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Mapping complementary genes in maize: positioning the *rf1* and *rf2* nuclear-fertility restorer loci of Texas (T) cytoplasm relative to RFLP and visible markers

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Abstract There are three major groups of cytoplasmic male-sterile cytoplasms in maize; C (Charrua), S (USDA), and T (Texas). These cytoplasms can be classified by the unique nuclear genes that suppress the male-sterility effects of these cytoplasms and restore pollen fertility. Typically, plants that carry Texas (T) cytoplasm are male fertile only if they carry dominant alleles at two unlinked nuclear restorer loci, *rf1* and *rf2*. To facilitate analysis of T-cytoplasm-mediated male sterility and fertility restoration, we have mapped *rf1* and *rf2* relative to closely-linked RFLP markers using five populations. The *rf1* locus and/or linked visible markers were mapped in four populations; the *rf2* locus was mapped in two of the populations. Data from the individual populations were joined with the aid of JoinMap software. The resulting consensus maps place *rf1* between umc97 and umc92 on chromosome 3 and *rf2* between umc153 and *sus1* on chromosome 9. Markers that flank the *rf1* and *rf2* loci have been used to identify alleles at *rf1* and *rf2* in segregating populations. These analyses demonstrate the possibility of tracking separate fertility restorer loci that contribute to a single phenotype.

Key words Maize · Cytoplasmic male sterility · Fertility restorer loci · Linkage mapping · Complementary genes

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Introduction

Cytoplasmic male sterility (cms) is a maternally inherited trait characterized by the inability to produce functional pollen. The cms trait is of significant value in the production of plant hybrids, eliminating the need for hand emasculation. In maize (*Zea mays* L.), there are three major groups of male-sterile cytoplasms, S (USDA), C (Charrua), and T (Texas), in addition to the N or normal male-fertile cytoplasm. These cytoplasms can be classified by the different nuclear genes that suppress their associated male-sterile phenotype, thereby allowing normal pollen development (Duvick 1965; Beckett 1971; Gracen and Grogen 1974; Laughnan and Gabay-Laughnan 1983), by mitochondrial DNA restriction endonuclease profiles (Levings and Pring 1976; Pring and Levings 1978; Borck and Walbot 1982), and by characteristic polypeptide patterns resulting from [^{35}S]-methionine incorporation by isolated mitochondria (Forde et al. 1978; Forde and Leaver 1980).

The normal N cytoplasm yields fertile plants in all known nuclear backgrounds, whereas the male-sterile C, S, and T cytoplasms only produce fertile plants in nuclear backgrounds carrying the appropriate restorer genes. These nuclear-encoded fertility-restorer genes compensate for cytoplasmic dysfunctions that are phenotypically expressed during microsporogenesis and/or microgametogenesis. Plants carrying S and C cytoplasm are restored to fertility by single dominant alleles of the *rf3* and *rf4* loci, respectively. The *rf4* locus maps approximately 2 cM from npi114A on chromosome 8 (Sisco 1991). Preliminary evidence suggests that the *rf3* locus is flanked by *whp* and bnl17.14 on chromosome 2L (T. Kamps and C. Chase, personal communication, Maize Genet Coop Newsl 66:45). In contrast, plants with T cytoplasm are restored to fertility by the dominant alleles of two loci, *rf1* and *rf2* (Laughnan and Gabay-Laughnan 1983). The mode of restoration of T cytoplasm is sporophytic; the genetic constitution of the diploid, sporophytic anther tissue, rather than that of the haploid, gametophytic pollen grain, determines pollen

development. Therefore, a T-cytoplasm plant that is heterozygous for both restorer gene loci (*Rf1/rf1*, *Rf2/rf2*) will produce all normal pollen even though only one-fourth of the pollen grains carry both *Rf1* and *Rf2*(Laughnan and Gabay-Laughnan 1983).

Texas-type cytoplasmic male sterility (cmsT) was widely used for hybrid seed production in the United States until the 1970 epidemic of southern corn leaf blight (Ullstrup 1972; Pring and Lonsdale 1989). At that time approximately 85% of the US maize crop was produced using cmsT. Maize that carries T cytoplasm is highly sensitive to the host-selective toxin (T toxin) produced by race T of *Cochliobolus heterostrophus* Drechsler (asexual stage *Bipolaris maydis* Nisikado and Miyake), the causal organism of southern corn leaf blight, and to the host-selective toxin (Pm toxin) produced by *Phyllosticta maydis*, Arny and Nelson, which causes yellow leaf blight (Hooker et al. 1970; Comstock et al. 1973; Yoder 1973).

Cms in many species is associated with the expression of novel open reading frames in the mitochondrial genome. Although each open reading frame is unique, the one common feature is that these open reading frames appear to have large hydrophobic domains (Dewey et al. 1987; Hanson et al. 1989; Nivison and Hanson 1989; Laver et al. 1991; Singh and Brown 1991). In T-cytoplasm maize, the unique mitochondrial gene, T-*urf13*, is associated with the cms and toxin sensitivity traits (Dewey et al. 1986; Rottmann et al. 1987; Wise et al. 1987a; Dewey et al. 1988; Braun et al. 1989, 1990; Pring and Lonsdale 1989; Fauron et al. 1990; Glab et al. 1990; Huang et al. 1990). T-*urf13* encodes a 13 kDa mitochondrial polypeptide (URF13) (Forde and Leaver 1980; Wise et al. 1987b) located in the mitochondrial membrane (Dewey et al. 1987). This polypeptide is not synthesized by deletion mutants (Dixon et al. 1982) and is truncated in the T4 frameshift mutant (Wise et al. 1987b). The URF13 protein binds to fungal pathotoxins (Braun et al. 1990) and appears to be present in the mitochondrial membrane in an oligomeric form (Korth et al. 1991).

The abundance of the URF13 protein is reduced by approximately 80% in plants that are restored to fertility (Forde and Leaver 1980; Dewey et al. 1987). Additionally, analysis of T-*urf13* specific transcripts in restored and nonrestored nuclear backgrounds has revealed an additional 1.6 kb transcript in mitochondria from these plants (Dewey et al. 1986; Kennell et al. 1987). The modification of the T-*urf13* transcription and the concurrent reduction of the URF13 protein has been shown to require the action of only *Rf1* and not *Rf2* (Dewey et al. 1987); however, other modifiers also appear to have an effect on T-*urf13* transcription depending on the nuclear background (Kennell et al. 1987; Rocheford et al. 1992). Little is known about *Rf2* except that, in addition to *Rf1*, it is essential for pollen restoration.

One of our long term objectives is to clone the fertility restorer genes, *rf1* and *rf2*, to aid in our understanding of their roles in fertility restoration. As a first step we have generated a collection of transposon tagged alleles of *rf2* (Schnable and Wise, 1994). However, our goal has been complicated by the interaction of the two complementary restorer loci. The ability to independently track alleles of the *rf1* and *rf2* loci was therefore essential in developing genetic constructs suitable for transposon tagging and subsequent analysis of tagged mutant alleles. The *rf1* and *rf2* restorer loci were previously mapped to chromosomes 3 and 9, respectively, using translocation breakpoint stocks (Blickenstaff et al. 1958; Duvick et al. 1961; Snyder and Duvick 1969). The order of markers for chromosome 3 was *d1-rf1-ts4* with 27 cM separating *d1* and *rf1* and 11 cM separating *rf1* and *ts4* (Duvick et al. 1961). The marker gene order for chromosome 9 was *sh1-wx1*–centromere-*rf2* with 10±2.4 cM between *wx1* and *rf2* (Snyder and Duvick 1969). Additional analyses placed these genes in the vicinity of the centromeres and relative to loosely linked visible markers (Coe et al. 1987). However, when tracking alleles of complementary loci, RFLP markers have several advantages over visible markers. Normally unaffected by dominance relationships, restriction fragment length polymorphism (RFLP) markers can be used in multiple populations without the introgression of visible markers. Additionally, identification of closely linked (and flanking) RFLP markers would simplify genetic analysis of the independently segregating *rf1* and *rf2* loci. Finally, some visible markers can influence the expression of pollen fertility. For example, the *d1* (dwarf plant 1, andromonecious) and *ts4* (tassel seed 4) markers that flank *rf1* are not suitable for studies involving male sterility, as they can affect tassel morphology and flowering. Although several detailed RFLP maps have been developed for maize (Burr et al. 1988; Burr and Burr 1991; Gardiner et al. 1993), most visible markers, including *rf1* and *rf2*, have not yet been integrated into these maps. For this reason, we have mapped the *rf1* and *rf2* nuclear restorer genes relative to a set of closely linked RFLP and visible markers. Interestingly, seemingly dominant *Rf1* was not expressed as expected in some populations, necessitating a unique transformation of the data to position the *rf2* locus. The molecular markers proved invaluble in this transformation and in subsequent analyses involving these two complementary genes.

