Fertility summary of the 1997 and 2008 *rf8* mapping population

Progenitor[†]	Progenitor Fertility Phenotype	Progeny Rows	No. of plants with the indicated phenotypes		Total No. of Plants
			S	"S"	
95 3233-2	"S"	97 2219-2226	118	44	166
97 2220-11	"S"	08 7243-7247	167	14	183
97 2220-12	"S"	08 7248-7250, 08 7301-7302	179	14	194
97 2220-17	S	08 7303-7307	117	7	125
97 2220-22	"S"	08 7308-7312	147	14	161
97 2221-3	S	08 7313-7316	129	32	163
97 2222-15	"S"	08 7327-7331	163	25	188
97 2222-3	S	08 7322-7326	162	12	174
97 2223-15	"S"	08 7332-7336	175	4	179
97 2224-3	"S"	08 7337-7341	164	12	176
97 2225-1	S	08 7342-7346	172	17	189
		2008 Total:	1575	151	1731

[†] Progenitor plants are (T) *Rf8-8703/rf8-W64A* crossed by (N) *rf8-W64A/rf8-W64A*. The genotype of all the progenitors was inferred by presence of the 1.42/0.42-kb T-*urf13* transcripts.

observation that fertility was environmentally sensitive; if the temperature was cool (24-28°C) during tassel development prior to anthesis, there was a high probability that plants with the potential for fertility would be fertile, whereas, if the temperature was hot (29-34°C) during the same period, plants with the identical genotype would be sterile (Dill et al., 1997). In order to further characterize these phenomena in the 2008 population, 126 partially male-fertile plants were genotyped for the tightly linked markers, csu811_p9 and umc36_p10. At the time of this analysis, it was believed csu811_p9 and umc36_p10 were the flanking

markers for *Rf8*. Since it was thought that *Rf8* was incompletely penetrant, it was expected that most or all partially male-fertile plants in the 2008 population would carry the dominant *Rf8-8703* allele and would be heterozygous (*Rf8-8703/rf8-W64A*) for the tightly markers. However, the plants displayed a 1:1 segregation ($\chi^2_{1:1} = 0.008$; P = 0.93) for these two markers—5 were recombinant, 61 were heterozygous (*Rf8-8703/rf8-W64A*), and 60 were homozygous recessive (*rf8-W64A/rf8-W64A*).

Because a 1:1 genotypic segregation was not expected in the partially fertile plants, we designed an experiment to test the partially fertile and sterile plants for the *Rf8*-associated transcripts. One-hundred eighty plants that were selected based on prior knowledge of their genotype score and fertility phenotype were assayed for presence of *Rf8*-associated 1.42/0.42-kb T-*urf13* transcripts. Table 6 and Figure 6 show co-segregation of partially male fertile and male sterile plants with and without the *Rf8*-associated transcripts. Of the 44 partially male fertile plants, 17 individuals did not contain the 1.42/0.42-kb transcripts while 27 individuals displayed the 1.42/0.42-kb transcripts. Of the 136 male sterile plants, 48 did not contain the 1.42/0.42-kb transcripts while 88 displayed the transcripts. The findings that partially male fertile individuals are segregating 1:1 for tightly linked markers and at least 17 of these partially male fertile plants do not display the transcripts suggest that fertility restoration could be under the control of additional unlinked locus. This suggests fertility and transcript accumulation could be uncoupled and therefore *Rf8* may not be incompletely penetrant as postulated previously. These observations further imply that accumulation of *Rf8*-associated T-*urf13* transcripts is not necessary or sufficient for fertility restoration.

