Theor Appl Genet (1997) 95:525–531 © Springer-Verlag 1997

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RFLP mapping of the maize gametophytic restorer-of-fertility locus (*rf3*) and aberrant pollen transmission of the nonrestoring *rf3* allele

Received: 30 September 1996 / Accepted: 21 March 1997

Abstract Cytoplasmic male sterility (CMS) is the maternally inherited inability to produce functional pollen. The *Rf3* allele of the nuclear gene *rf3* gametophytically restores male fertility to maize plants with the S-type of CMS. The *rf3* locus is on the long arm of maize chromosome two (2L). Using 2L RFLPs and three-point mapping analysis we showed that the *rf3* locus is located an estimated 4.3 cM distal to the *whp* locus and 6.4 cM proximal to the *bnl17.14* locus. This information was used in combination with RFLPs on two additional maize chromosomes to show that *Rf3*/*rf3* CMS-S plants may aberrantly transmit the nonrestoring allele, *rf3*, through the male gametophyte.

Key words Cytoplasmic male sterility · Mitochondria · *Zea mays*

Introduction

Cytoplasmic male sterility (CMS) is the maternally inherited inability to produce functional pollen and occurs widely throughout the plant kingdom (Edwardson 1970; Laser and Lersten 1972). The CMS character was initially exploited as a means to economically produce F_1 hybrids for improved crop production (Duvick 1959), but subsequently these male-sterile-inducing cytoplasms proved to be valuable in basic biological research on nuclear-mitochondrial interactions as well (reviewed by Newton 1988). In maize there are three major CMS systems, designated T (Texas), C (Charrua), and S (USDA) (Beckett 1971), that have been utilized for both purposes.

The T, C, and S male-sterile-inducing cytoplasms are distinguishable by the nuclear genes necessary to restore male fertility (Beckett 1971). Known as *rf* genes, they differ in both the number required to restore fertility and their mode of action. Maize plants with S-type cytoplasm are classically restored by a single locus, *rf3*. The *rf*3 locus exhibits a gametophytic mode of restoration (Buchert 1961). In the S cytoplasm, the nonrestoring allele (*rf3*) does not transmit through the pollen. Heterozygous (*Rf3*/*rf3*) CMS-S maize plants are semifertile, shedding approximately 50% collapsed pollen grains that do not contain starch and 50% starch-filled pollen grains. *Rf3*/*Rf3* CMS-S plants are fully fertile, shedding predominantly starch-filled pollen. Allelic designations for pollen grains are, therefore, *Rf3* for the starch-filled phenotype and *rf3* for the collapsed phenotype. In addition to an ability to easily assign alleles to pollen grains with respect to the nuclear restorer-of-fertility gene, the gametophytic mode for CMS fertility restoration provides a distinctive experimental approach for assessing nuclear-mitochondrial interactions.

Changes in either the mitochondrial DNA (mtDNA) (Schardl et al. 1985; Wise et al. 1987) or a favorable nuclear-cytoplasmic interaction (Hanson and Conde 1985) can result in the production of functional pollen by CMS plants. Unexpected male-fertile plants have been found in some CMS plant populations. These exceptional plants are reported to occur with varying frequency in CMS-S maize populations generated to be homozygous for the nonrestoring *rf3* allele (Laughnan and Gabay 1978). The instability of the male-sterile phenotype made this CMS system unsuitable for

This is paper number R-05135 in the University of Florida Agricultural Experiment Station Journal Series

Communicated by G. E. Hart

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widespread use in producing hybrid seed. Conversely, the CMS-S system is ideal for conducting research designed to test factors that can affect the expression of a male-fertile phenotype. Genetic testcrosses showed that although some exceptional male-fertile plants do arise from mutations in the nuclear genome, the majority are due to cytoplasmic changes (Laughnan and Gabay 1978). These plants are referred to as nuclear and cytoplasmic revertants, respectively. Molecular investigations of the cytoplasmic revertants by Schardl et al. (1985) revealed that spontaneous complex reorganizations of the mtDNA occurred. The complexity of these reorganizations has contributed to the difficulty in identifying a sequence specifically correlated with male sterility.