Materials and methods

Gene symbols and phenotypes

T cytoplasm conditions male sterility in the absence of dominant alleles at a pair of complementary nuclear restorer loci, *rf1* and *rf2*. Unless otherwise noted, stocks used in this study have the genotype *rf1/rf1 Rf2/Rf2*.

The *rg1* and *gl6* loci have previously been mapped near *rf1* (Coe et al. 1987). Seedling leaves on plants homozygous for the *gl6* mutant allele are shiny (or "glossy") (Emerson et al. 1935). Adult plants carrying the dominant mutant, *Rg1*, have regions of defective tissue between the veins of mature leaves; this results in a "ragged" appearance (Brink and Senn 1931). The *wx1* locus maps near the *rf2* locus (Snyder and Duvick 1969). Homozygous *wx1* kernels have a distinctive "waxy" appearance as a result of an increased proportion of amylopectin versus amylose starch. IKI stain accentuates the difference between mutant and wild type starch. The *wx-m8* allele used in this

Table 1 Maize populations used for mapping *rf1* and *rf2* with RFLP and visible markers

Population	Cross type	Number of progeny	Parent 1[a]	Parent 2[a]	Traits scored
RF1					
92 1267-68[b]	BC_1	96	R213-T/*gl6* [*Rf1rf1*, *rf2Rf2*][e]	*gl6*-N [*rf1rf1*, *Rf2Rf2*]	*Rf1*-mediated male fertility
Gl6					
92 1140-43 92 2117-18	F_2	102[c]	Q66-N [*Gl6Gl6*]	*gl6*-N [*gl6gl6*]	*gl6*
RG1					
92g 5029-63	TC	89 (6 selected)[d]	R213-T/Acc731 [*Rf1rf1 rgRg*, *rf2Rf2*]	*gl6*-N [*rf1rf1 rgrg*, *Rf2Rf2*]	*Rf1*-mediated male fertility *Rg1*
RF2A					
91g 6222-30	BC_1	41	R213-T [*Rf1Rf1*, *rf2rf2*]	*rf2-m 8904*/R213-N [*rf1rf1*, *rf2Rf2*]	*Rf2*-mediated male fertility
RF2B					
92 1101-05	BC_1	903 (86 evaluated for RFLP markers)	R213-T/*wx-m8* [*rf1Rf1*, *Rf2rf2*]	R213-N [*Rf1Rf1*, *rf2rf2*]	*Rf1*- and *Rf2*-mediated male fertility

[a] See text for sources of genetic stocks
[b] Pedigree numbers associated with this population
[c] Selected for homozygous *gl6*
[d] Ragged, male-fertile plants, carrying a recombination between the *rg* and *rf1* loci were selected
[e] Parental genotype, see Materials and methods

study arose via a *dSpm* insertion into the *Wx1* allele (McClintock 1961, Schwarz-Sommer et al. 1984).

Genetic stocks

Allelism tests have established that our *gl6* and *wx-m8* stocks have (like most maize lines) the genotype *rf1/rf1 Rf2/Rf2* (data not shown). The *gl6* stock was obtained from the Maize Genetics Cooperation Stock Center, University of Illinois (our accession 245). Restorer loci alleles from this *gl6* line are designated *rf1-Acc245* and *Rf2-Acc245*. The *wx-m8* stock was developed by selfing an F_1 between a line homozgyous for *wx-m8* (originally developed by B. McClintock) and Line C (a color-converted W22 developed by R. Brink, University of Wisconsin) and selecting for *wx-m8/wx-m8* kernels. Plants from this derived line carry *rf1-Mc* (McClintock) and/or *rf1-LC*, and are expected to be homozygous for *Rf2-Mc* based on the close linkage between the *wx1* and *rf2* loci. The inbred line R213, which was derived from WF9 (*rf1rf1, rf2rf2*) and Ky21 (*Rf1Rf1, Rf2Rf2*), has the genotype *Rf1-Ky21Rf1-Ky21 rf2-R213/rf2-R213* (D. Duvick, personal communication, Maize Gen. Coop. Newsl. 33:95). T- and N- cytoplasm versions of R213 were obtained from M. Albertsen, Pioneer Hi-Bred International (our accessions 298 and 299, respectively). The *RgRg rf1rf1, Rf2Rf2*N cytoplasm stock was obtained from the Maize Genetics Cooperation Stock Center, University of Illinois (our accession 731). The inbred line Q66 was a gift from A. Hallauer, Iowa State University (Hallauer 1967).

Scoring visible traits

Plants were grown at either the Iowa State University Curtiss Research Farm in Ames, Iowa (summer season) or at the Hawaiian Research Ltd facility on Molokai, Hawaii (winter season). In these populations and under these environmental conditions classification of male-fertile and male-sterile plants was straight-forward; anthers on male-fertile plants exerted, anthers on male-sterile plants did not. The glossy (*gl6/gl6*) phenotype was scored at the seedling stage either in the genetics nursery or in greenhouse sand benches. The genotypes of the plants that had been scored as glossy in the field assay were usually confirmed by examining their respective F_3 families in greenhouse sand benches. The glossy phenotype is most easily discerned by misting seedlings with water; the water adheres to the leaves of the glossy seedling, but is repelled from wild-type seedling leaves. The ragged (*Rg1*) phenotype was scored visually at the time of flowering.

Origin of segregating populations

The five populations used to map *rf1* and *rf2* relative to RFLP and visible markers are presented in Table 1.

1) The **RF1** population was generated by crossing the inbred line R213 (T cytoplasm *Rf1 rf2*) by the *gl6* stock. The resulting F_1 was backcrossed (as female) by the *gl6* stock (as male) (cross 1), resulting in a population segregating for *rf1*-mediated male sterility, which could then be mapped relative to linked RFLP markers.

Cross 1: T cytoplasm *Rf1/rf1 rf2/Rf2*×*rf1/rf1 Rf2/Rf2*

2) The **GL6** population was generated by selfing (cross 2) 2 F_1 plants created by crossing the inbred line Q66 (*Gl6/Gl6*) by the relatively inbred *gl6* line (*gl6-ref/gl6-ref*). The resulting F_2 families were planted in either the summer genetics nursery or greenhouse sand benches. Seedlings with the glossy phenotype (*gl6/gl6*) were identified and scored for linked RFLP markers.

Cross 2: N cytoplasm *Gl6/gl6* self

3) The **RG1** population was generated by crossing T cytoplasm plants with the genotype *Rf1-R213 rg1/rf1-Acc731 Rg1 rf2-R213/Rf2-Acc731* (or *Rf2-Acc245/Rf2-Acc731*) by our *gl6* stock (cross 3).

Cross 3: T cytoplasm *Rf1 rg1/rf1 Rg 1 rf2/Rf2*(or *Rf2/Rf2*)×*rf1 rg1/rf1 rg1 Rf2/Rf2*

4) The **RF2A** population was developed by crossing a plant with the genotype *rf1-Mc/Rf1-R213 rf2-m8904/Rf2-Mc* to T cytoplasm R213 (cross 4). This generated a population segregating for *Rf2*-mediated male fertility, which was scored for RFLP markers. The *rf2-m8904* allele was generated in an *Spm* transposon tagging experiment (Schnable and Wise, 1994).

Cross 4: T cytoplasm *Rf1/Rf1 rf2/rf2r*×*f1/Rf1 rf2/Rf2*

5) The **RF2B** population was generated by backcrossing an F_1 between T cytoplasm R213 and our *wx-m8*stock (*rf1-Mc/rf1-Mc Rf2-Mc/Rf2-Mc*) to R213 (cross 5). This generated a population segregating for *Rf2*-mediated male fertility. From this backcross 86 random plants were scored for male-fertility, and RFLP were markers linked to both the *rf1* and *rf2*loci.