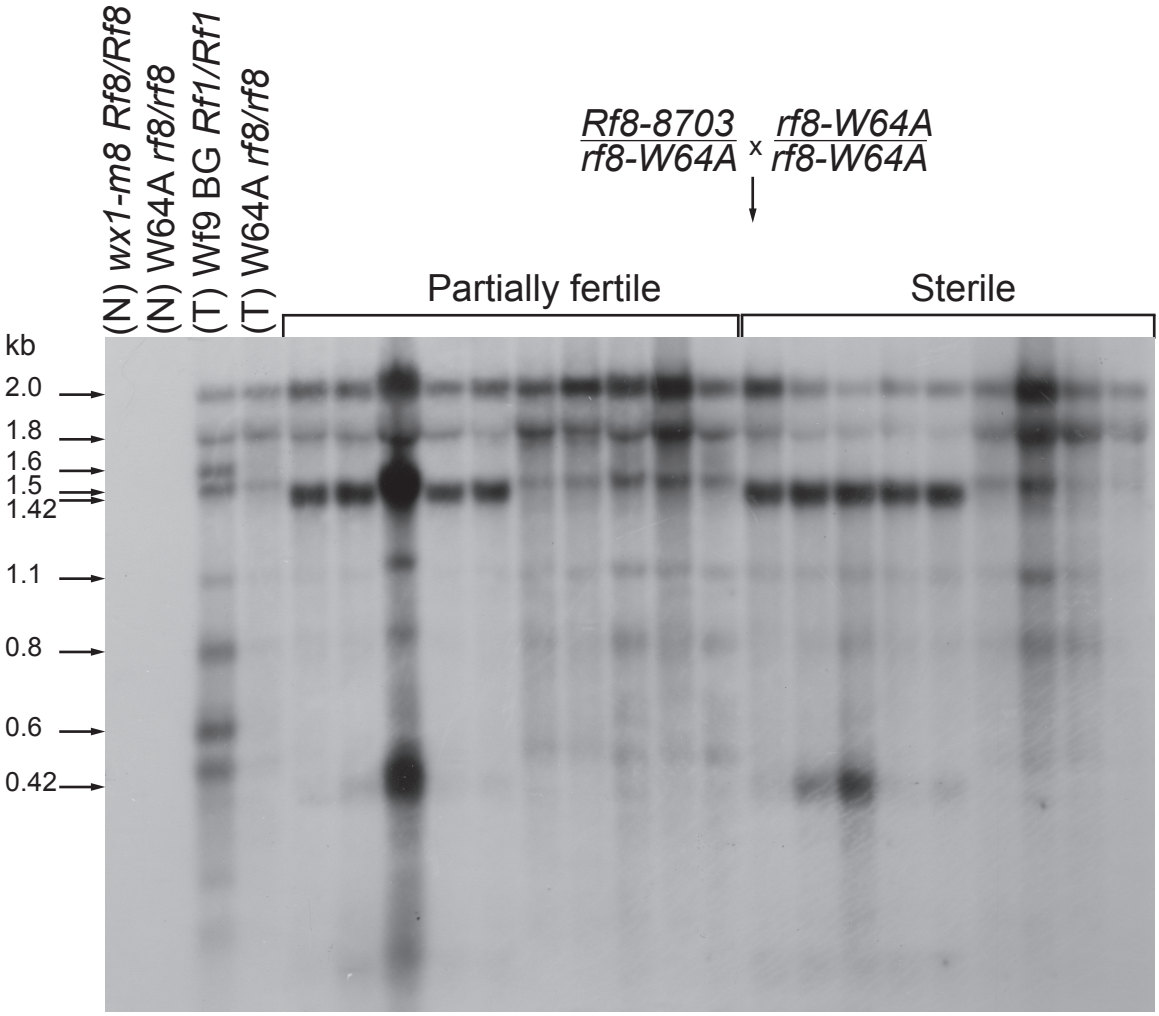

Figure 6. RNA gel blot of partially male fertile and sterile plants showing segregation between fertility and the *Rf8*-associated 1.42 kb transcript. The probe is st308, a portion of the T-*urf13* gene (Wise et al., 1996). No hybridizing bands are expected on the RNA blot in lanes 1 and 2 because the probe is specific to T cytoplasm while the samples are N cytoplasm. Lane 3 contains the *Rf1* control illustrasting the 1.6 kb transcript. Lanes 5-14 are partially male fertile with lanes 5-9 displaying the 1.42 kb transcript. Lanes 10-14 illustrate the absence of the transcript. Lanes 15-23 are male sterile with lanes 15-19 demonstrating the 1.42 kb transcript. Lanes 20-23 display the absence of the transcript.

Table 6. Segregation of the 1.42/0.42-kb T-*urf13* transcripts and partial male fertility

T-*urf13* transcript accumulation	No. of partially male fertile plants	No. of male sterile plants	Total
1.42/0.42 kb present	27	88	115
1.42/0.42 kb absent	17	48	65
Total	44	136	180[†]

[†]Plants are a subset of the 2008 mapping population. Plants were chosen for RNA blot analysis based on prior knowledge of their genotype scores and fertility phenotype.

Molecular marker genotypes in the *rf8* region segregate as expected but fertility phenotypes do not

In order to determine the number of factors responsible for transcript accumulation and the observed fertility phenotypes, segregation data was examined. To test the hypothesis that *rf8* is a single locus, tightly linked genotypic markers should segregate 1:1 for heterozygous and homozygous recessive in a backcrossed population. Table 7 demonstrates that the *P* value for segregation of the tightly linked PCR marker 66902p1 was greater than 0.05, and therefore not significantly different from a 1:1. Marker 66902p1 was chosen because at the time of the analysis, it was believed to be the distal flanking marker. All progenitor plants of the 2008 population displayed the *Rf8*- associated transcripts and were heterozygous for RFLP markers csu811 and umc36. Progeny from one progenitor, 97 2220-22, did not segregate for 66902p1. This could be explained by a crossover occurring at meiosis in the 97 2220-22 plant between 144635p4 and 66902p1. The adjusted population total listed in Table 7 removes these progenies.

Dill (1997) reported environmental sensitivity in *Rf8* plants. Greater anther exertion is observed at lower temperatures while less is observed at higher temperatures. Segregation

for fertility categories in the 2008 mapping population is reported in Table 5. This population displayed a ratio of 1:10 (χ21:10 = 0.244; P = 0.62) partially fertile to sterile plants. This ratio does not align with the hypothesis of one, two, three, or four completely dominant, independently assorting genes, however flowering is a complex process and other phenomena are likely. A 1:10 ratio lies between a 3-gene test cross (1:7) and a 4-gene test cross (1:15). Linkage, additivity, epistatis, or incomplete dominance could be involved in skewing a standard ratio to 1:10.

Table 7. Genotypic segregation of PCR marker 66902p1 in the *Rf8* population

		No. of plants with the indicated 66902p1 score			
Progenitor[†]	Progeny Rows	Heterozygous	Recessive	$\chi^2_{1:1}$	*P* value
97 2220-11	08 7243-7247	60	67	0.386	0.535
97 2220-12	08 7248-7250, 08 7301-7302	33	41	0.865	0.352
97 2220-17	08 7303-7307	37	42	0.316	0.574
97 2220-22[‡]	08 7308-7312	0	121	121.000	0.000
97 2221-3	08 7313-7316	34	47	2.086	0.149
97 2222-15	08 7327-7331	54	71	2.312	0.128
97 2222-3	08 7322-7326	82	67	1.510	0.219
97 2223-15	08 7332-7336	61	67	0.281	0.596
97 2224-3	08 7337-7341	57	76	2.714	0.099
97 2225-1	08 7342-7346	43	51	0.681	0.409
	Adjusted Total[§]:	461	529	4.671	0.792

[†]All progenitor plants were crossed by (N) W64A to derive the 2008 plants.

[‡]Progeny did not segregate for marker 66902p1, however 76755p2 segregated 47 homozygous to 28 heterozygous; *P* value = 0.028. This can be explained by a crossover event happing between 144635p4 and 66902p1 during meiosis in plant 97 2220-22.

[§]Total reported is excluding progeny of 97 2220-22 which did not segregate for 66902p1. Eight degrees of freedom were used to calculate the adjusted total *P* value.

