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Molecular mapping of the *rf1* gene for pollen fertility restoration in sorghum (*Sorghum bicolor* L.)

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Abstract We report the molecular mapping of a gene for pollen fertility in A1 (milo) type cytoplasm of sorghum using AFLP and SSR marker analysis. DNA from an F_2 population comprised of 84 individuals was screened with AFLP genetic markers to detect polymorphic DNAs linked to fertility restoration. Fifteen AFLP markers were linked to fertility restoration from the initial screening with 49 unique AFLP primer combinations (+3/+3 selective bases). As many of these AFLP markers had been previously mapped to a high-density genetic map of sorghum, the target gene (*rf1*) could be mapped to linkage group H. Confirmation of the map location of *rf1* was obtained by demonstrating that additional linkage group-H markers (SSR, STS, AFLP) were linked to fertility restoration. The closest marker, AFLP *Xtxa2582*, mapped within 2.4 cM of the target loci while two SSRs, *Xtxp18* and *Xtxp250*, flanked the *rf1* locus at 12 cM and 10.8 cM, respectively. The availability of molecular markers will facilitate the selection of pollen fertility restoration in sorghum inbred-line development and provide the foundation for map-based gene isolation.

Keywords Sorghum (*Sorghum bicolor* L.) · Fertility restoration · AFLP · Microsatellite

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Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait in which pollen development or normal anther dehiscence is impaired but female fertility is normal (Pring et al. 1995). CMS is widely distributed having been observed in approximately 150 plant species (Laser and Lersten 1972) and is often associated with the expression of novel (e.g., chimeric) mitochondrial open reading frames (Pring et al. 1998; Schnable and Wise 1998; Tang et al. 1999; Wen and Chase 1999). In sorghum (*Sorghum bicolor*), as in maize (*Zea mays*), CMS is observed when a cytoplasm is transferred to a different nuclear background. In many CMS systems, male fertility can be restored by a series of fertility restorer (*rf*) genes encoded in the nucleus. It has been proposed that the *rf* genes block or compensate for mitochondrial dysfunctions that are phenotypically expressed during pollen development (Schnable and Wise 1998). Thus, CMS-restorer systems appear to result from specific nuclear-mitochondrial interactions though little is known about the mechanism of fertility restoration in even the best-characterized systems (Cui et al. 1996; Wise et al. 1996; Pring et al. 1998; Schnable and Wise 1998; Tang et al. 1999).

The identification and development of male sterile and fertility restorer lines was a major step in the success of hybrid breeding programs in sorghum (Schertz and Dalton 1980). The first CMS-restorer system discovered in sorghum involved milo (designated as A1) cytoplasm and a kafir nuclear background (Stephens and Holland 1954). The A1 cytoplasm has been used in nearly all females in hybrid sorghum production. CMS plants of A1 cytoplasm have small pointed anthers and typically meiosis is normal but the microspores remain uninucleate and abort (Singh and Hadley 1961). Twenty one additional sources of cytoplasms that confer male sterility in sorghum have been described (Schertz et al. 1989). Despite these discoveries, A1 remains the primary CMS system used for hybrid seed production.

The restoration of pollen fertility in A1 cytoplasm has been reported to be controlled by two major genes and

several modifying genes. Maunder and Pickett (1959) reported that a single gene (*Msc1*) caused fertility restoration in milo (A1) cytoplasm. Subsequently, Erichsen and Ross (1963) described a second locus, *Msc2*, interacting complementary with *Msc1* to restore fertility in A1 cytoplasm lines. Miller and Pickett (1964) reported epistatic interactions between two fertility restoration genes for A1 cytoplasm, and intra-allelic interaction within the locus accounted for most of the variability in male fertility. Schertz et al. (1989) conducted a detailed investigation of fertility restoration in CMS sorghums and determined that the inheritance of fertility restoration in hybrids with A1 cytoplasm varied depending on the nuclear backgrounds of the female and male parents. Depending on the parental lines, a single major fertility restorer gene was observed while in other A1 cytoplasm crosses two or more major genes (or multiple genes with minor effects) controlled fertility restoration. Hence, the inheritance of fertility restoration in A1 cytoplasm lines can be complex although one or two major genes appear to operate in many crosses.