Research investigating the cause of the cytoplasmic male-sterile phenotype in CMS-S maize has largely focused on the mitochondrial genome. Determining the role of the *Rf3* allele in restoring male fertility to these plants is an appealing alternative to approach this basic question. The *rf3* locus has been located to the long arm of maize chromosome 2 (2L) using translocation and inversion heterozygotes (Laughnan and Gabay 1978). Here we report on the use of restriction fragment length polymorphisms (RFLPs) to locate the approximate chromosomal position of the *rf3* gene, and consequently, to increase the accuracy and reliability of identifying the *rf3* genotype of CMS-S maize plants. We have tested the linkage between the male-fertile phenotype associated with the *Rf3* allele and six RFLP markers positioned along the length of 2L (Burr and Burr 1991; Franken et al. 1991). Three-point analysis identified close proximal and distal linkage of male fertility with the *whp* and *bnl17.14* loci, respectively. This information enabled us to demonstrate clearly that the nonfunctional allele, *rf3*, (Kamps et al. 1996) can aberrantly transmit through pollen from a CMS-S plant. This supplies an explanation for the occurrence of some exceptional male-sterile progeny within CMS-S populations expected to be uniformly semifertile. Additional applications are proposed for the linked markers in research related to fertility restoration by the *rf3* locus.

Materials and methods

Plant materials

The USDA (S) cytoplasm and its subgroups are presented in parenthesis in association with genomic designations. CA and S are two of the five S cytoplasm subgroups; the VG cytoplasm is a member of the CA subgroup (Sisco et al. 1985). The restored inbreds KY21(S) and CE1(VG) were provided by J. Laughnan and S. Gabay-Laughnan (University of Illinois). The nonrestored inbreds W182BN(CA) and NYD410(CA) as well as W182BN in a normal cytoplasm (LF) were provided by the Cornell Cytoplasm Bank (Cornell University). The nonrestored inbred WF9(S) was provided by P. Chourey (University of Florida). Four backcross populations were generated for transmission analysis of the *rf3* alleles (Table 1). Experiments identifying linkage between CMS fertility restoration and previously mapped RFLP loci (Burr and Burr 1991; Franken et al. 1991) are described below and were conducted using 47 BC1KY21^{W182BN} progeny.

Table 1 Genetic testcross populations

Population	Source
BC1KY21^{W182BN}	W182BN(CA) × [(W182BN(CA) × KY21(S)]
BC1CE1^{W182BN}	W182BN(CA) × [(W182BN(CA) × CE1(VG)]
BC1KY21^{WF9}	WF9(S) × [(WF9(S) × KY21(S)]
BC1KY21^{NYD410}	NYD410(CA) × [(NYD410(CA) × KY21(S)]

Separate RFLP analyses were conducted following the identification of loci linked to fertility restoration. This was to ascertain the genotypes of exceptional male-sterile plants with respect to *rf3*. We investigated 4 male-sterile plants that had been identified among the semifertile progeny in the BC1KY21^{W182BN} population and 6 plants found in the BC1CE1^{W182BN} population. RFLPs of eight and four DNA probes (described below) were used to test transmission of the *rf3* alleles to the BC1KY21^{W182BN} and BC1CE1^{W182BN} populations, respectively. Progeny from two additional testcross populations, BC1KY21^{NYD410} and BC1KY21^{WF9}, were grown and scored for phenotype with respect to male fertility.

Male fertility

The ability to shed pollen was recorded for all plants. Shed pollen from the BC1KY21^{W182BN} and BC1CE1^{W182BN} progeny was collected in acetocarmine stain (Sisco et al. 1985) and examined at 100 × magnification. No less than 500 pollen grains from each plant were classified as either normal (starch-filled) or aborted.