Effect of genetic background on *Rf8*-mediated fertility

The observation was made from 1994 to 2000 that fertility of plants carrying Rf8 decreased with each successive generation backcrossed to (N) *rf8-W64A/rf8-W64A*. Starting in 2000, plants were crossed by (N) *wx1-m8* in addition to (N) W64A (see Figure 1) in order to test if the *wx1-m8* background would increase fertility compared to (N) W64A. As shown in Table 8, five generations were observed for fertility in our 2009 summer Ames nursery. When backcrossed to (N) W64A, fertility decreased, whereas, crossing to (N) *wx1-m8* increased fertility. A subset of the plants showing fertility, including the one partially male fertile plant from the BC4 cross, were used as pollen donors and seed was obtained, demonstrating their fertility. The generations tested generally displayed the expected trend of increased fertility with greater amounts of (N) *wx1-m8* in the pedigree as opposed to (N) W64A. This is suggestive of other factors involved in fertility present in the background of (N) *wx1-m8* yet absent in the background of (N) W64A.

Delay of fertility in partially male-fertile plants

In addition to the background affecting the amount of fertility in *Rf8* plants, the timing of anther exertion affects the amount of fertility observed. The observation was made by Schnable and Wise (1994) that partially male fertile *rf1*/*rf1*, *Rf2*/*Rf2* plants accumulate novel T-*urf13* transcripts (later characterized as the 1.42/0.42-kb *Rf8*-associated transcripts by Dill et al. (1997) and flower one to two weeks later than near-isogenic siblings. To test if mostly male fertile plants flower earlier than partially male fertile plants, five generations were grown and observed in 2009. All plants were observed daily for exertion of anthers. Four generations, BC_5TC_1, BC_5TC_2, BC_6TC_1, and BC_6TC_2, segregated for three of the fertility categories—sterile, partially male fertile, and mostly male fertile. As shown in Table 9, the

Table 8. Phenotypic ratios associated with various amounts of backcrossing

Cross[†]	Parental Genotype	Pollen Donor[‡]	Parental Fertility Phenotype	Parental Transcript Accumulation	2009 Progeny Rows	No. of plants with the indicated phenotypes			Total No. of plants
						S	"S"	"F"	
08 7330-1 / 7319	BC_4	(N) W64A	S	1.42/0.42 present	8132, 8136, 8147, 8148	53	1	0	54
01 4136-4 / 4018	BC_6TC_1	(N) W64A	"S"	1.42/0.42 present	8123, 8126, 8130, 8145	23	20	11	54
02 5230-21 / 5229-3	BC_6TC_2	(N) *wx1-m8*	"S"	1.42/0.42 present	8131, 8141, 8146, 8150	8	18	22	48
00 3437-1 / 3446	BC_5TC_1	(N) *wx1-m8*	NA[§]	1.42/0.42 present	8124, 8133, 8140, 8142	7	23	17	47
01 4136-3 / 4149	BC_5TC_2	(N) *wx1-m8*	"S"	1.42/0.42 present	8135, 8137, 8139, 8144	13	20	2	35

[†]Crosses are diagrammed in Figure 1 and were planted in 2009.

[‡]Pollen donor lines are listed, which correspond to the second plant number in the Cross column.

[§]Parental fertility phenotype not available.

average day after planting to the first flowering (DAPFF) was calculated for all cross types, with DAPFF defined as the first day an exerted plump yellow anther was observed. A two-tailed, paired t test was used to calculate significance between flowering time of partially fertile and mostly fertile plants. Within a given genotype, all mostly male fertile plants showed a significantly earlier DAPFF of flowering than the partially male fertile plants. Flowering in 2009 was delayed by 3.7 to 2.4 days depending on the genotype. This could be explained by other factors responsible for flowering time segregating in the partially fertile and mostly fertile plants. These results suggest that there is another factor in the genome responsible for the differences observed in the partially fertile and mostly fertile plants.

Table 9. Average DAPFF in partially male-fertile and mostly male-fertile plants

Genotype	Phenotype	Average DAP of flowering	P value
BC_5TC_1	Mostly fertile	63.9	0.0014**
	Partially fertile	67.3	
BC_5TC_2	Mostly fertile	67.5	0.0092**
	Partially fertile	70.0	
BC_6TC_1	Mostly fertile	66.5	0.0030**
	Partially fertile	68.9	
BC_6TC_2	Mostly fertile	65.4	0.0113**
	Partially fertile	68.1	

Mostly male fertile plants flowered significantly earlier than partially male fertile plants of a given genotype.

Discussion

rf8 is located in a 4.56 Mb region on *2L*

Understanding cytoplasmic male sterility has a clear economic advantage. The production of hybrid maize seed would benefit from a viable CMS system. The precise

mechanisms of CMS and fertility restoration need to be uncovered. Mapping the restorers of fertility with easily assayed molecular markers is a step towards the understanding of fertility restoration because individual factors can then be tracked in the progeny of various crosses. Here we demonstrate *rf8* is located in the 4.56 Mb region on *2L* between PCR markers 76755p2 and 135087p1 corresponding to contig 108 in Maizesequence.org release 4a.53.

Dill et al. (1997) reported *Rf8* was incompletely penetrant based on fertility data and transcript accumulation of 79 individuals. All partially male fertile plants in their study showed the presence of the 1.42/0.42-kb transcripts. However, our results demonstrate the partially male fertile individuals in the 2008 mapping population segregate 1:1 for tightly linked genotypic markers and 17 of these did not display the *Rf8*-associated transcripts. Thus, our results do not coincide with the incomplete penetrance conclusion of Dill et al. (1997). *Rf8* is responsible for transcript accumulation while fertility appears to be at least partially controlled by other genes. Given the existence of partially male fertile individuals without the 1.42/0.42-kb transcripts, transcript presence does not appear to be necessary or sufficient for fertility restoration. This could indicate the presence of at least one other factor in the genome responsible for partial fertility restoration.

To test the hypothesis that genetic background affects fertility, five generations of *Rf8* plants were grown in 2009. The fertility observations suggest a difference in the backgrounds of W64A and *wx1-m8* because plants reintroduced with *wx1-m8* displayed greater fertility. This could be interpreted as *wx1-m8* harboring other unlinked genes favorable to fertility that W64A does not possess. In order to test for differences between partially fertile and mostly fertile plants, the day after planting to the first flowering (DAPFF) was recorded. This demonstrated that partially fertile plants flower significantly later than

mostly fertile plants. This suggests the presence of other factors segregating for the timing of flowering.