Molecular markers tightly linked to *rf* loci have several applications in sorghum breeding programs. In many situations, sorghum geneticists do not know whether a new breeding line (or germplasm accession) should be classified as a B or R line. Currently, the only method to determine the status of these lines is to test cross the lines to a male-sterile line and score the resulting F_1 for male sterility/fertility. The identification of molecular markers tightly linked to *rf* loci in sorghum would permit the classification of lines as either B or R without the need for test crosses. Molecular tags for *rf* loci have been identified in a number of other crop species (Schnable and Wise 1994; He et al. 1995; Ichikawa et al. 1997; Jia et al. 1997; Kamps and Chase 1997; Kojima et al. 1997; Yao et al. 1997; Borner et al. 1998) although similar linkage analyses have not been reported in sorghum. We have developed a series of F_2 populations in sorghum to permit gene tagging of fertility restorer loci for use in inbred-line development. A segregating population for *rf1* was derived from a cross between inbred lines ATx623 (*rf1rf1*) and RTx432 (*Rf1Rf1*). Additional populations that segregate for *rf2* and other fertility restoration loci have been developed to also permit tagging of these loci.

We report herein the mapping and tagging of the *rf1* locus in sorghum by amplified fragment length polymorphism (AFLP) and microsatellite simple sequence repeat (SSR) genetic markers. A regional linkage map around the *rf1* locus was established with the locus mapping to a position 2.4 cM from AFLP marker *Xtxa2582*. Furthermore, we have determined that the *rf1* locus maps to linkage group (LG) H of the high-density genetic map of sorghum. Finally, we present a set of SSR genetic markers flanking the *rf1* locus that are available for marker-assisted selection during sorghum inbred-line development.

Materials and methods

Plant materials

A mapping population of 373 F_2 plants was produced from a single F_1 plant heterozygous for *rf1* by crossing the two lines ATx623 (*rf1rf1*) and RTx432 (*Rf1Rf1*). ATx623 has an A-type of male-sterile-inducing cytoplasm and is a common female parent while RTx432 is a common R line of Texas hybrids (Miller 1984). ATx623 and RTx432 have been previously observed to differ by a single major gene (designated *rf1*) for fertility restoration (Schertz, unpublished results). Both lines are homozygous at the locus of interest, having been either backcrossed (ATx623) or selfed (RTx432) for numerous generations with concurrent selection for sterility/fertility.

Phenotypic classification of male fertility

Plants were grown in the field at the College Station, Texas, and scored 4–5 weeks after planting. One panicle of each plant was covered with a paper bag prior to anthesis. Thirty to forty days after bagging, each plant was classified as fertile (selfed-seed) or male-sterile (< 1% seed set). Seed from each fertile F_2 plant was planted to establish F_3 progeny rows in order to identify F_2 plants homozygous for the dominant, fertility restorer allele (*Rf1*). F_3 progeny rows (minimum of 40 plants) were bagged prior to flowering and fertility was based on seed set. F_3 progeny rows with 100% seed set were concluded to have come from F_2 plants homozygous for *Rf1*.

DNA extraction

Genomic DNA was isolated from the youngest leaves of each F_2 plant at 4–5 weeks after planting. Leaf tissue was freeze-dried and genomic DNA was isolated as previously described (Klein et al. 2000). DNA was quantified by flourometry. Genomic DNA was isolated from 373 F_2 plants that were phenotyped for male fertility/sterility. A subset (84) of these F_2 plants was used for the initial screening with AFLP markers (see below).

AFLP and microsatellite analysis

DNA from 84 F_2 individuals was used for AFLP analysis. DNA samples were digested with the restriction endonucleases *Eco*RI and *Mse*I. AFLP template preparation and AFLP PCR reaction conditions were as described (Klein et al. 2000). Forty nine +3/+3 AFLP primer combinations were examined in 84 F_2 progeny (54 sterile/30 fertile). Detection of AFLP products was conducted using a LiCor 4200 IR gel detection system. Sorghum SSRs (primer sequences obtained from Dr. Gary Hart, Texas A&M University) were initially examined for polymorphism between ATx623 and RTx432 genomic DNA. SSR markers displaying polymorphism between the parents and mapping to the same LG as *rf1* were utilized to genotype F_2 individuals. Detection of SSR markers was conducted using either a LiCor 4200 IR gel detection system or an ABI 3700 DNA sequencer.