Preparation of genomic DNA and Southern hybridization

Genomic DNA was isolated from leaf tissue (Dellaporta et al. 1983), digested with restriction endonucleases, fractionated by electrophoresis through 0.8% agarose gels, and blotted onto a nylon membrane support (Hybond™-N Amersham) according to previously described procedures (Maniatis et al. 1982). Hybridizations were performed according to the protocol described by Church and Gilbert (1984). Membranes were hybridized a minimum of 16 h, washed three times for 15 min in 1% SDS, 0.04 *M* Na_2HPO_4, pH 7.2 at 65°C. Membranes were exposed to X-OMAT™ AR film (Kodak) with an intensifying screen at − 80°C for 2–8 days. Re-used membranes were stripped of probes between each hybridization by submersion in a 100°C solution of 0.1% SDS followed by cooling to room temperature. Clones of the *npi271*, *npi297*, *npi122*, *npi456*, and *npi298* loci were provided by Native Plants Inc. and are currently available through Pioneer HiBred Inc. Dr. B. Burr (Brookhaven National Lab) supplied the clones of the *bnl12.09* and *bnl17.14* (*pio200075*) loci. Clones of the *c2* and *whp* loci were obtained from Dr. U. Weinand (Max-Planck-Institut). Clones of the *vp1* and *wx1* loci were obtained from Dr. D.R. McCarty (University of Florida) and Dr. S. Wessler (University of Georgia), respectively. Cloned DNAs were radiolabeled by randomly primed DNA synthesis in the presence of alpha-[^{32}P]dCTP (Feinberg and Vogelstein 1984).

Labeled probe and unincorporated nucleotides were separated either chromatographically through a G-50-50 Sephadex column or by precipitation in 1 volume isopropanol and 1/5 volume 5 *M* ammonium acetate.

Results

Mapping the *rf3* locus

A heterozygous semifertile CMS-S F_1 plant was used to pollinate a W182BN(CA) inbred to generate the BC1KY21^{W182BN} progeny. All progeny were expected to be heterozygous (semifertile) since only the restoring allele (*Rf3*) should transmit through the F_1 pollen. Of the 51 progeny grown and evaluated, 47 were semifertile. Unexpectedly, 4 progeny displayed a male-sterile phenotype. Only the semifertile BC_1 progeny were used in RFLP linkage experiments. The nature of the unexpected male-sterile progeny will be discussed below.

Sampled DNA from each of the semifertile BC1KY21^{W182BN} plants was hybridized with DNA clones corresponding to six RFLP loci previously mapped to chromosome 2. For loci unlinked to the *rf3* locus, the BC_1 population was expected to segregate (in a 1:1 ratio) individuals homozygous for W182BN alleles or heterozygous for W182BN and KY21 alleles. For loci tightly linked to the *rf3* locus, only the heterozygous class was expected. The frequency of recombination between an RFLP locus and the *rf3* locus was therefore indicated by the frequency of individuals heterozygous for alleles at the RFLP locus. Gene order and linkage estimates among all loci were determined by three-point mapping analysis (Table 2). The loci *bnl12.09*, *npi271*, and *npi456* were unlinked to fertility restoration. Loci linked to fertility restoration were *npi298*, *whp* and *bnl17.14*. Most tightly linked to fertility restoration were the *whp* and *bnl17.14* loci. The identification of 2 and 3 recombinant progeny between these loci and the *rf3* locus resulted in an estimated distance of 4.3 cM for the *whp-rf3* interval and 6.4 cM for the *rf3-bnl17.14* interval. Double recombination events between genetically linked loci were detected within the *bnl12.09-npi298* interval only; all others occurred over map distances greater than 50 cM. These results are summarized in Fig. 1.

Table 2 Recombination among maize chromosome 2 loci for the 47 semifertile progeny in the BC1KY21^{W182BN} population

Map interval	Number of single recombinant progeny	Number of double recombinant progeny
bnl12.0 9-npi271	4	0
bnl12.09-npi456	11	0
bnl12.09-npi298	22	3
bnl12.09-whp	26	5
bnl12.09-rf3	28	5
bnl12.09-bnl17.14	29	6
npi271-npi456	7	0
npi271-npi298	18	3
npi271-whp	26	3
npi271-rf3	28	3
npi271-bnl17.14	29	4
npi456-npi298	17	0
npi456-whp	25	0
npi456-rf3	27	0
npi456-bnl17.14	28	1
npi298-whp	9	0
npi298-rf3	11	0
npi298-bnl17.14	13	0
whp-rf3	2	0
whp-bnl17.14	5	0
rf3-bnl17.14	3	0