Clusters of linked restorer of fertility genes are conserved across plant taxa

Fine mapping of *ZmRf8* (T cytoplasm) as well as *ZmRf3* (S cytoplasm) suggest both genes map to the same cluster of PPR genes on *2L*. The phenomenon of linked restorer genes is not unique to maize (see Table 10). Rice contains a PPR cluster spanning 450 kb on chromosome *10L* that contains six *Rf* genes that restore four different cytoplasms (Tan et al., 2010). *OsRf4* and *OsqRf-10-2* restore WA and DA cytoplasm, respectively, which are characterized by sporophytic restoration (Xie et al., 2002; Yao et al., 1997). *OsRf1a* and *OsRf1b* restore BT cytoplasm, whereas *OsRf5* and *OsRf6* restore HL cytoplasm (Akagi et al., 2004; Komori et al., 2004; Liu et al., 2004; Wang et al., 2006). Both BT and HL cytoplasms are characterized by gametophytic restoration. This rice *10L* locus is analogous to the maize *2L* locus. Both species contain linked *Rf* genes capable of restoring cytoplasms with different modes of restoration. Similar to rice and maize, cotton has two linked *Rf* genes, $GhRf_1$ and $GhRf_2$. These genes restore two different cytoplasms that also utilize different modes of restoration. $GhRf_1$ sporophyticly restores D2 cytoplasm while $GhRf_2$ gametophyticly restores D8 cytoplasm (Meyer, 1975; Zhang and Stewart, 2001). Two studies have mapped these genes within 1 cM of each other (Wang et al., 2007; Wang et al., 2009). Likewise, common bean (*Phaseolus vulgaris*) contains two linked fertility restorer genes *PvFr* and $PvFr_2$ on linkage group K (He et al., 1995; Jia et al., 1997). Interestingly, sorghum, maize's closest relative, does not contain PPR clusters around its unlinked *Rf* genes. *SbRf1* and *SbRf2* both are lone PPR encoding genes (Jordan et al., 2010; Klein et al.,

2005). Thus, precedent exists for linked *Rf* genes to exist in one PPR cluster capable of restoring multiple cytoplasms characterized by various modes of restoration.

Canola (*Brassica napus*) and petunia (*Petunia hybrida*) contain *Rf* genes present in PPR clusters containing only one known *Rf* gene and multiple pseudogenes. *BnRfo* of canola is located in a cluster with two other PPR encoding genes (Brown et al., 2003; Feng et al., 2009). *Phrf-PPR592* of petunia is adjacent to another PPR gene, *PhPPR591* (Bentolila et al., 2002; Bentolila et al., 1998). The nonrestoring allele of *Phrf-PPR592* contains a promoter deletion and most likely a recombination event involving similar PPR genes (Bentolila et al., 2002). Currently, it is unknown if sunflower (*Helianthus annuus*) contains linked *Rf* genes residing in a PPR cluster. *HaRf1* restores PET1 cytoplasm in sunflower, however the landscape of this fertility locus needs to be elucidated (Yue et al., 2010).

The mapping of *ZmRf8* in this study places it in a PPR cluster on *2L*. Fine mapping of *ZmRf3* for S cytoplasm maize positions *ZmRf3* 4.3 cM distal to *whp1* (Kamps and Chase, 1997). Further investigations of the *whp1* region revealed a cluster of rice *OsRf1*-orthologus PPR genes in B73 (Xu et al., 2009). This is the same PPR cluster to which *ZmRf8* maps. It is likely that *ZmRf3* and *ZmRf8* reside in the same cluster of PPR encoding genes.

ZmRf8's molecular phenotype is the accumulation of the additional 1.42/0.42-kb T-*urf13* transcripts. Based on this phenotype, the predicted function of *ZmRf8* is RNA editing or cleavage. PPR encoding genes are the most promising candidates because of previous cloned restorer of fertility genes and the molecular phenotype of *Rf8*. We have mapped *ZmRf8* to a region on *2L* that contains seven PPR encoding genes. Fujii et. al. (2011) identified *Rf*-like (RFL) PPR genes in many species. There are five RFL-identified annotated genes in maize B73. Finer mapping in the region containing the PPR encoding genes could

elucidate whether *ZmRf3* and *ZmRf8* map to the same RFL PPR encoding gene. If *ZmRf3* and *ZmRf8* are alleles, this would be one of the first *Rf* gene with alleles capable of restoring two different types of cytoplasm with different modes of restoration. Even if they are not alleles, these loci will provide insight into the evolution of CMS/*Rf* systems. Clearly this complex locus is a hotspot for fertility restoration.

Table 10. Comparisons of species and their fertility restorer genes.

Species	Cytoplasm	Mode of Restoration	Restorer Genes	Gene Location	Reference
Oryza sativa (Rice)	WA	Sporophytic	*OsRf4*	*10L*	(Yao et al., 1997)
	DA	Sporophytic	*Osq-Rf-10-2*	*10L*	(Xie et al., 2002)
	BT	Gametophytic	*OsRf1a, OsRf1b*	*10L*	(Akagi et al., 2004; Komori et al., 2004; Wang et al., 2006)
	HL	Gametophytic	*OsRf5, OsRf6*	*10L*	(Liu et al., 2004)
Zea mays (Maize)	T	Sporophytic	*ZmRf1*	*3*	(Schnable and Wise, 1994)
			ZmRf2	*9S*	(Schnable and Wise, 1994)
			*ZmRf8, ZmRf**	*2L*	(Pei, 2000)
	S	Gametophytic	*ZmRf3*	*2L*	(Kamps and Chase, 1997; Xu et al., 2009)
Gossypium hirsutum (Cotton)	D2	Sporophytic	$GhRf_1$	*D5*	(Meyer, 1975; Wang et al., 2007; Wang et al., 2009; Zhang and Stewart, 2001)
	D8	Gametophytic	$GhRf_2$	*D5*	
Sorghum bicolor (Sorghum)	A1	Sporophytic	*SbRf1*	*SBI-08L*	(Klein et al., 2005)
			SbRf2	*SBI-02*	(Jordan et al., 2010)
Brassica napus (Canola)	Ogura	Sporophytic	*BnRfo*[†]	*CN19*	(Brown et al., 2003; Feng et al., 2009)
Petunia hybrida (Petunia)	RM	Gametophytic	*Phrf-PPR592*	*4*	(Bentolila et al., 2002; Bentolila et al., 1998)
Phaseolus vulgaris (Bean)	Sprite	NA[‡]	*PvFr*, $PvFr_2$	*K*	(He et al., 1995; Jia et al., 1997)
Helianthus annuus (Sunflower)	PET1	Sporophytic	$HaRf_1$	13	(Yue et al., 2010)