Segregation and linkage analysis

Allelic segregation data for AFLP and SSR markers was either scored manually or by Genotyper software (ABI Applied Biosystems) for ABI 3700 data. Recombinant fractions between pairs of linked markers was calculated using the software package MapMaker Macintosh (v2.0) and MapMaker/exp (v3.0) on a Sun Microsystems II workstation. The Kosambi mapping function (Kosambi 1944) was used in calculating genetic distances. Map order was based on maximum-likelihood estimates. LG nomenclature is according to Peng at al. (1999). Additional MapMaker functions utilized during map construction were as previously detailed (Klein et al. 2001).

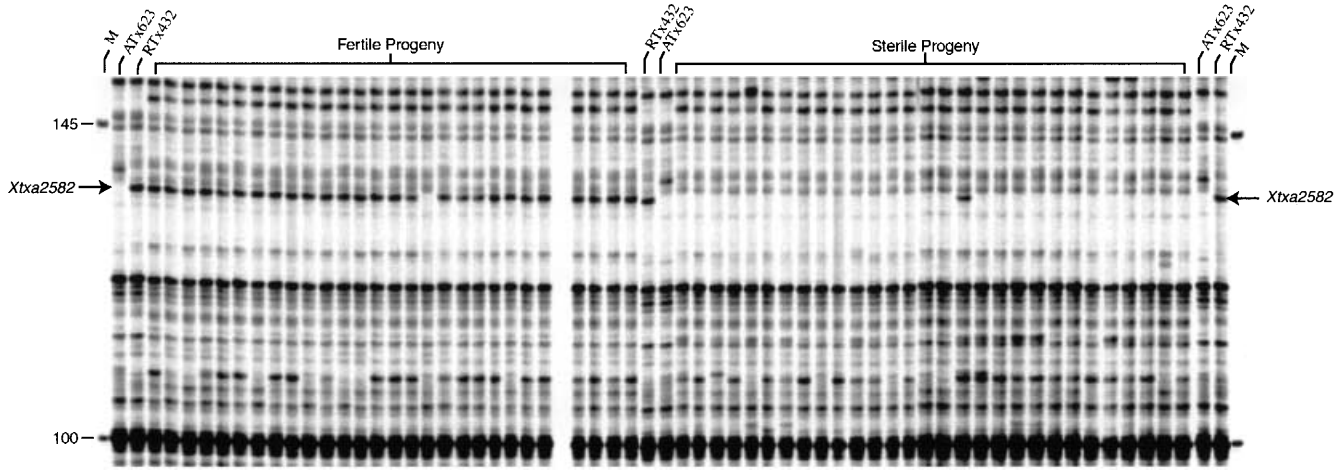

Fig. 1 Cosegregation of AFLP marker *Xtxa2582* and the *rf1* locus in F_2 progeny derived from the cross of ATx623 and RTx432. AFLP templates from parental inbreds ATx623 (*rf1rf1*) and RTx432 (*Rf1Rf1*) were run as controls to aid in the identification of polymorphic bands. The *blank lane* represents a failed PCR reaction. The *arrow* to the left indicates the position of AFLP marker *Xtxa2582*. Fluorescent-labelled molecular-weight markers (LiCor) were run in lanes marked *M* and their sizes (bp) are shown at the margins of the gel

Results

F_2 analysis

Of the F_2 plants scored for seed set, 253 were classified as male fertile and 105 were male sterile. The population segregated in a normal Mendelian ratio (χ^2=3.57, n.s.) indicating that a single major dominant locus controlled fertility restoration. This result is consistent with observations previously noted in crosses between ATx623 and RTx432.