RFLP analysis of unexpected male-sterile BC_1 progeny

RFLP markers were used to determine the genetic basis for the unexpected male-sterile plants in our mapping BC_1 population. DNA from the 4 unexpected male-sterile plants in the BC1KY21^{W182BN} population was hybridized with probes corresponding to the six 2L RFLP loci, as well as the cDNA clones of the *vp1* gene located on chromosome 3 and the *wx1* gene located on chromosome 9. Only *rf3*-associated alleles were transmitted for the six 2L markers tested (Table 3). Hybridization with the *whp* locus clone is shown in Fig. 2a. Only the allele associated with the nonrestoring parent, W182BN(CA), was present in the male-sterile progeny. Hybridization of these DNA samples with the *vp1* locus cDNA clone is shown in Fig. 2b, and hybridization with the *wx*1 locus cDNA clone is shown in Fig. 2c. Parental allele assignment was determined in the tests for polymorphism (not shown). Lanes 1, 3 and 4 in Fig. 2b show the presence of both parental alleles in the exceptional male-sterile progeny as do lanes 2 and 3 in Fig. 2c. The absence of any allele other than the W182BN(CA) and KY21(S) parental alleles in either the unexpected male-sterile plants or the BC1KY21^{W182BN} mapping progeny is a strong indication that pollen contamination from an outside source

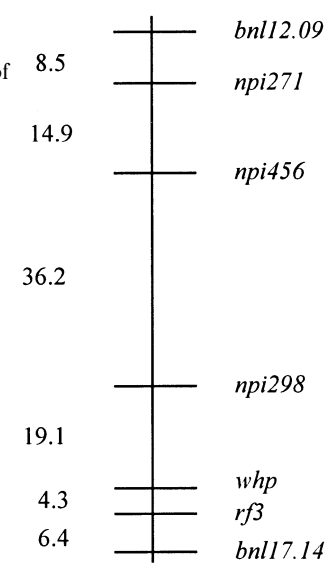

Fig. 1 Map of the maize chromosome 2 long arm (2L) showing the relative positions of RFLP loci and the CMS-S restorer-of-fertility gene *rf3*. Estimated map distances are based on data from 47 BC1KY21^{W182BN} progeny and are presented in centiMorgans. The map is oriented with the centromere proximal region at the *top* of the figure

Table 3 RFLP genotypes of exceptional CMS-S male-sterile progeny

BC_1 population	Plant no.	Locus							
		bnl2.09	*npi271*	*npi456*	*npi298*	*whp*	*bnl17.14*	*vp1*	*wx1*
$KY21^{W182BN}$	1	r[a]	r	r	r	r	r	R[c]r	r
$KY21^{W182BN}$	2	r	r	r	r	r	r	r	Rr
$KY21^{W182BN}$	3	r	r	r	r	r	r	Rr	Rr
$KY21^{W182BN}$	4	r	r	r	r	r	r	Rr	r
$CE1^{W182BN}$	1	—[b]	—	—	—	Rr	Rr	r	r
$CE1^{W182BN}$	2	—	—	—	—	r	r	r	Rr
$CE1^{W182BN}$	3	—	—	—	—	r	r	r	Rr
$CE1^{W182BN}$	4	—	—	—	—	r	r	r	Rr
$CE1^{W182BN}$	5	—	—	—	—	Rr	Rr	r	Rr
$CE1^{W182BN}$	6	—	—	—	—	r	r	r	Rr

[a] r designates an allele from the nonrestoring parent, W182BN(CA)
[b] —designates an untested locus
[c] R designates an allele from the restoring parent, KY21(S) or CE1(VG)

did not occur. Furthermore, the examination of six additional lines in our maize program showed these lines to be polymorphic (with respect to W182BN and KY21) for at least one of the eight loci employed in our study (not shown). The absence of these or any allele other than the W182BN(CA) and KY21(S) parental alleles in either the unexpected male-sterile plants or the $BC1KY21^{W182BN}$ mapping progeny is a strong indication that pollen contamination from an outside source did not occur. Taken together, these molecular data demonstrate that the 4 exceptional male-sterile BC_1 progeny arose from a pollination by a heterozygous F_1 parent and that the nonfunctional (*rf3*) allele was aberrantly transmitted through the male gametophyte.