[†]Three research groups cloned this gene simultaneously and assigned different names. Brown et al. (2003) named it *Rfo*, Koizuka et. al. (2003) named it *orf687*, and Desloire et. al. (2003) named it *Ppr-B*. For simplicity, this manuscript refers to it as *BnRfo*.

[‡]The *Fr*-mediated restoration of bean is permanent. The presence of *Fr* causes the permanent elimination of the mitochondrial sterility-associated gene *pvs* from reproductive tissue (He et al., 1995).

Acknowledgements

The authors recognize Karin Werner and Greg Fuerst for expert technical assistance in the maize nursery and for nucleic acid isolation and analysis. Also, the authors recognize Ian Small for PPR informatics data prior to publication. This research was supported by USDA-National Research Initiative (NRI) grant nos. 99-35300-7752 and 2002-35301-12064. This article is a joint contribution of The Iowa Agriculture and Home Economics Experiment Station and the Corn Insects and Crop Genetics Research Unit, USDA-Agricultural Research Service. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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CHAPTER 3. GENERAL CONCLUSIONS

Cytoplasmic male sterility is a unique system to study nuclear-plastid genome interactions. The fact that CMS is found in so many different species, points to the importance of mitochondria in male gamete development. The sequence of T-*urf13* is also very unique. Multiple recombinations of mitochondrial genes are needed to derive its sequence. Here we show *rf8* maps to a 4.56 Mb region on the long arm of chromosome 2 in contig 108 near *whp1*. Partially male-fertile plants segregated for the presence or absence of the *Rf8*-associated transcripts, indicating that presence of the 1.42/0.42 kb transcript is not necessary for anther exertion. Previously, *Rf8* was reported to be incompletely penetrant for fertility restoration (Dill et al. 1997). A new, larger data set demonstrating that partially fertile plants segregate for the presence or absence of the *Rf8*-associated transcripts indicate that presence of the 1.42/0.42 kb transcripts is not necessary for anther exertion. Thus, fertility appears to be controlled by at least one other factor in the genome.

rf3 of S cytoplasm and *rf** of T cytoplasm also map near *whp1* indicating a fertility hotspot on *2L*. An allelism test between *Rf8* and *Rf3* would increase our understanding of this locus. It would be very interesting if genes located very close could restore two different types of cytoplasm with different modes of restoration. T cytoplasm is sporophytic while S cytoplasm is gametophytic. Understanding the genes in this locus would help uncover the mechanism of fertility restoration for cytoplasmic male sterility.

Future studies could also include searching for the cause of fertility in these *Rf8* plants. Since fertility and 1.42 kb transcript accumulation did not cosegregate, there might be more than one locus responsible for partial male fertility. A quantitative trait loci study could be used to find other unlinked fertility loci in the population. This leads into another

question. With such similar molecular phenotypes, why do *Rf1* and *Rf8* differ in the ability to restore fertility to T cytoplasm plants?

A bioinformatics approach could be taken to locate candidate genes for *Rf1* and *Rf8*. Genes with RNA editing, cleavage, and stabilization functions especially PPR genes are the most likely candidates. Even though B73 either contains the recessive alleles or does not contain these loci, regions suggesting gene duplication could be of interest. It is interesting to note that maize's close relative, sorghum, has a different subclass and locus structure of its *Rf* gene. Elucidating the loci in maize and comparing them to sorghum will yield clues to the evolution of the CMS/*Rf* system.

APPENDIX A. LIST OF RECOMBINANT PLANTS

Table 1. List of recombinant 2008 *Rf8* plants including plants with RNA gel blot analysis

1997 Cross[1]	2008 Plant No.		76755p2	1.42 kb	135087p1	144635p4	66902p1	Csu811_p9	J04p6	J04p8	18p4	87p2	10p2	26p3	umC36_p10
97 2221-3 / 2202	7316-16	"S"	B[2]	--[3]			B	B							B
97 2221-3 / 2202	7317-36	"S"	B	--			B	B			B	B	B	B	B
97 2222-3 / 2149	7325-39	"S"	B	--			B	B			B	B	B	B	B
97 2224-3 / 2150	7339-28	"S"	B	--			B	B					B	B	B
97 2225-1 / 2228-8	7346-34	"S"	B	--			B	B			B		B	B	B
97 2220-11 / 2150	7245-11	"S"		--				B	B		B		B	H	H
97 2220-12 / 2228-11	7249-39	"S"		--			B	B			B	B	B	B	B
97 2220-12 / 2228-11	7301-20	"S"		--				B					B	B	
97 2221-3 / 2202	7314-28	"S"		--			B	B			B		B	B	B
97 2221-3 / 2202	7313-31	"S"		--				B					B	B	B
97 2222-15 / 2228-15	7331-17	"S"		--			B	B			B	B	B	B	H
97 2222-3 / 2149	7323-2	"S"		--			B	B	B	H	H		H	H	H
97 2223-15 / 2230	7334-42	"S"		--				B				B	B	B	B
97 2224-3 / 2150	7337-2	"S"		--			B	B					B	B	B
97 2224-3 / 2150	7337-3	"S"		--			B	B					B	B	B
97 2224-3 / 2150	7341-1	"S"		--			B								X
97 2225-1 / 2228-8	7346-35	"S"		--				B					B	B	B
97 2225-1 / 2228-8	7342-11	S	B	--	A	H	H	H							H
97 2220-11 / 2150	7245-16	S	B	--	B	B	H	X							X
97 2222-3 / 2149	7322-16	S	B	--	B	H	H	H					H		
97 2223-15 / 2230	7332-28	S	B	--	B	B		H							X
97 2225-1 / 2228-8	7346-16	S	B	--	B	B	H	H							H
97 2225-1 / 2228-8	7346-37	S	B	--	B		H	H							H
97 2225-1 / 2228-8	7346-38	S	B	--	B		H	H							H
97 2220-11 / 2150	7244-11	S	B	--			B	B	A	H	B		B	H	H
97 2220-17 / 2202	7303-19	S	B	--			B	H							X
97 2220-17 / 2202	7307-17	S	B	--			B	X							X
97 2222-3 / 2149	7325-37	S	B	--		B	H	X							
97 2222-3 / 2149	7323-9	S	B	--			B	H					H		
97 2224-3 / 2150	7338-37	S	B	--			B	B	B	B	H				H
97 2224-3 / 2150	7339-17	S	B	--			B	B							B
97 2220-11 / 2150	7245-1	S	H	--	B	B	B	B							B
97 2220-11 / 2150	7247-15	S	H	--	B	B	B	B							