Cross 5: T cytoplasm *rf1/Rf1 Rf2/rf2*×*Rf1/Rf1 rf2/rf2*

DNA isolation and Southern analysis

Total DNA was isolated from either fresh or lyophilized maize tissue using a modified CTAB extraction (Saghai-Maroof et al. 1984). Freeze-dried tissue was powdered with a mechanical mill, and 0.4 g was suspended in 8 ml 1X CTAB extraction buffer [100 mM TRIS-

Table 2 RFLP probes and sources

Probe	Enzyme used to release insert	Insert size	Source	Reference
Chromosome 3				
umc50	*Pst*I	770 bp	M. Lee	Gardiner et al. 1993
umc92	*Pst*I	1180 bp	M. Lee	Gardiner et al. 1993
umc10	*Pst*I	1100 bp	S. Hulbert	Gardiner et al. 1993
umc97	*Pst*I	700 bp	D. Hoisington	Gardiner et al. 1993
umc102	*Pst*I	1010 bp	M. Lee	Gardiner et al. 1993
bnl6.06	*Pst*I	2400 bp	M. Lee	Burr et al. 1988
bnl5.37	*Pst*I	2300 bp	B. Burr	Burr et al. 1988
Chromosome 9				
5′ *wx1* cDNA[a]	*Eco*RI/*Bam*HI	700 bp	B. Bowen	Wessler and Varagona 1985
bnl5.10	*Pst*I	2200 bp	E. Coe	Burr et al. 1988
umc153	*Pst*I	700 bp	M. Lee	Gardiner et al. 1993
p3′*sus1*[b]	*Hin*dIII/*Bam*HI	1100 bp	L. C. Hannah	McCarty et al. 1986
umc95	*Pst*I	700 bp	E. Coe	Gardiner et al. 1993

[a] A 700-bp *Eco*RI-*Bam*HI fragment representing the 5′ end of the waxy cDNA was isolated from pPHI1735 (a gift from Pioneer Hi-Bred) for use as a *wx* RFLP marker
[b] A 1.1-kb *Hin*dIII-*Bam*HI fragment representing the 3′ end of the *sus1* genomic clone (previously designated *Css*) was isolated and subcloned from p21.2 (a gift from L.C. Hannah) for use as a *sus1* RFLP marker

Table 3 Enzyme/probe combinations necessary to detect polymorphisms in the mapping populations[a]

Mapping population[c]	Chromosome 3 RFLP markers[b]						
	bnl5.37	bnl6.06	umc10	umc97	umc102	umc92	umc50
RF1	*Eco*RV	*Eco*RV	*Eco*RI	*Eco*RV	*Bam*HI	NP[d]	*Eco*RI
GL6	*Xba*I	*Eco*RI	*Xba*I *Eco*RI	NP	*Eco*RI	*Xba*I *Eco*RI	NP
RG1	*Eco*RV	*Eco*RV	NP	*Eco*RV	*Eco*RV	–[e]	*Eco*RV
RF2A	*Hin*dIII	*Bam*HI	–	*Bam*HI	*Dra*I	*Dra*I	–
RF2B	*Hin*dIII	*Hin*dIII	–	*Hin*dIII	*Apa*I	*Eco*RV	*Eco*RV
Mapping populations	Chromosome 9 RFLP markers						
	5′ *wx1*	bnl5.10	umc153	3′ *sus1*	umc95		
RF2A	*Hin*dIII *Bam*HI	*Hin*dIII	*Hin*dIII *Bam*HI	*Hin*dIII *Bam*HI	*Bam*HI		
RF2B	*Hin*dIII	NP	*Apa*I	*Hin*dIII	*Hin*dIII		

[a] The restriction endonucleases, *Apa*I, *Bam*HI, *Bcl*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Sst*I, *Taq*I, and *Xba*I were used to survey parents for potential polymorphisms. Enzymes for mapping were chosen based on the greatest number of polymorphic probes per enzyme in the mapping populations
[b] See Table 2 for description of the RFLP probes used in these analyses
[c] See Table 1 for description of the mapping populations used in these analyses
[d] NP indicates that the probe did not reveal polymorphisms between the parents with several of the restriction enzymes listed above
[e] A dashed line indicates that data were not collected for the probe/mapping population combination

HCl, pH 7.5; 0.7 M NaCl; 10 mM Na_2EDTA; 1.0% (w/v) hexadecyl-trimethylammonium bromide; 1% (v/v) 2-mercaptoethanol]. Samples were incubated at 65°C for 60 min with mixing by tube inversion at 15 min intervals. After cooling for 10 min at room temperature, 4 ml of chloroform:octanol, 24:1 (v/v) was added and mixed for 10 min. The resulting emulsion was centrifuged at room temperature at 3500 rpm in a HS-4 rotor (Sorvall) for 20 min at 20°C to separate the phases. The upper aqueous phase was precipitated with 4 ml of ice-cold isopropanol and the nucleic acids were spooled out with a glass hook and transferred to a microcentrifuge tube containing 76% ethanol, 0.2 M sodium acetate. After 20 min the DNAs were transferred to a new tube containing 76% ethanol, 10 mM ammonium acetate. After 5 min the DNAs were transferred to 400 ul TE (10 mM TRIS-HCl, pH 8.0; 1.0 mM Na_2EDTA) containing 10 µl of RNAse A (10 mg/ml)+RNAse T1 (2500 u/ml) and dissolved overnight at 4°C or room temperature. Fresh tissue was processed the same way except that it was ground under liquid nitrogen and 2X CTAB buffer (100 mM TRIS-HCl, pH 8.0; 1.4 M NaCl; 20 mM Na_2EDTA; 2.0% (w/v) hexadecyl-trimethylammonium bromide; 1% (v/v) 2-mercaptoethanol) was used in the initial extraction.

Individual DNAs (10 ug) were digested with 3 units of enzyme per microgram DNA for 5 h or overnight and precipitated by the addition of 0.1 volume of 8 M ammonium acetate and 2 volumes of absolute ethanol. The precipitates were centrifuged for 20 min at 15 000 *g* at 4°C, washed with 70% ethanol, centrifuged for 10 min at 15 000 *g* and dried and rehydrated in 40 µl 10 mM TRIS, 1 mM Na_2EDTA, pH 8.0. Either one or two 30-sample rows of the digested DNAs were subjected to electrophoresis through 0.9% agarose (Seakem LE, FMC) in 20×24 cm gels (IBI) for 16 h at 45 V (sin-

gle row) or 30 V (double row) in 1×TPE buffer (36 mM TRIS, 3 mM NaH_2PO_4, 1 mM Na_2EDTA) and transferred to Hybond N+ nylon (Amersham).

RFLP plasmid inserts (Table 2) were labeled with [^{32}P] dCTP by the random hexamer method (Feinberg and Vogelstein 1983), and membranes were hybridized in a Robbins Scientific Hybridization incubator at 65°-67°C for 18-20 h in 7% SDS, 1% BSA, 1 mM Na_2EDTA, 0.5 M NaH_2PO_4, pH 7.2 (Church and Gilbert 1984). The membranes were washed 3×30 min at 65°C in 1×SSPE (20X=3.6 M NaCl, 0.2 M NaH_2PO_4, 20 mM Na_2EDTA, pH 7.4), 30 min at 65°C in 0.5× or 0.1×SSPE, and exposed to Kodak X-Omat AR film for 3–5 days at –80°C with one or two Lightning Plus (Dupont) intensifying screens.

Data analysis

RFLP and visible markers found to be polymorphic in the respective mapping populations (Table 3) were analyzed for marker order and distance using a combination of the computer software RI Plant Manager version 2.4 (licensed from Kenneth Manley, Roswell Park Cancer Institute) and JoinMap version 1.3 (a gift from Piet Stamm, Wageningen). RI Plant Manager facilitates mapping in backcross as well as recombinant inbred populations. JoinMap facilitates the merging of linkage data from different populations and cross types, such as backcross, F_2, and recombinant inbreds (Stamm 1993). Following the calculation of pairwise recombination frequencies and the corresponding LOD (log10) scores, JoinMap builds the map starting with marker pairs with the highest LOD score. The default critical LOD scores of 3.0 for linkage groups and 0.05 for mapping were used with the JoinMap program. Recombination values were transformed into map distances by using the Kosambi function (Kosambi 1944). The method is described in detail by Stamm (1993), with an accompanying example (Hauge et al. 1993) demonstrating the usefulness of the program.