The source of 6 unexpected male-sterile plants identified among 39 normal, semifertile progeny in a CMS-S $BC1CE1^{W182BN}$ population was also investigated. DNA from these exceptional progeny was hybridized with clones of the *rf3*-linked loci *whp* and *bnl17.14* and with cDNA clones of the unlinked *vp1* and *wx1* loci. The test with the linked loci revealed that 4 of the 6 progeny carried only alleles associated with the nonrestoring parent, W182BN(CA). The remaining 2 progeny carried alleles from both the restoring and nonrestoring parents. Both parental alleles of the *wx1* gene were transmitted to 5 of the 6 progeny. Among these 5 progeny were the 4 progeny with only the W182BN(CA) alleles at the *whp* and *bnl17.14* loci. Hybridizations with the *vp1* cDNA revealed that all 6 progeny inherited only the allele from the W182BN(CA) parent (Table 3).

Plants from the CMS-S testcrosses $BC1KY21^{NYD410}$ and $BC1KY21^{WF9}$ were visually scored to gain additional information on nuclear-cytoplasmic genomic combinations and the occurrence of unexpected male sterility. Plants which shed pollen were designated as male fertile. Pollen was not examined microscopically, however, to determine semifertility. Twelve progeny in the $BC1KY21^{NYD410}$ population were male-sterile and 36 plants were male-fertile. The $BC1KY21^{WF9}$ population was composed of 3 male-sterile progeny and 48 male-fertile plants.

Discussion

The KY21 inbred carries the restoring allele *Rf3-KY21*, which first defined the gametophytic system of restoration for the maize S-type sterility-inducing cytoplasm (Buchert 1961). Previous mapping studies indicated that the *rf3* locus was proximally located to the 2L.80 breakpoint of maize chromosome 2 (Laughnan and Gabay 1978). To more accurately estimate the relative position of *the rf3-KY21* locus, we performed Southern analysis with probes corresponding to previously mapped loci (Burr and Burr 1991; Franken et al. 1991) on a BC_1 population generated from the fertilization of a male-sterile CMS-S W182BN(CA) inbred plant by a semifertile CMS-S W182BN(CA) X KY21(S) F_1 hybrid plant.

Recombination among RFLP markers and male-fertility restoration of W182BN(CA) × [W182BN/CA X KY21(S)] progeny showed the proximal to distal linkage order *bnl12.09*, *npi271*, *npi456*, *npi298*, *whp*, and *bnl17.14* to be consistent with the maize universal genetic map presented in the 1991 Maize Genetics Cooperation Newsletter (Coe et al. 1991). The *rf3-KY21* locus was localized to an estimated 10.7-cM region between the *whp* and *bnl17.14* loci through three-point analysis of RFLP markers. Either or both the *whp* and *bnl17.14* markers can now be utilized to effectively track *rf3* alleles in future genetic and breeding programs.

To demonstrate one use of this linkage information, we assayed exceptional male-sterile plants identified in two genetically distinct backcross populations to determine their genotype with respect to the *rf3* locus. Each