Table 1. (continued)

1997 Cross[1]	2008 Plant No.		76755p2	1.42 kb	135087p1	144635p4	66902p1	Csu811_p9	J04p6	J04p8	18p4	87p2	10p2	26p3	umC36_p10
97 2220-22 / 2228	7309-20	S	H	--	B	B	B	B							B
97 2220-22 / 2228	7310-7	S	H	--	B	B	B	B							B
97 2222-3 / 2149	7324-31	S	H	--	B		B	B							B
97 2225-1 / 2228-8	7343-20	S	H	--	B	B	B	B							B
97 2225-1 / 2228-8	7343-4	S	H	--	B	B	B	B							B
97 2221-3 / 2202	7317-35	S	H	--		B	B	B					B		
97 2221-3 / 2202	7314-27	S	H	--			C	B							B
97 2220-11 / 2150	7245-25	S		--			B	H							
97 2220-11 / 2150	7244-31	S		--			B	B	B	B	B	B	B	X	H
97 2220-11 / 2150	7243-11	S		--				B				B		H	H
97 2220-11 / 2150	7244-3	S		--				B		B	B		B	B	B
97 2220-12 / 2228-11	7250-25	S		--			B	H				H			X
97 2220-17 / 2202	7306-9	S		--			B	B	B	B				B	B
97 2221-3 / 2202	7314-15	S		--			H	B							B
97 2221-3 / 2202	7315-11	S		--				H	A	H	A		H	H	H
97 2222-15 / 2228-15	7329-30	S		--			B	B	B		B	B	B	H	H
97 2222-15 / 2228-15	7329-34	S		--			B	B				B	B	B	B
97 2222-15 / 2228-15	7330-24	S		--			B	B	B		B	H		B	B
97 2222-15 / 2228-15	7331-6	S		--			B	B			B	B	B	B	B
97 2222-15 / 2228-15	7330-13	S		--				B			B	B	B	B	B
97 2222-3 / 2149	7326-20	S		--			B	B					B		B
97 2222-3 / 2149	7325-15	S		--				B	B	B			B	H	H
97 2222-3 / 2149	7326-16	S		--				B	B	B	B	C	B	C	B
97 2222-3 / 2149	7326-29	S		--				B	B	B	B		B	C	B
97 2223-15 / 2230	7334-27	S		--			B	B		B	B				H
97 2223-15 / 2230	7334-41	S		--			B	B	B	B	B	B		H	H
97 2225-1 / 2228-8	7342-17	S		--			B	B	B	B	B		B	H	H
97 2225-1 / 2228-8	7345-22	S		--				B	B	B	B	B	B	H	H
97 2225-1 / 2228-8	7344-23	"S"	B	+	A	H	H								X
97 2220-11 / 2150	7244-26	"S"	H	+			H	X			A		H	H	H
97 2220-11 / 2150	7244-1	"S"	H	+			H	X			A		H	H	X
97 2220-22 / 2228	7309-25	"S"	H	+			B	B				B	B	B	B
97 2220-22 / 2228	7310-15	"S"	H	+			B	B			B	B	B	B	B
97 2220-22 / 2228	7312-38	"S"	H	+			B	B			B		B	B	B
97 2222-3 / 2149	7322-26	"S"	H	+			H	H			A	H	H	H	H
97 2220-12 / 2228-11	7302-28	"S"		+				X					H		

Table 1. (continued)