Identifying AFLP markers linked to the *rf1* locus

Genomic DNA from 84 F_2 individuals (54 sterile, 30 fertile) was examined for AFLP genetic markers linked to fertility restoration. Forty nine AFLP primer combinations (+3/+3 selection) revealed 15 markers that could be visually scored as segregating with fertility restoration. A gel image of a representative AFLP marker, designated *Xtxa2582*, is shown in Fig. 1. Linkage analysis of 84 F_2 individuals indicated that *Xtxa2582* is approximately 2.4 cM from the *rf1* locus. Construction of a LOD ≥ 3.00 regional linkage map initially placed 12 AFLP markers within a 50-cM segment encompassing the *rf1* locus. Most of the AFLP markers displaying linkage to *rf1* have been previously mapped in a F_{6-8} recombinant inbred line population of sorghum and the map position of the AFLPs has been determined (P.E. Klein, unpublished information). Of the eight *rf1*-linked AFLP markers previously assigned to the genetic map of sorghum, all clustered to a 60-cM segment of sorghum LG H (Fig. 2). To confirm the map location of *rf1*, four additional AFLP markers that mapped to this region of LG H were examined for polymorphism between the parents of the F_2 population. Of the four AFLP markers examined, one, *Xtxa335*, was polymorphic and mapped within 7 cM of the *rf1* locus (see Fig. 2).

To develop *rf1* tags for marker-assisted breeding, SSR (*Xtxp* prefix) and STS, sequence tagged site, (*Xtxs* prefix) markers that map to this region of LG H were examined for linkage to fertility restoration. Of the seven genetic markers examined, *Xtxp18*, *Xtxp250* and *Xtxs560* were polymorphic in the cross between ATx623 and RTx432 and were subsequently determined to be linked to fertility restoration (see Fig. 2). Figure 3 shows a gel image of two SSRs, *Xtxp18* and *Xtxp250*, that were linked to fertility restoration. Linkage analysis revealed that the *rf1* locus is flanked by *Xtxp18* and *Xtxp250* at a genetic distance of 12 cM and 10.8 cM, respectively (Fig. 2). In addition, STS marker *Xtxs560* mapped at a distance of 6.8 cM from *rf1*.

A comparison of the regional map of *rf1* and the dense map of sorghum LG H reveals that recombination fractions between markers proximal to the *rf1* locus are, in general, similar in the two maps, while markers most distal to the *rf1* locus show greater recombination fraction values in the common sorghum map. The order of markers flanking the *rf1* locus is similar in the two genetic maps. Differences in the recombination fraction between markers may reflect differences in the population types used to create the two maps (F_2 vs F_{6-8}) and differences in the densities of the two maps. In addition, several of the markers linked to *rf1* (marked with an asterisk) exhibit a low ripple score when placed in a framework map of LG H (eg., *Xtxp18*). As a consequence, not all of the genetic markers linked to *rf1* could be accurately placed on the high-density map at a LOD threshold ≥ 3.00. Nevertheless, all *rf1*-linked markers were assigned to LG H at a LOD threshold ≥ 10.00 supporting the observation that the *rf1* locus resides in this region of LG H.

To more-accurately predict the recombination fractions between the *rf1* locus and the SSRs *Xtxp18* and *Xtxp250*, DNA was isolated from 373 F_2 individuals and two-point analysis was conducted. Two-point analysis