Results

Mapping of *rf2*

Two populations segregating for *rf2*-mediated male-sterility (RF2A and RF2B) were established (Materials and methods and Table 1). In the RF2A population (41 gametes), *rf2* cosegregated with bnl5.10 and umc153, and these 3 loci were flanked by *wx1* and *sus1* (Table 4). We also analyzed 304 individuals, carrying 3 independent *rf2* alleles. These individuals were derived from three *Spm*-containing F_2 populations and confirmed the results of Burr et al. (1988) and Gardiner et al. (1993), in that bnl5.10 lies between *wx1* and umc153. To more precisely determine the position of *rf2* relative to these previously positioned markers, we developed a larger *rf2* mapping population (RF2B, Table 1). In the RF2B population (cross 5, Materials and methods), male sterility would be expected to segregate 1:1 in conjunction with *rf2*. However, in this population, an excess of male steriles was observed (655 out of 903), resulting in a ratio significantly different from 1:1 (χ^2=183), but not significantly different from 3:1 (χ^2=2.92).

The RFLP patterns of 86 random individuals from population RF2B were determined at *wx1*, umc153, *sus1*, and umc95. In the RF2B population, plants heterozygous for *wx1* and *sus1*, markers that flank *rf2* (based on the analysis of the RF2A population), are expected to have the genotype *Rf1/Rf1*, *Rf2/rf2*, or *Rf1/rf1*,*Rf2/rf2*, and therefore to be male fertile. Many of these plants were unexpectedly male sterile, apparently resulting from an inordinate number of double cross-overs. Shown in Fig. 1 B are the raw data from this population assuming that *rf2* resides between umc153 and *sus1* (one of the two possible *rf2* positions consistent with the results from population RF2A). Note the apparent double cross-overs between umc153 and *sus1* in plants nos. 5, 7, 9, 12, 26, 36, 38, 41, 46, 48, 52, 55, 66, and 86 (Fig. 1 B). The other *rf2* position consistent with results from population RF2A, i.e., between *wx1* and umc153, results in even more apparent double cross-overs (analysis not shown).

To help explain the large number of apparent double cross-overs and the 3:1 ratio of male-sterile to male-fertile plants in this population, plants in the RF2B population were also scored for RFLP markers linked to *rf1* (Fig. 1 A). As described below, *rf1* is flanked by umc97 and umc92. Plants that were heterozygous for both umc97 and umc92 were almost always sterile, while those that were homozygous for both markers segregated 1:1 male sterile to male fertile. On the basis of the cross used to generate the RF2B population (cross 5, Materials and methods), plants that were heterozygous for umc97 and umc92 would be expected to have the genotype *Rf1/rf1*, and plants that were homozygous for both of these markers would be expected to have the genotype *Rf1/Rf1*. It was therefore concluded that in this population, plants with the predicted genotype *Rf1/rf1*, *Rf2/rf2* were male sterile, while plants with the genotype *Rf1/Rf1*, *Rf2/rf2* were male fertile. Regardless of the genotype at the *rf1* locus, *rf2/rf2* plants would be male sterile. This explanation is consistent with the 3:1 ratio of male-sterile to male-fertile plants in this population. To clarify these unexpected results, additional

Table 4 Comparison of recombination estimates among markers flanking *rf2* on chromosome 9[a]

Interval	Population			
	RF2A (41)[b]	RF2B (86)[c]	F_2 Intercross (304)	Consensus[d] (431)
wx1 to bnl5.10	2.44±2.41		3.62±0.91	3.62±0.91
wx1 to umc153		4.71±2.30[e]		
bnl5.10 to umc153	0.00±2.44		1.36±0.56	1.36±0.56
umc153 to *rf2*	0.00±2.44	10.26±4.86	–	5.00±2.44
rf2 to *sus1*	4.88±3.36	7.69±4.27	–	6.25±2.70
*sus*1 to umc95	2.44±2.41	1.18±1.17	–	1.59±1.11

[a] Map distance±standard error, derived from pairwise estimates
[b] Population size in brackets
[c] Population size for umc153 to *rf2* and *rf2* to *sus1*=39
[d] Consensus estimate based on pairwise estimates
[e] No polymorphism at bnl5.10

```
A                    1 11111 11112 22222 22223 33333 33334 44444 44445 55555 55556 66666 66667 77777 77778 88888 8
                 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 6
UMC50            BBBHB HHHHB HHHBB BBBHB HHBHB BHBBB BBBBH BBHBH HBHBB HHHHB BHBHH BBHBB BHHHB HBBHB HBBHH BHHBB BBHBH H
                                  x
UMC92            BBBHB HHHHB HHHBH BBBHB HHBHB BHBBB BBBBH BBHBH HBHBB HHHHB BHBHH BBHBB BHHHB HBBHB HBBHH BHHBB BBHBH H
                   x  x              x    x   xx          x              x           x     x      x  xx x          x
UMC97            BBBBB BHHHB HHHBH BBBBB HHHHB HBBBB BBBBH HBHBH HBHBB HHBHB BHBHH BBBBB BHBHB HBBBB BHBBH BHHB- BHHBH H
                          x
UMC102           BBBBB BHHHH HHHBH BBBBB HHHHB HBBBB BBBBH HBHBH HBHBB HHBHB BHBHH BBBBB BHBHB HBBBB BHBBH BHHBB BHHBH H
                        x                                    x                 x                 x
BNL606           BBBBB BBHHH HHHBH BBBBB HHHHB HBBBB BBBBH HBHBH HBHBH HHBHB BHBHH BBBBB HHBH- HBBBB BHBBH HHHBB BHHBH H
                              x          x          x          xx                x           x          xx     x
BNL537a          BBBBB BBHHH HBHBH BBBBB BHHHB HBBBH BBBBH HBHBH HBHHB HHBHB BHBHH BBBHB HHBH- HBBBH BHBBH HHBHB BHHHH H

B                    1 11111 11112 22222 22223 33333 33334 44444 44445 55555 55556 66666 66667 77777 77778 88888 8
                 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 6
wx               HHBHB HHBHH BHHBH HBBBB BBHBB HBBHH HHHHB HBHBH HBBBB HBHBB BHBHB BHHHH HBBB- HBHHH HHHBB BHBHB HBBBB H
                              x          x                                        x                 x
UMC153           HHBHB HHBHH BHBBH HBBBB BBBBB HBBHH HHHHB HBHBH HBBBB HBHBB BHBHH BHHHH HBBBB HHHHH HHHBB BHBHB HBBBB H
                   x x  x x   x  x              x          x x x x     x x    x xx           x              x x  x
rf2              HHHHH HBBB- BBBBB -BBBB BBBBB BBBHH HHHHB BBBBB BBBBB BBBBB BBBBB BHHHH HBBBB BHHHH HHHBB BBBBB BBBBB B
                     x  x x   x              x          x  x x   x     x  x   x  x           x  x   x                x
sus1             HHHHB HHBHH BHBBB HBBBB BBBBB HBBHH HHHBB HBHBB HBBBB HBHBB BHBBH BHHHH HBBHB HHHHB HHHBB BBBBB BBBBB H
                                                                          x
UMC95            HHHHB HHBHH BHBBB HBBBB BBBBB HBBHH HHHBB HBHBB HBBBB HHHBB BHBBH BHHHH HBBH- HHHHB HHHBB BBBBB BBBBB H

C                    1 11111 11112 22222 22223 33333 33334 44444 44445 55555 55556 66666 66667 77777 77778 88888 8
                 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 6
wx               HHBHB HHBHH BHHBH HBBBB BBHBB HBBHH HHHHB HBHBH HBBBB HBHBB BHBHB BHHHH HBBB- HBHHH HHHBB BHBHB HBBBB H
                              x          x                                        x                 x
UMC153           HHBHB HHBHH BHBBH HBBBB BBBBB HBBHH HHHHB HBHBH HBBBB HBHBB BHBHH BHHHH HBBBB HHHHH HHHBB BHBHB HBBBB H
                   x x                                                                                          x  x
rf2              HHH-H ----- ---B- -BB-B ----B --BHH HHHH- -B-B- -B-BB ----B B-B-- BH-HH H---B -HH-H --H-- B--BB B--B- -
                     x                                  x                                        x
sus1             HHHHB HHBHH BHBBB HBBBB BBBBB HBBHH HHHBB HBHBB HBBBB HBHBB BHBBH BHHHH HBBHB HHHHB HHHBB BBBBB BBBBB H
                                                                          x
UMC95            HHHHB HHBHH BHBBB HBBBB BBBBB HBBHH HHHBB HBHBB HBBBB HHHBB BHBBH BHHHH HBBH- HHHHB HHHBB BBBBB BBBBB H
```