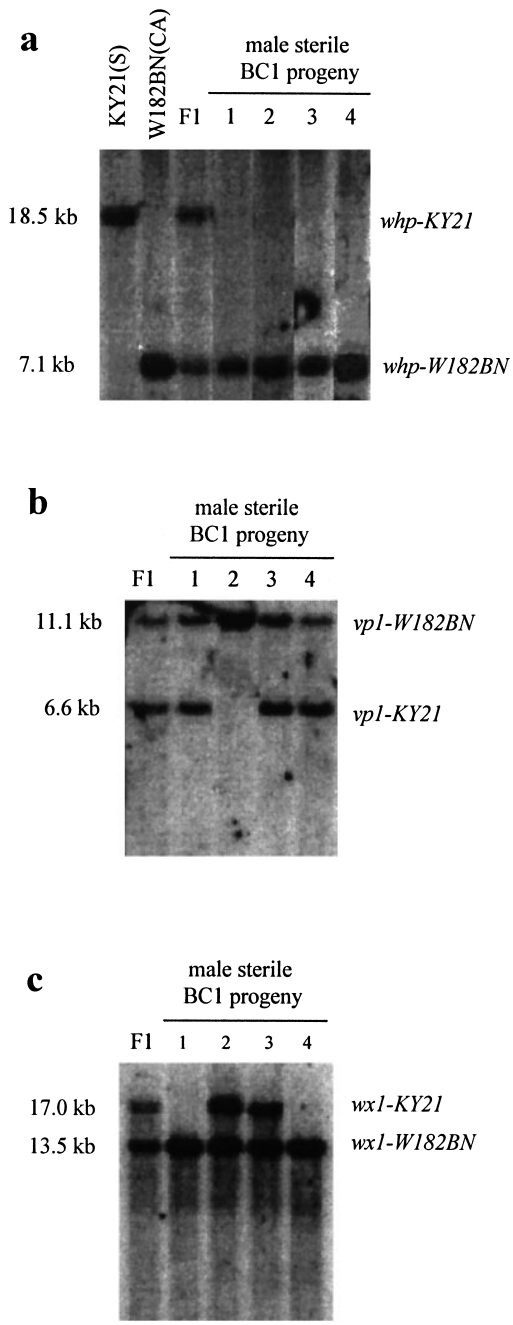

Fig. 2 Southern blot analysis of the F_1 heterozygote male parent and the 4 exceptional male-sterile progeny in the BC1KY21^{W182BN} population. **Panels a, b** and **c** are genomic DNA digested with the restriction endonucleases *Sst*I, *Hin*dIII and *Eco*RI, respectively. The cDNA clone of the *whp* duplicate gene *c*2 was used to hybridize the blot shown in **panel a**. Band identification was achieved by a separate hybridization of parental DNA samples with the *whp*-specific cDNA (not shown). **Panels b** and **c** show hybridizations with the cDNA clones corresponding to the *vp1* and *wx1* loci, respectively

of the 2L markers in combination with two unlinked genes, *vp1* and *wx1*, was used to conduct this investigation on the 4 exceptional male-sterile plants found among the 51 BC1KY21^{W182BN} progeny. A similar analysis, to be discussed sequentially to the data from the BC1KY21^{W182BN} progeny, was conducted on 6 exceptional male-sterile plants identified in the backcross population, BC1CE1^{W182BN}. Exceptional male-sterile plants may be either: (1) *Rf3*/*rf3* heterozygotes that displayed a male-sterile phenotype due to some other unidentified factor(s); (2) progeny from a cross contaminated with pollen from the maintainer line, W182BN(LF); (3) progeny from a cross contaminated by pollen from other maize lines that do not carry restoring alleles; or (4) progeny produced from an aberrant transmission of the nonrestoring *rf3* allele from the CMS-S F_1 male parent. Our expectations with regard to Southern analysis for these possibilities were as follows: (1) presence of both parental alleles at either or both the *whp* and *bnl17.14* loci; (2) alleles from the restoring parent absent at all loci and only alleles associated with the W182BN parent present; (3) presence of non-parental alleles at any loci; and (4) the linkage group *whp-rf3-bnl17.14* inherited from the W182BN parent and alleles from the fertility-restoring parent represented at other loci.

Southern analysis of the 4 BC1KY21^{W182BN} male sterile progeny with the six 2L RFLPs showed that these plants carried alleles associated only with the nonrestoring (*rf3-W182BN*) allele (Table 3). Fertilization by *Rf3* pollen from the CMS-S F_1 plant used to generate this population would require a double recombination event (with recombinations in the *whp-rf3* and the *rf3-bnl17.14* intervals) for both the RFLP and male sterile phenotypes to have occurred. The likelihood of double recombinations can be tested by calculating interference (*I*). We calculated the coefficient of coincidence and the value for *I* from the recombination data on the remaining 47 semifertile progeny. The coefficient of coincidence is zero since no double recombinants were detected for the *whp-bnl17.14* interval. Therefore, $I = 1$, and interference is complete. This indicates that no double recombination events would be expected for this interval. For this to have occurred in approximately 8% of the population makes this possibility appear even less feasible.