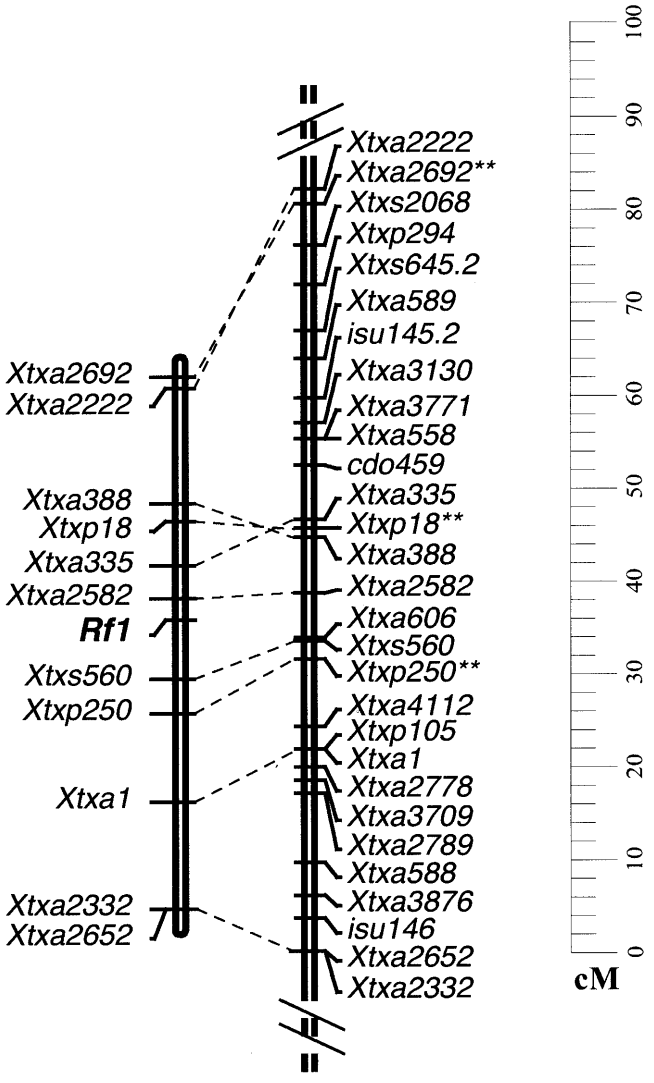

Fig. 2 Partial linkage map of the segment of sorghum LG H around the *rf1* locus. The regional *rf1* map developed from F_2 progeny derived from the cross of ATx623 and RTx432 (*left panel*) and the map derived from F_{6-8} progeny from the cross of BTx623 and IS3620C (*right panel*) have been aligned with markers common to both maps (markers connected with *dashed lines*). Genetic distances in cM (Kosambi 1944) are shown at the right. *Asterisks* beside selected markers indicate that these markers could not be ordered on LG H at a LOD ≥ 3.00

indicated that the recombination fraction between *rf1* and *Xtxp250* and *Xtxp18* was 6.5% and 6.8%, respectively. These recombination values are slightly less than that predicted from the smaller F_2 population. Nevertheless, screening germplasm with SSRs *Xtxp18* and *Xtxp250* should tag the genomic region flanking the *rf1* locus.

Discussion

The identification and development of male-sterile and fertility restorer lines is required for the exploitation of CMS systems for hybrid seed production. The A1 (milo) cytoplasm, one of approximately 21 known CMS systems for sorghum, remains the primary CMS system used for hybrid-seed production (Schertz et al. 1989). The inheritance of fertility restoration in A1 cytoplasm crosses is dependent on the parental lines involved although one or two major genes appear to operate in many crosses (Schertz et al. 1989). In the present study, we have constructed a regional map encompassing the *rf1* locus with some markers being tightly linked to fertility restoration. These molecular markers provide a clear indication of the map location of the *rf1* locus on LG H (LG designation according to Peng et al. 1999) and also identify several PCR-based markers that could be used in marker-assisted selection of fertility restoration.

The strategy utilized to map *rf1* involved the use of an F_2 population segregating for this trait and AFLP high-volume marker technology. AFLPs, and previously RAPDs, have been used with considerable success in plant species. In our laboratory, greater success has been achieved with AFLP markers owing to better reproducibility as compared to RAPDs. By screening 84 F_2 individuals with 49 AFLP primer combinations, a regional map of *rf1* was constructed that initially consisted of only AFLP markers. As many of these markers have been mapped on a dense genetic map of sorghum (P.E. Klein, unpublished information), the *rf1* locus could be assigned to LG H. To increase the density of the regional *rf1* map and to confirm the map location of the *rf1* locus, genetic markers clustering to this region of LG H were examined for co-segregation with fertility restoration in the F_2 population. Markers *Xtxa335*, *Xtxp18*, *Xtxp250*, *Xtxp294* and *Xtxs560* were determined to be linked to fertility restoration in the F_2 population, thereby confirming the accuracy of the *rf1* locus map location, and also increase the density of markers flanking the *rf1* locus. The resulting LOD threshold ≥ 3.0 map of *rf1* spanned a 60-cM region of LG H and consisted of 11 markers including several SSRs that are well-suited for marker-assisted selection (see below).