Fig. 1A–C RI plant manager output illustrating transformed data from population RF2B used to map *rf2* on chromosome 9. **A** RFLP marker data for chromosome 3 RFLP markers, **B** RFLP data for *rf2* linked markers on chromosome 9, **C** transformed data used to map *rf2* on chromosome 9. For RFLP markers, *H* denotes heterozygotes, *B* denotes homozygous backcross parent (R213: *Rf1 Rf1, rf2 rf2*). For the *rf2* marker, *H* denotes male-fertile phenotype, *B* denotes male-sterile phenotype. *Numbers* across the *top* denote individuals in the population. The symbol *X* denotes a crossover

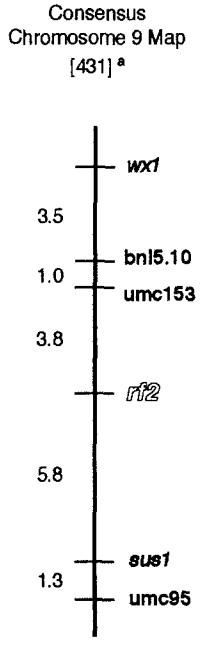

Fig. 2 Consensus genetic map of *rf2* region on chromosome 9L. The map was derived from joining RFLP and fertility data from the RF2A and RF2B populations, in addition to RFLP data from an additional 304 F_2 individuals. The inclusion of the F_2 individuals aided the more precise positioning of bnl5.10 and umc153. [a]Population size

plants from several sublines of R213 were crossed to *rf1/rf1* lines. Analysis of the progeny from these crosses established that some, but not all, R213 sublines carry *rf1*. Therefore, it is possible that the excess proportion of male-sterile plants in the RF2B population resulted from the use of an *rf1/rf1 rf2/rf2* version of R213 as the male parent in Cross 5 during the development of the RF2B population.

To permit the mapping of *rf2* in this population where male fertility/sterility is confounded by the segregation of the complementary *rf1* locus, we transformed the data in the RF2B population. The data transformation was accomplished by removing from the analysis all plants with the predicted genotype *Rf1/rf1*, i.e., those that were heterozygous for umc97, umc92, or both. This transformation does not bias our *rf2* mapping results because umc97 and umc92 on chromosome 3 segregate independently of *rf2* and RFLP markers on chromosome 9. The 47 confounding plants that were removed from the analysis are coded by a dash (-) at the *rf2* locus in the transformed data set representing chromosome 9 (Figure 1 C). By means of this transformed data set it was then possible to map *rf2* relative to chromosome 9 RFLP markers by comparing the RFLP patterns of the remaining 21 male-sterile and 18 male-fertile plants.

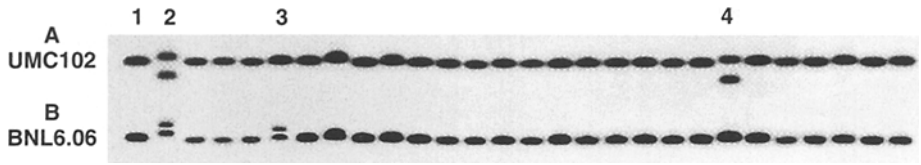

Fig. 3A, B Southern analysis of GL6A interval mapping population. **A** *Eco*RI restriction digestion of DNA isolated from plants in the row 92 1142–43 (Table 1), hybridized with umc102. **B** Nylon membrane from **A** stripped and hybridized with bnl6.06. *Lane 1* homozygous *gl6* control, *lane 2* F_1 parent between Q66 (*Gl6 Gl6*) and inbred *gl6* line (*gl6 gl6*), *lane 3* recombinant between *gl6* and bnl6.06, distal to *gl6*, *lane 4* recombinant between *gl6* and umc102, proximal to *gl6*.

The resulting map based on transformed data from population RF2B was then joined to that derived from populations RF2A and the three pooled F_2 populations to generate a consensus map for chromosome 9 (Table 4, Fig. 2). The position of *rf2* relative to chromosome 9 RFLP markers was determined from a total of 80 individuals, 41 from the RF2A population and 39 from the RF2B population. On this consensus map, *rf2* is flanked by umc153 and *sus1*. Because previous research has established that *wx1* is in the short arm of chromosome 9 and that the centromere is located between *wx1* and umc153 (Weber and Helentjaris 1989), *rf2* must be on the long arm of chromosome 9.

Mapping of *rf1*

The *rf1* locus, linked RFLPs, and/or visible markers were mapped in all five of our populations. It was possible to directly map *rf1* in three of these populations (RF1, RF2A, and RG1). In these three populations *rf1* is invariably flanked by umc50 and/or umc92 on one side and umc97 and/or umc10 on the other.

Population RF1 (cross 1, Materials and Methods) was generated via an *rf1* testcross that segregated 1:1 for *rf1*-mediated male sterility. It was also possible to map *rf1* in the RF2B population. RF2B was the *rf2* testcross population discussed above (in reference to mapping *rf2*), in which presumed *rf1/Rf1 Rf2/rf2* plants were unexpectedly sterile, while presumed *Rf1/Rf1 Rf2/rf2*plants were male fertile. However, because *rf2*-mediated male sterility was also segregating in this population, a data transformation similar to the one described above for mapping *rf2* was used to remove *rf2/rf2* plants. A conservative assay for *rf2/rf2* genotypes was used; plants were removed if they were homozygous for either umc153 or *sus1* or both. Following transformation, the RF2B population contained 33 plants for which fertitility was segregating. The complete RF2B population (86) was assayed for chromosome 3 RFLP markers, in which *rf1* was flanked by umc97 and umc92.

To place *rf1* on the classical genetic map we positioned *rf1* relative to *rg1* in population RG1. The position of *rf1* and *rg1*, in reference to the chromosome 3 RFLP markers between them, was determined from a total of 89 individuals. Six male-fertile, ragged recombinants were obtained from 89 ragged plants derived from cross 3 (the non-ragged plants were not examined). This places *rg1* approximately 7 cM from *rf1*. These 6 male-fertile, ragged recombinants, in addition to 12 male-sterile, ragged and 12 male-fertile, non-ragged controls were scored for chromosome 3 RFLP markers to position *rg1* on the RFLP map. Because of the pre-selection for recombination between *rg1* and *rf1* inherent in the analysis, it was only possible to estimate genetic distances within the interval defined by *rg1* and *rf1*. However, it was possible to confirm that the order of RFLPs outside this interval was consistent with our other analyses.

Two additional populations (RF2A and GL6) were analyzed with RFLP markers from chromosome 3. Although it was not possible to map *rf1* or any other visible markers in RF2A, the inclusion of RFLP results from this population provided greater precision to the estimates of genetic map distances between the RFLP markers used in these studies. The GL6 population (cross 2, Materials and methods) was produced to position the visible marker, *gl6,* relative to *rf1*. From this segregating F_2 population, 102 *gl6* homozygotes were selected for RFLP analysis. In this way, scoring for recombinants between linked markers and the *gl6* phenotype was easily accomplished by identifying rare heterozygous banding patterns, indicating recombination between the respective RFLP marker and the *gl6* phenotype (Fig. 3). Hybridization analysis of the GL6 population with chromosome 3 RFLP markers placed *gl6* between umc102 and bnl6.06.

Because the GL6 population was not segregating for *rf1* it was not possible to directly map *gl6* relative to *rf1*. To circumvent this limitation, we first positioned *gl6* relative to the RFLP markers used to map *rf1*. By joining the resulting GL6 map with the RFLP maps that included *rf1*, it was possible to establish that *gl6* is approximately 3 cM from *rf1* (Fig. 4).

A comparison of the Missouri and Brookhaven RFLP maps to chromosome 3 maps derived from each of the five mapping populations, and a JoinMap-derived consensus map, is presented in Fig. 4.