1997 Cross[1]	2008 Plant No.		76755p2	1.42 kb	135087p1	144635p4	66902p1	Csu811_p9	J04p6	J04p8	18p4	87p2	10p2	26p3	umC36_p10
97 2220-12 / 2228-11	7302-30	"S"		+				X					H		
97 2220-17 / 2202	7305-20	"S"		+			B	B			B		B	B	B
97 2220-17 / 2202	7305-10	"S"		+			H	H	A		B		B		B
97 2220-17 / 2202	7305-18	"S"		+			H	X					H	H	X
97 2220-22 / 2228	7310-2	"S"		+			B	B			B	B	B	B	B
97 2220-22 / 2228	7310-5	"S"		+			B	B			B		B	B	B
97 2221-3 / 2202	7315-21	"S"		+			H	H			A		H	H	H
97 2221-3 / 2202	7315-2	"S"		+				H							X
97 2221-3 / 2202	7316-22	"S"		+				H							X
97 2222-15 / 2228-15	7328-5	"S"		+			H	B							B
97 2222-15 / 2228-15	7331-5	"S"		+			H	H			H		H	H	H
97 2222-15 / 2228-15	7329-35	"S"		+				H			H	H	H	H	H
97 2222-15 / 2228-15	7331-19	"S"		+				B			B		B	B	B
97 2222-15 / 2228-15	7331-7	"S"		+							H		H	H	H
97 2224-3 / 2150	7338-1	"S"		+			H	H					H	H	H
97 2225-1 / 2228-8	7346-30	"S"		+			B	B			B		B	B	B
97 2225-1 / 2228-8	7342-21	"S"		+											X
97 2225-1 / 2228-8	7346-19	"S"		+				H					H		H
97 2220-11 / 2150	7244-7	S	B	+	A	H		H							X
97 2220-12 / 2228-11	7248-27	S	B	+	A	H	H	H							X
97 2221-3 / 2202	7317-27	S	B	+	A	H	H	H					H		
97 2222-3 / 2149	7323-28	S	B	+	A	H	H	H							H
97 2222-3 / 2149	7323-6	S	B	+	A	H	H	H					H		
97 2222-3 / 2149	7324-12	S	B	+	A	H	H	H							H
97 2222-3 / 2149	7326-15	S	B	+	A	H	H								X
97 2223-15 / 2230	7333-30	S	B	+	A	H	H	H			H				H
97 2225-1 / 2228-8	7346-24	S	B	+	A	A	B	C							
97 2225-1 / 2228-8	7343-29	S	B	+	A	H	H	H							H
97 2225-1 / 2228-8	7344-1	S	B	+	A	H	H	H							H
97 2225-1 / 2228-8	7344-25	S	B	+	A	H	H								X
97 2225-1 / 2228-8	7344-8	S	B	+	A	H	H								X
97 2225-1 / 2228-8	7342-8	S	B	+	A	H		H							X
97 2225-1 / 2228-8	7344-21	S	B	+	A	H		H							X
97 2220-17 / 2202	7307-9	S	B	+		H	H	X							X
97 2221-3 / 2202	7317-29	S	B	+			H	H					H		
97 2222-3 / 2149	7323-30	S	B	+			H	H							H

Table 1. (continued)

1997 Cross[1]	2008 Plant No.		76755p2	1.42 kb	135087p1	144635p4	66902p1	Csu811_p9	J04p6	J04p8	18p4	87p2	10p2	26p3	umC36_p10
97 2220-11 / 2150	7247-23	S	H	+	A	H	B	B							
97 2220-12 / 2228-11	7248-10	S	H	+	A	H	B	B							C
97 2220-17 / 2202	7303-24	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7308-12	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7308-14	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7308-25	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7308-5	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7309-1	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7309-14	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7311-19	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7311-6	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7311-8	S	H	+	A	H	B	B							
97 2220-22 / 2228	7312-16	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7309-11	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7309-22	S	H	+	A	H	B	B							B
97 2220-22 / 2228	7312-30	S	H	+	A	H		B							B
97 2220-22 / 2228	7308-4	S	H	+	A		B	B							B
97 2220-22 / 2228	7312-29	S	H	+	A		B	B							B
97 2220-22 / 2228	7312-40	S	H	+	A		B	B							B
97 2220-22 / 2228	7308-21	S	H	+	A		B	B							B
97 2221-3 / 2202	7317-34	S	H	+	A	H	B	B					B		
97 2221-3 / 2202	7313-15	S	H	+	B	B	B	B							C
97 2220-11 / 2150	7246-16	S	H	+			H	B							B
97 2220-11 / 2150	7244-25	S	H	+			H	B							B
97 2220-22 / 2228	7308-3	S	H	+		H	B	B							B
97 2220-22 / 2228	7310-11	S	H	+		H	B	B							B
97 2225-1 / 2228-8	7344-13	S	H	+			H	B							B
97 2220-11 / 2150	7243-5	S		+				A				H		H	H
97 2220-12 / 2228-11	7248-13	S		+			H	H	A	H	B	B	B		B
97 2220-12 / 2228-11	7301-1	S		+				H					H		
97 2220-12 / 2228-11	7301-30	S		+				H	A				B		B
97 2220-17 / 2202	7305-2	S		+			H								B
97 2221-3 / 2202	7314-16	S		+				H	A					H	H
97 2222-15 / 2228-15	7329-3	S		+			H	H	A	H	H	H	B	B	B
97 2222-15 / 2228-15	7330-1	S		+			H	H			H	H	H	H	H
97 2222-15 / 2228-15	7330-28	S		+			H	H	A		H		B	H	A

Table 1. (continued)

1997 Cross[1]	2008 Plant No.		76755p2	1.42 kb	135087p1	144635p4	66902p1	Csu811_p9	J04p6	J04p8	18p4	87p2	10p2	26p3	umC36_p10
97 2222-15 / 2228-15	7331-15	S		+			H	H			H	H	H	H	H
97 2222-15 / 2228-15	7330-10	S		+				H			H	H	H	B	B
97 2222-15 / 2228-15	7331-10	S		+				H			H			H	H
97 2222-15 / 2228-15	7331-9	S		+				H	A		H			B	B
97 2222-3 / 2149	7324-33	S		+			H	H	A	H	H	H	H	B	B
97 2222-3 / 2149	7322-24	S		+				H	A	H	A	H	B	B	B
97 2222-3 / 2149	7325-29	S		+				H	A	H	H	H	B	B	B
97 2223-15 / 2230	7333-29	S		+			H	H	A	H	H		H		H
97 2223-15 / 2230	7335-27	S		+			H	H	A	H	A	H	H	B	B
97 2224-3 / 2150	7337-36	S		+			H	B							B
97 2225-1 / 2228-8	7345-33	S		+			H	B							B
97 2220-12 / 2228-11	7249-11	S		+			H	B							B
97 2220-17 / 2202	7306-19	S		+			H	B							B
97 2222-15 / 2228-15	7329-5	S		+			H	B			B	B	B	B	B
97 2222-3 / 2149	7325-14	S		+			H	B							
97 2223-15 / 2230	7335-9	S		+			H	B							B
97 2223-15 / 2230	7333-18	S		+				H	A	H					H
97 2224-3 / 2150	7339-29	S		+			H	H					H	H	H
97 2224-3 / 2150	7337-16	S		+				H	A	H	A				B
97 2225-1 / 2228-8	7344-2	S		+				H	A	H	A	H	H	C	B
97 2220-11 / 2150	7245-17	"S"	B		A	H	H								X
97 2225-1 / 2228-8	7345-35	"S"	B				H	X							
97 2220-11 / 2150	7246-7	S	B				H	H							
97 2220-17 / 2202	7305-1	S	B				H								X
97 2220-17 / 2202	7305-25	S	B				H								X
97 2221-3 / 2202	7315-16	S	B				H								X
97 2221-3 / 2202	7317-13	S	B				H	H					H		
97 2221-3 / 2202	7317-22	S	B				H	H					H		
97 2222-15 / 2228-15	7327-27	S	B				H								X
97 2222-15 / 2228-15	7327-19	S	B				H	H							X
97 2222-15 / 2228-15	7328-27	S	B				H								
97 2222-15 / 2228-15	7328-30	S	B				H	H							X
97 2222-3 / 2149	7325-27	S	B				H	H							H
97 2222-3 / 2149	7326-10	S	B				H	X							
97 2222-3 / 2149	7322-18	S	B				H						H		
97 2222-3 / 2149	7326-19	S	B				H	H							H