The second possibility we suggest is that the male-sterile plants arose from contaminating pollen of a W182BN inbred with normal cytoplasm (W182BN maintainer). This was tested using clones of the *vp1* and *wx1* genes as RFLP markers. These genes have previously been mapped to chromosomes 3 and 9, respectively (Coe et al. 1991). Southern analysis showed that the 4 male-sterile progeny carried *vp1* and *wx1* alleles from both the W182BN and KY21 genomes and that *vp1* and *wx1* assorted independently as would be expected for unlinked loci (Fig. 2). We can conclude from this evidence that contaminant pollen from a W182BN maintainer line was not the source of the *rf3* allele in these exceptional progeny.

The likelihood that these plants arose from some other contaminating source is also remote. Additional

polymorphism data on the 2L RFLP markers used in this study was examined to address this possibility. Although we were able to identify appropriate polymorphisms between W182BN(CA), KY21(S), and six additional lines in our maize program, only the parental alleles were seen within the BC1KY21^{W182BN} population. This information can only be considered indicative of purity for the pollination which generated the BC1KY21^{W182BN} population due to the limited number of genotypes examined. However, maize is highly polymorphic, and the absence of alleles other than those matching the W182BN and KY21 parental genomes at any of the eight loci tested supports the conclusion that the unexpected male-sterile progeny did not result from contaminant pollen.

RFLP analysis of unexpected male-sterile progeny was therefore consistent with our proposal of aberrant pollen transmission of the *rf3* allele. To test whether this phenomenon was unique to this BC1KY21^{W182BN} population or occurred in other genetic backgrounds as proposed by Duvick (1965), we performed a similar analysis on 6 exceptional male-sterile plants found in a BC1CE1^{W182BN} population. RFLP linkage analysis of the semifertile male progeny in this population confirmed that the *Rf3* allele from the CE1(VG) inbred parent was within the *whp-bnl17.14* interval (unpublished data). This is in agreement with the report by Laughnan and Gabay (1978) that the *rf3* genes from KY21(S) and CE1(VG) are either allelic or very tightly linked. Based on this information and our previous results we confined our 2L linkage analysis of these exceptional progeny to the *whp* and *bnl17.14* loci.

For 2 of the 6 male-sterile progeny, the 2L markers from both parental genomes were represented. These results suggest that the male-sterile phenotype was due to factors unrelated to fertility restoration by the *Rf3* allele. In the remaining 4 exceptional male-sterile BC1CE1^{W182BN} progeny, the *whp* and *bnl17.14* alleles associated with the restoring allele from the CE1(VG) parent were absent. This observation indicates that these 4 progeny may have resulted from the aberrant pollen transmission of the *rf3* allele from the F_1 parent. The likelihood that double recombination events (i.e. recombination between the *whp* and *rf3* loci and recombination between the *rf3* and *bnl17.14* loci) resulted in the male-sterile phenotype was tested by calculating interference (I) as described for the BC1KY21^{W182BN} population. No double recombinants were detected in the *whp-bnl17.14* interval for BC1CE1^{W182BN} semifertile progeny. Therefore, the coefficient of coincidence is zero, $I = 1$, and interference is complete. This is consistent with the results obtained for the BC1KY21^{W182BN} population and provides additional evidence that double recombination events within the *whp-bnl17.14* interval are unlikely to occur.

An analysis of the unlinked *wx1* and *vp1* loci was employed to test the source of the male gametes that generated the exceptional male-sterile BC1CE1^{W182BN} progeny. An absence of the alleles from the CE1(VG) parent for both genes or the presence of a new allele(s) would indicate that the progeny arose from a contaminate pollen source. Both parental alleles of the *wx1* gene were inherited in the 4 male-sterile progeny carrying only W182BN alleles for the *whp1* and *bnl17.14* loci. These same 4 progeny inherited only the *vp1* allele from the genome of the nonrestoring parent, W182BN(CA). This information suggests pollen contamination by a genetically unrelated source is unlikely. Taken alone, the *vp1* genotypes are those we would expect if the male-sterile progeny arose from pollen contamination by a maintainer line of the W182BN inbred (W182BN with a normal cytoplasm). Taken together with the *wx1* data, however, these data verify that pollen from the CMS-S F_1 parent, not a contaminating pollen source, produced all 6 male-sterile plants identified in this BC1CE1^{W182BN} population. Furthermore, the data support the conclusion that 4 of these progeny did indeed arise from the aberrant transmission of the *rf3* allele through pollen from a CMS-S F_1 plant. The genotypes of the unexpected male-sterile progeny might be further tested by pollinating such plants with a maintainer line. In the absence of reversion to fertility, these pollinations should produce only male-sterile progeny.