Discussion

A number of cytoplasmic male sterility (cms) and fertility restoration systems have been well characterized at the genetic and molecular levels. Examples of these include petunia (Nivison and Hanson 1989), common bean (Mackensie and Chase 1990; Johns et al. 1992), *Brassica napus* (Singh and Brown 1991), sunflower (Laver et al. 1991), S-cytoplasm maize (Schardl et at. 1984, Schardl et al. 1985; reviewed in Levings and Brown 1989), and T-cytoplasm

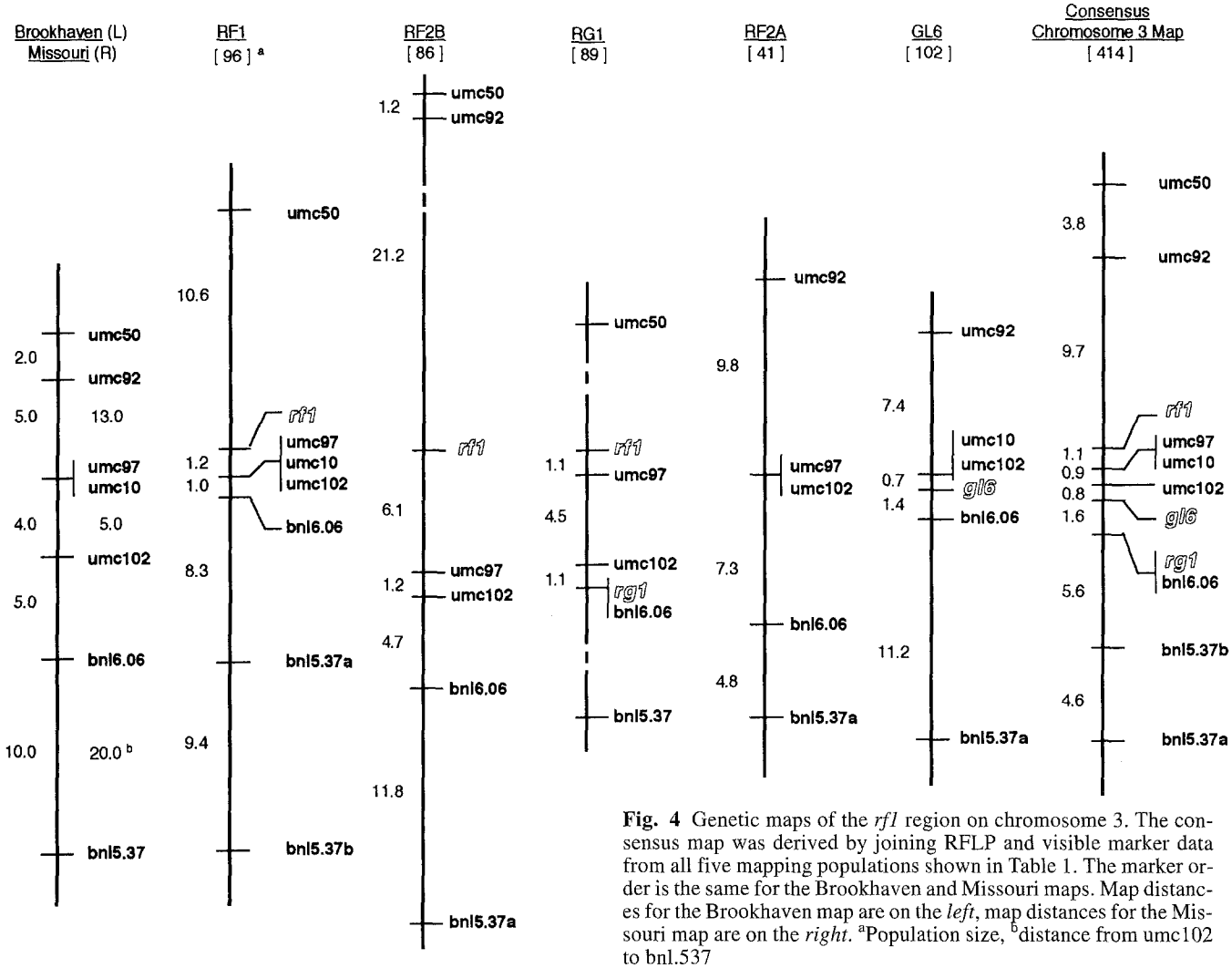

Fig. 4 Genetic maps of the *rf1* region on chromosome 3. The consensus map was derived by joining RFLP and visible marker data from all five mapping populations shown in Table 1. The marker order is the same for the Brookhaven and Missouri maps. Map distances for the Brookhaven map are on the *left*, map distances for the Missouri map are on the *right*. [a]Population size, [b]distance from umc102 to bnl.537

maize (Pring and Lonsdale 1989). Cms in petunia, bean, *Brassica*, C-cytoplasm maize, and S-cytoplasm maize can be restored to fertility by single dominant nuclear genes. In contrast to other male-sterile cytoplasms, T-cytoplasm is restored by the combination of dominant alleles at two nuclear restorer loci, *rf1* and *rf2* (Laughnan and Gabay-Laughnan 1983). In this study of 718 individuals, we have confirmed and extended prior classical mapping data with the complementary results of positioning *rf1* and *rf2* relative to more generally useful markers. These analyses also provide data that better integrate the classical and RFLP maps in the vicinity of the centromeres of chromosomes 3 and 9.

The 8.6±3.9 cM distance from *wx1* to *rf2* reported here is in agreement with the 10±2.4 cM reported previously by Snyder and Duvick (1969) (Table 4). However, in addition, we have identified 4 RFLP markers that are more tightly linked to *rf2* than *wx1*. The 2 closest of these RFLP markers, umc153 and *sus1*, flank *rf2* and are separated from *rf2* by 3.8 and 5.8 cM, respectively. The use of these closely linked flanking markers would remove the risk of a single crossover invalidating a *rf2* allele tracking experiment. The placement of *rf2* relative to RFLP markers which have themselves been mapped relative to the centromere (Weber and Helentjaris 1989) allowed us to determine that *rf2* is on the long arm of chromosome 9.

Previous research had placed *rf1* between *d1* and *ts4*. However, these markers are not suitable for following *rf1* alleles. First, they are not closely linked to *rf1*; Duvick et al. (1961) placed them 27 and 11 cM away from *rf1*, respectively. Second, both markers require special handling to propagate because they can influence male or female fertility. In this study, mapping data from five genetically distinct populations were joined, resulting in a detailed map of the region of chromosome 3 that flanks *rf1*. This consensus map includes both RFLP and closely linked, easily maintained visible markers that do not influence male fertility. On one side of *rf1*, umc97 and umc10 are only 1.2 cM from *rf1*. On the other side, umc92 is 9.5 cM from *rf1*. Two visible markers (*gl6* and *rg1*) were mapped to within 3.0 and 4.5 cM of *rf1*, respectively. It was not possible to position *rf1* to a chromosome arm because the chromosome 3 centromere has not been located with high resolution to the RFLP map. Additionally, many of the RFLP markers used in this study have not been mapped relative to the centromere (Weber and Helentjaris 1989).

Table 5 Comparison of recombination estimates among markers flanking *rf1* on chromosome 3[a]

Interval	Population					
	RF1 [96][b]	RF2B [86][c]	RG1 [89][d]	RF2A [41]	GL6 [102]	Consensus[e] [414]
umc50 to *rf1*	10.59±3.34	21.21±7.12	–	–	–	13.56±3.15
umc92 to umc97/umc10	–	17.65±4.13	–	9.75±4.63	7.38±1.91	15.08±3.19
rf1 to umc97/umc10	1.18±1.17	6.06±4.15	1.12±1.12	–	–	1.93±0.96
umc97/umc10 to umc102	0.00±1.04	1.18±1.17	4.49±2.20	0.00±2.44	0.00±0.014	1.61±0.71[g]
umc102 to bnl6.06[f]	1.04±1.04	4.71±2.30	1.12±1.12	7.32±4.07	1.96±0.97	2.52±0.69
bnl6.06 to bnl5.37a	8.33±2.82	11.76±3.49	–	4.76±4.65	11.18±2.28	10.60±1.53

[a] Map distance ± standard error, derived from pairwise estimates
[b] Population size in brackets
[c] Population size of *rf1* to RFLP markers=33
[d] Population size of *rf1* to RFLP markers=89
[e] Consensus estimate based on pairwise estimates
[f] bnl6.06 cosegregated with *rg1* in the RGA population
[g] Consensus estimate based on recombination between umc97 and umc102

Differences in recombination frequency among populations

A striking feature of this study is the large amount of variability in genetic distances observed among the different mapping populations and relative to the Missouri and Brookhaven maps (Table 5 and Fig. 4). Most notably, in the RF1 and GL6 populations, there was no recombination among umc97, umc10, and umc102, and very little between these 3 markers and bnl6.06. In contrast, in the RF2B, RG1 and RF2A populations, we observed significantly more recombination between umc97, and umc102 and/or between umc102 and bnl6.06 (Table 5).