Table 1. (continued)

1997 Cross[1]	2008 Plant No.		76755p2	1.42 kb	135087p1	144635p4	66902p1	Csu811_p9	J04p6	J04p8	18p4	87p2	10p2	26p3	umC36_p10
97 2222-3 / 2149	7326-3	S	B				H	H							H
97 2222-3 / 2149	7326-5	S	B				H								X
97 2222-3 / 2149	7326-14	S	B					H							H
97 2223-15 / 2230	7334-13	S	B				H								X
97 2223-15 / 2230	7334-22	S	B				H	H							H
97 2223-15 / 2230	7334-38	S	B				H	H							A
97 2225-1 / 2228-8	7345-30	S	B				H	H							H
97 2222-15 / 2228-15	7331-28	"S"	H			B	B	B							B
97 2223-15 / 2230	7333-3	"S"	H				B	B							B
97 2220-11 / 2150	7244-28	S	H				H	B							
97 2220-12 / 2228-11	7250-19	S	H				H	H				B			B
97 2220-22 / 2228	7309-19	S	H				B	B							B
97 2220-22 / 2228	7310-16	S	H				B	B							B
97 2220-22 / 2228	7311-29	S	H				B	B							B
97 2220-22 / 2228	7312-10	S	H				B	B							B
97 2221-3 / 2202	7317-16	S	H				B	B					B		
97 2222-15 / 2228-15	7331-27	S	H				H	B							B
97 2222-3 / 2149	7322-11	S	H				B	B					B		
97 2222-3 / 2149	7325-34	S	H				B	B							B
97 2223-15 / 2230	7332-12	S	H					B							B
97 2224-3 / 2150	7340-13	S	H				B	B							B
97 2224-3 / 2150	7341-15	S	H				B	B							B
97 2224-3 / 2150	7341-27	S	H				B	B							B
97 2224-3 / 2150	7341-6	S	H				B	B							B
97 2225-1 / 2228-8	7346-6	S	H				B	B							B
97 2220-11 / 2150	7244-9	S					H	B							B
97 2220-12 / 2228-11	7250-4	S					B		B	B	B	B	B	H	H
97 2220-12 / 2228-11	7248-38	S					H	H				B			B
97 2220-12 / 2228-11	7248-15	S						H	A	H		H		B	B
97 2220-12 / 2228-11	7249-20	S							A	H		H	H	B	B
97 2220-17 / 2202	7305-6	S						B	B	B	B		H	H	H
97 2222-15 / 2228-15	7330-18	S					B	B	B		B	B		B	H
97 2222-15 / 2228-15	7331-18	S					B	B			B	B	B	B	H
97 2222-15 / 2228-15	7328-35	S					H	B							
97 2222-3 / 2149	7326-22	S						B	B	B	B		B	C	H
97 2223-15 / 2230	7335-15	S					B	B	B	B		B	H		H

Table 1. (continued)

1997 Cross[1]	2008 Plant No.		76755p2	1.42 kb	135087p1	144635p4	66902p1	Csu811_p9	J04p6	J04p8	18p4	87p2	10p2	26p3	umC36_p10
97 2223-15 / 2230	7336-18	S					B	H							H
97 2223-15 / 2230	7336-19	S					B	H							H
97 2223-15 / 2230	7332-17	S						B	B	B					H
97 2224-3 / 2150	7337-29	S						X	A	H	H		H	C	C
97 2224-3 / 2150	7339-21	S						B	B		B		B	H	H
97 2225-1 / 2228-8	7342-9	S					B	B	B	B	B		B	H	H
97 2225-1 / 2228-8	7342-7	S					H								B
97 2225-1 / 2228-8	7346-5	S						H			A		B		B

[1]Cross corresponds to (T) *Rf8-8703/rf8-W64A* x (N) *rf8-W64A/rf8-W64A*. All samples in this table are from the 2008 mapping population.

[2]Genotype scores are summarized as A = (N) *wx1-m8* pattern, B = (N) W64A pattern, H = heterozygous pattern, C = heterozygous or B pattern, X = heterozygous or other pattern.

[3]The + and – correspond to the presence or absence of the *Rf8*-associated 1.42/0.42-kb T-*urf13* transcripts.

ACKNOWLEDGEMENTS

I would like to thank my family and coworkers who made my Masters studies possible. My parents always encouraged my pursuit in the sciences. I would also like to say thanks to my coworkers. As laboratory technicians, Greg Fuerst and Karin Werner helped me learn and perfect protocols. Greg assisted me with the RNA extractions and Northern analyses. My work would definitely not have been possible without Karin. She helped me with every question I had and with all the field work. Her years of experience in Dr. Wise's lab made her an invaluable resource of knowledge and her enthusiastic personality made her very enjoyable to work with. I would also like to thank the undergraduate workers, especially Charles Warwick. My experiments benefited from his thoroughness and attention to detail. I would like to thank my fellow graduate student, Matt Moscou, for giving me guidance and teaching me the basics of marker development. Finally, I would like to thank my major professor, Dr. Roger Wise. He runs an excellent lab. He is always willing to discuss experimental design and results. I am glad for the opportunity he has given me.