The reports of both nuclear and cytoplasmic spontaneous revertants to male fertility in CMS-S maize suggest one feasible mechanism that would allow for the transmission of the nonrestoring *rf3* allele through pollen from the F_1 hybrid parent. To produce the relatively few exceptional plants observed in these BC_1 populations, however, we would expect that any reversion event(s) would need to be restricted to micro sectors within the anther(s). Although our present research does show that the *rf3* allele was aberrantly transmitted in two distinct BC_1 populations, to determine whether reversion to fertility or some other mechanism permitted this phenomenon to occur requires additional research. The feasibility of this research is now enhanced by the identification of the *rf3*-linked RFLP markers.

The exceptional CMS-S male-sterile plants might also be beneficial in creating new approaches to investigate the control of pollen development. Important new insight into nuclear-mitochondrial interactions is possible if the mechanism of *rf3* leakage can be ascertained. As discussed above, the *rf3*-linked RFLP markers are the necessary primary tools needed to proceed in these types of studies. A further consideration is that research in this area might be enhanced if there is a widespread occurrence of this phenomenon in CMS-S maize populations. To gain some comprehension of this aspect we assayed two additional backcross populations for the presence of unexpected male-sterile progeny. These populations differed genetically in the source of the nonrestoring *rf3* allele. Additionally, the WF9 parent

contributed the S-type cytoplasm, which differs from the CA subgroup (Sisco et al. 1985) contributed by the other three inbreds included in this report. As with the two populations discussed above, exceptional male-sterile progeny appeared in both the BC1KY21^{NYD410} and the BC1KY21^{WF9} populations. These data further support the proposal by Duvick (1965) that some maize genotypes with CMS-S cytoplasm may produce a small number of fertile pollen grains with the nonrestoring (*rf3*) allele. The four populations described in the present work also show that the frequency with which unexpected male-sterile plants arise is variable. This is not surprising since male fertility has been shown to be influenced by genetic background, the environment, and their interaction in maize plants with male-sterile inducing cytoplasms (Duvick 1965; Duvick and Noble 1978). The late timing of pollen abortion in the CMS-S system (Lee et al. 1980) combined with other factors affecting the male-fertile phenotype may result in increased abnormal inheritance. Consequently, genetic studies can be confounded significantly. Examination of the genotypes at the *whp* and *bnl17.14* loci may be used with confidence as an early and objective screening technique for the presence of different alleles at the *rf3* locus.

In the study presented here, genotyping the exceptional male-sterile plants with respect to the *rf3* locus enabled us to improve the accuracy of our linkage mapping study and to test hypotheses concerning the origin of these exceptional plants. Beyond the genetic characterization of unexpected CMS-S male-sterile plants, the tight linkage between the *rf3* locus and the *whp* and *bnl17.14* loci can now help clarify and expand research into fertility restoration, providing the capacity for pre-tassel screening, new transmission experiments (Kamps et al. 1996), simpler linkage testing, a more direct method to examine *rf3* inheritance anomalies, and a means to confirm insertional inactivation by a transposable element.

Acknowledgements We acknowledge and thank L.C. Shaw and R. Okagaki for their valuable assistance in the preparation of this manuscript and V. Ortega for technical assistance. We thank L.C. Hannah and G. Moore for their critical review. We thank S. Gabay-Laughnan, D.R. Pring, U. Weinand, T. Helentjaris, S. Wessler, B. Burr and the Maize Genetics Cooperation Stock Center for providing maize stocks and probes. T.L.K. was supported in part by a Graduate Research Assistantship from the University of Florida Horticultural Sciences Department.

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