A possible explanation for the map compression observed in these regions is that mapping populations RF1 and GL6 are heterozygous for a small inversion in the vicinity of umc10, umc97 and umc102 relative to the RF2B, RG1, and RF2A populations and those populations used by the Brookhaven and Missouri laboratories. Crossovers within the inversion loop in an inversion heterozygote produce gametes containing duplications and/or deletions of regions encompassing (or flanking) the inversion. These gametes would almost certainly not be transmitted, since this putative inversion is near the centromere. The loss of these cross-over gametes would result in map compression relative to maps derived from populations homozygous for either the normal or the inversion chromosome. The RF1 and GL6 populations both carry the chromosome 3 derived from our *gl6* stock, suggesting that the chromsome 3 from this stock may be responsible for the map compression observed in these two populations.

Further evidence suggesting that an inversion exists in this region comes from an analysis of an *Rf1*-converted version of the inbred B37 obtained from Pioneer Hi-Bred. A chromosome 3 from this line carries *Rf1* and the umc102 allele provided by the *Rf1* donor parent, but not the B37 alleles of umc97, umc10, and bnl6.06 (data not shown). These results suggest that in this line umc10 and umc97 are not flanked by *rf1* and umc102, or that a double crossover occurred during the backcross procedure. A double crossover in this interval is unlikely because the genetic distance is very small (Fig. 4). If the former is true, this would represent a gene order reversal relative to our consensus map and those of the Brookhaven and Missouri groups. Variability in recombination frequencies among populations has been observed for other maize chromosomes as well (Tulsieram et al. 1992, Beavis and Grant 1991). Interestingly, other reports have shown that variability in recombination frequencies may be associated with heterochromatic regions (Robertson 1984, Nel 1973, Chang and Kikudome 1973). Since the markers in the *rf1* region of chromosome 3 are tightly linked to the centromere (Weber and Helentjaris 1989), this may account for some of the observed differences, although our study was not set up to test this hypothesis.

An additional cytological polymorphism was identified in this study. The R213 parent of the RF1 population carries a tandem duplication of bnl5.37. *Eco*RV restriction endonuclease digestion of segregating progeny from the RF1 population, followed by DNA hybridization analysis with bnl5.37, revealed that the R213 stock in this cross had two linked, hybridizing bands. This duplication appears to have a profound effect on the apparent rate of double crossovers. Four double crossovers (out of 96 gametes) were recorded within the 17.7 cM interval defined by bnl5.37a and bnl6.06. A reversal of the gene order of bnl5.37a and bnl5.37b would result in five double cross-overs. The two bnl5.37-related sequences (bnl5.37a and bnl5.37b) from the R213 parent are approximately 9.4 cM apart (RF1 map, Fig. 4). bnl5.37a-R213 was determined to be allelic to the single copy band in our *gl6* stock because the copy number of bnl5.37-hybridizing sequences was one-half as intense in heterozygotes as in homozygotes. The intensity of bnl5.37b-R213 was identical in both homozygotes and heterozygotes. Similar analyses for each of the other populations represented in Table 1 demonstrated that the single copy of a bnl5.37-related sequence that is present in the *gl6*, Q66, and *wx-m8* stocks is allelic to bnl5.37a (data not shown).

Considerations in joining mapping populations using JoinMap

Differences in recombination frequencies among populations generate difficulties when creating consensus maps with computer programs such as JoinMap (Beavis and Grant 1991, Hauge et al. 1993). JoinMap compiles the data from all entered populations and calculates pairwise estimates of recombination frequencies with standard errors. On the basis of these calculations, the JoinMap program "reshuffles" pairwise data starting with the pair of markers with the highest LOD score (Stamm 1993). This marker order can then be used as a guide for marker placement and distance. When many populations are used, each having pairwise differences as described above, caution is warranted in interpreting the final result. For example, JoinMap would not allow the placement of *gl6* within the umc102 - bnl6.06 interval, even though a "fixed sequence" command was given and results from the GL6 populations had clearly positioned *gl6* in this interval (Fig. 3). JoinMap also separated *rg1* from bnl6.06 and umc97 from umc10, even though we did not observe any crossovers in any of the five populations between the members of these two sets of markers. In addition, in our consensus chromosome 3 map, it appears that *gl6* and *rg1* are reversed relative to the maize chromosome 3 classical map. This is most likely the result of merging two populations, GL6 (where *gl6* is flanked by bnl6.06 and umc102) and RG1 (where *rg1* cosegregates with bnl6.06). Pairwise estimates of the recombination distance between bnl6.06 and *gl6* and between bnl6.06 and *rg1* were 1.96±0.97 and 0.00±1.12, respectively, indicating some overlap between the two estimates. However, because these two visible markers were mapped in populations carrying different chromosome 3s, and because we have not directly mapped *rg1* relative to *gl6*, this order may be an artifact resulting from the interaction of limited population sizes and the JoinMap algorithm. If a composite map is required of a genome, Morton's (1956) test of homogeneity coupled with the computational capabilities of computer software such as MAPMAKER (Lander et al. 1987), as suggested by Beavis and Grant (1991), should be applied. We found that it is possible to create integrated maps of a particular chromosomal region using JoinMap. The resulting map must be evaluated relative to previously established map orders, however. Thus, we have successfully integrated the *rf1* and *rf2* fertility restorers in reference to RFLP and visible markers. These markers, which do not affect tassel morphology or pollen fertility, provide a foundation upon which the biology of fertility restoration can be investigated involving these two complementary genes in T-cytoplasm maize.

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References

Beavis WD, Grant D (1991) A linkage map based on information from four F_2 populations of maize (*Zea mays* L.). Theor Appl Genet 82:636–644

Beckett JB (1971) Classification of male-sterile cytoplasms in maize (*Zea mays* L.). Crop Sci 11:721–726

Blickenstaff JD, Thompson DL, Harvey PH (1958) Inheritance and linkage of pollen fertility restoration in cytoplasmic male-sterile crosses of corn. Agron J 50:430–434

Borck KW, Walbot V (1982) Comparison of the restriction endonuclease digestion patterns of mitochondrial DNA from normal and male-sterile cytoplasms of *Zea mays*L. Genetics 102:109–128.

Braun CJ, Siedow JN, Williams, ME, Levings CS III (1989) Mutations in the maize mitochondrial T-*urf13* gene eliminate sensitivity to a fungal pathotoxin. Proc Natl Acad Sci USA 86:4435–4439

Braun CJ, Siedow JN, Levings CS III (1990) Fungal toxins bind to the URF13 protein in maize mitochondria and *Escherichia coli*. Plant Cell 2:153–161

Brink RA, Senn PH (1931) Heritable characters in maize XL-ragged, a dominant character, linked with *A1*, *Ts4*, and *D1*. J Hered 22:155–161

Burr B, Burr FA (1991) Recombinant inbreds for molecular mapping in maize: theoretical and practical considerations. Trends Genet 7:55–60

Burr B, Burr FA, Thompson KH, Albertsen MC, Stuber CW (1988) Gene mapping with recombinant inbreds in maize. Genetics 118:519–526

Chang CC, Kikudome GY (1973) The interaction of knobs and B chromosomes of maize in determining the level of recombination. Genetics 77:45–54

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Coe EH, Hoisington DA, Neuffer MG (1987) Linkage map of corn (maize) (*Zea mays* L.) (2N=10). In: O'Brien ST (ed) Genetic Maps, a compilation of linkage and restriction maps of genetically studied organisms, vol 4. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp 685–708

Comstock JC, Martinson CA, Gengenbach BG (1973) Host specificity of a toxin from *Phyllosticta maydis*for Texas cytoplasmically male-sterile maize. Phytopathology 63:1357–1361

Dewey RE, Levings CS III, Timothy DH (1986) Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell 44:439–449

Dewey RE, Timothy DH, Levings CS III (1987) A mitochondrial protein associated with cytoplasmic male sterility in the T cytoplasm of maize. Proc Natl Acad Sci USA 84:5374–5378

Dewey RE, Siedow JN, Timothy DH, Levings CS III (1988) A 13-kilodalton maize mitochondrial protein in *E. coli* confers sensitivity to *Bipolaris maydis*toxin. Science 239:292–295

Dixon LK, Leaver CJ, Brettell RIS, Gengenbach BG (1982) Mitochondrial sensitivity to *Drechslera maydis* T-toxin and the synthesis of a variant mitochondrial polypeptide in plants derived from maize tissue cultures with Texas male-sterile cytoplasm. Theor Appl Genet 63:75–80

Duvick DN (1965) Cytoplasmic pollen sterility in corn. Adv Genet 13: 1–56

Duvick DN, Snyder RJ, Anderson EJ (1961) The chromosomal location of *Rf1*, a restorer gene for cytoplasmic pollen sterile maize. Genetics 46: 1245–1252

Emerson RA, Beadle GW, Fraser AC (1935) A summary of linkage studies in maize. Cornell Univ Agric Exp Stat Mem 180:1–72

Fauron D M-R, Havlik M, Brettel RIS (1990) The mitochondrial genome organization of a maize fertile cmsT revertant line is generated through recombination between two sets of repeats. Genetics 124:423–428

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA fragments to high specific activity. Anal Biochem 132: 6–13

Forde BG, Leaver CJ (1980) Nuclear and cytoplasmic genes controlling synthesis of variant mitochondrial polypeptides in male sterile maize. Proc Natl Acad Sci USA 77:418–422

Forde BG, Oliver RJC, Leaver CJ (1978) Variation in mitochondrial translation products associated with male-sterile cytoplasms in maize. Proc Natl Acad Sci USA 75:3841–3845

Gardiner JM, Coe EH, Melia-Hancock S, Hoisington DA, Chao S (1993) Development of a core RFLP map in maize using an immortalized F_2 population. Genetics 134:917–930

Glab N, Wise RP, Pring DR, Jacq C, Slonimski P (1990) Expression in *S. cerevisiae* of a gene associated with cytoplasmic male sterility from maize: respiratory dysfunction and uncoupling of yeast mitochondria. Mol Gen Genet 223:24–32

Gracen VE, Grogan CO (1974) Diversity and suitability for hybrid production of different sources of cytoplasmic male sterility in maize. Agron J 65:417–421

Hallauer AR (1967) Development of single-cross hybrids from two-eared maize populations. Crop Sci 7:192–195

Hanson MR, Pruitt KD, Nivison HT (1989) Male sterility loci in plant mitochondrial genomes. Oxford Surv Plant Mol Cell Biol 6:61–85

Hauge BM, Hanley SM, Cartinhour S, Cherry JM, Goodman HM, Koornneef M, Stamm P, Chang C, Kempin S, Medrano L, Meyerowitz E (1993) An integrated genetic/RFLP map of the *Arabidopsis thaliana* genome. Plant J 3: 745–754

Hooker AL, Smith DR, Lim SM, Beckett JB (1970) Reaction of corn seedlings with male-sterile cytoplasm to *Helminthosporium maydis*. Plant Dis Rep 54:708–712

Huang J, Lee S-H, Lin C, Medice R, Hack E, Myers AM (1990) Expression in yeast of the T-URF13 protein from Texas male-sterile maize mitochondria confers sensitivity to methomyl and to Texas-cytoplasm-specific fungal toxins. EMBO J 9:339–247

Johns C, Lu M, Lyznik A, Mackensie SA (1992) A mitochondrial DNA sequence is associated with abnormal pollen development in cytoplasmic male-sterile bean plants. Plant Cell 4:435–449

Kennell JC, Wise RP, Pring DR (1987) Influence of nuclear background on transcription of a maize mitochondrial region associated with Texas male-sterile cytoplasm. Mol Gen Genet 210:399–406

Korth KL, Kaspi CI, Siedow JN, Levings CS III (1991) URF13, a maize mitochondrial pore-forming protein, is oligomeric and has a mixed orientation in *Escherichia coli*plasma membranes. Proc Natl Acad Sci USA 88:10865–10869

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12: 172–175

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Laughnan JR, Gabay-Laughnan S (1983) Cytoplasmic male sterility in maize. Rev Genet 17:27–48

Laver HK, Reynolds SJ, Moneger F, Leaver CJ (1991) Mitochondrial genome organization and expression associated with cytoplasmic male sterility in sunflower (*Helianthus annus*). Plant J 1:185–193

Levings CS III, Brown GG (1989) Molecular biology of plant mitochondria. Cell 56:171–179

Levings CS III, Pring DR (1976) Restriction endonuclease analysis of mitochondrial DNA from normal and Texas cytoplasmic male sterile maize. Science 193:158–160

Mackensie SA, Chase CD (1990) Fertility restoration is associated with loss of a portion of the mitochondrial genome in cytoplasmic male-sterile common bean. Plant Cell 2:905–912

McCarty DR, Shaw JR, Hannah LC (1986) The cloning, genetic mapping, and expression of the constitutive sucrose synthase locus of maize. Proc Natl Acad Sci USA 83:9099–9103

McClintock B (1961) Further studies of the *Suppressor-Mutator* system of control of gene action of maize. Carnegie Inst Wash Yearb 60:469–476

Morton NE (1956) The detection and estimation of linkage between genes for elliptocytosis and the Rh blood type. Am J Hum Genet 8:80–96

Nel PM (1973) The modification of crossing over in maize by extraneous chromosomal elements. Theor Appl Genet 43:196–202

Nivison HT, Hanson MR (1989) Identification of a mitochondrial protein associated with cytoplasmic male sterility in petunia. Plant Cell 1:1121–1130

Pring DR, Levings CS III (1978) Heterogeneity of maize cytoplasmic genomes among male-sterile cytoplasms. Genetics 89:121–136

Pring DR, Lonsdale DM (1989) Cytoplasmic male sterility and maternal inheritance of disease susceptibility in maize. Rev Phytopathol 27:483–502

Robertson DS (1984) Different frequency of recovery of crossover products from male and female gametes of plants hypoploid for B-A translocations in maize. Genetics 107:117–130

Rocheford TR, Kennell JC, Pring DR (1992) Genetic analysis of nuclear control of T-*urf13/orf221*transcription in T cytoplasm maize. Theor Appl Genet 84:891–898

Rottmann WH, Brears T, Hodge TP, Lonsdale DM (1987) A mitochondrial gene is lost via homologous recombination during reversion of CMS T maize to fertility. EMBO J 6:1541–1546

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley. Proc Natl Acad Sci USA 81: 8014–8018

Schardl CL, Lonsdale DM, Pring DR, Rose KR (1984) Linearization of maize mitochondrial chromosomes by recombination with linear episomes. Nature 310:292–296

Schardl CL, Pring DR, Lonsdale DM (1985) Mitochondrial DNA rearrangements associated with fertile revertants of S-type male-sterile maize. Cell 43:361–368

Schnable PS, Wise RP (1994) Recovery of heritable, transposon-induced, mutant alleles of the *rf2* nuclear restorer of T-cytoplasm maize. Genetics 136:1171–1185

Schwarz-Sommer ZS, Gierl A, Kloesgen B, Wienand U, Peterson PA,Saedler H (1984) The *Spm* (*En*) transposable element controls the excision of a 2-kb insert at the *wx-m8* allele of *Zea mays*. EMBO J 3:1021–1028

Singh M, Brown GG (1991) Suppression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. Plant Cell 3:1349–1362

Sisco PH (1991) Duplications complicate genetic mapping of *Rf4*, a restorer for cms-C cytoplasmic male sterility in corn. Crop Sci 31:1263–1266

Snyder RJ, Duvick DN (1969) Chromosomal location of *Rf2*, a restorer gene for cytoplasmic pollen sterile maize. Crop Sci 9:156–157

Stamm P (1993) Construction of integrated linkage maps by means of a new computer package: JoinMap. Plant J 3: 739–744

Tulsieram LW, Compton WA, Morris R, Thomas-Compton M, Eskridge K (1992) Analysis of genetic recombination in maize populations using molecular markers. Theor Appl Genet 84:65–72

Ullstrup AJ (1972) The impacts of the Southern Corn Leaf Blight epidemics of 1970–1971. Rev Phytopathol 10:37–50

Weber D, Helentjaris T (1989) Mapping RFLP loci in maize using B-A translocations. Genetics 121:583–590

Wessler SR, Varagona MJ (1985) Molecular basis of mutations at the waxy locus in maize: correlation with the fine structure genetic map. Proc Natl Acad Sci USA 82:4177–4181

Wise RP, Pring DR, Gengenbach BG (1987a) Mutation to male fertility and toxin insensitivity in T-cytoplasm maize is associated with a frameshift in a mitochondrial open reading frame. Proc Natl Acad Sci USA 84:2858–2862

Wise RP, Fliss AE, Pring DR, Gengenbach BG (1987b) *urf13-* T of T-cytoplasm maize mitochondria encodes a 13-kDa polypeptide. Plant Mol Biol 9:121–126

Yoder OC (1973) A selective toxin produced by *Phyllosticta maydis*. Phytopathology 63:1361–1366