The closest marker to the *rf1* locus was the AFLP marker *Xtxa2582* mapping at a distance of 2.4 cM from *rf1*. If 100 F_2 plants were selected using *Xtxa2582*, nearly all of them would be expected to have the *rf1rf1* genotype, with a predicted error rate of 4.7%[1] (see Koh

[1] Let ρ equal the crossover frequency between two loci *A* and *B*, such that the proportion of the phenotype groups *aaB_* (a recombinant type) and *aabb* (a parental type having homozygous recessive alleles) in an F_2 population will be $\rho(2-\rho)/4$ and $(1-\rho)^2/4$, respectively. In this study, since the recombination fraction between *Xtxa2582* and *rf1* is 0.024 (2.4%), the proportion of each *aaRf1_* and *aarf1rf1* phenotypic group will be 0.0119 and 0.2381. Therefore, the possible selection error rate using *Xtxa2582* to select for *rf1* will be $[0.0119/(0.0119 + 0.2381)] \times 100 = 4.7\%$. The recombination fraction for *Xtxp18* and *Xtxp250* is 6.8% and 6.5%, respectively. Therefore the possible selection error rate when using *Xtxp18* and *Xtxp250* will be 13.1% and 12.3%, respectively, when these markers are used singularly. When used in combination, the selection error rate for *Xtxp18* plus *Xtxp250* is $0.0131 \times 0.0123 \times 100 = 1.61\%$

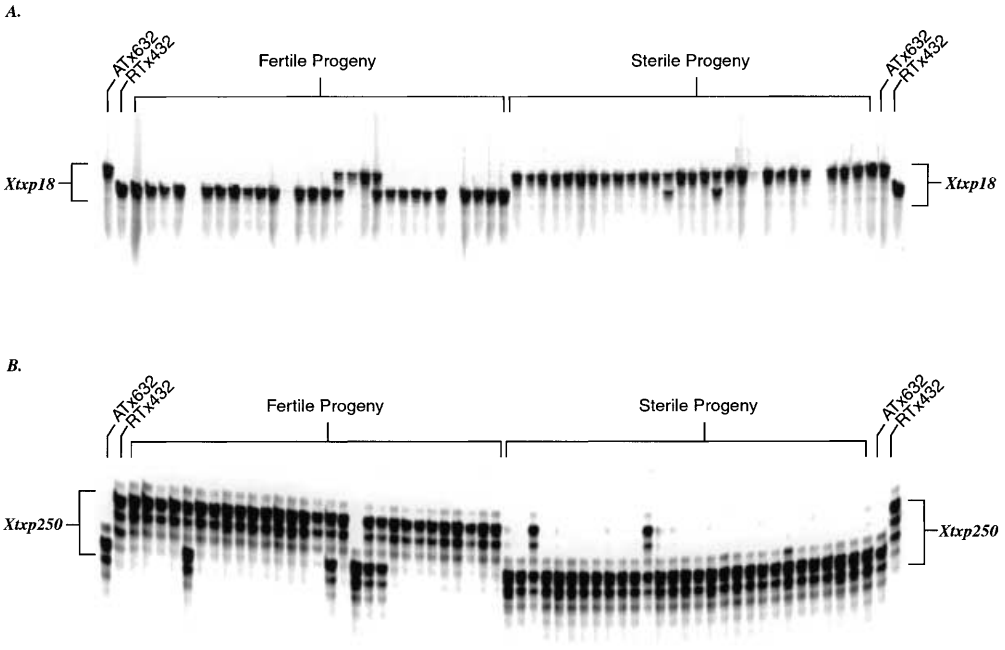

Fig. 3 Cosegregation of co-dominant markers *Xtxp18* (**A**) and *Xtxp250* (**B**) and the *rf1* locus in F_2 progeny derived from the cross of ATx623 and RTx432. Genomic DNA from parental inbreds ATx623 (*rf1rf1*) and RTx432 (*Rf1Rf1*) were run to aid in the identification of parental alleles for SSRs *Xtxp18* and *Xtxp250*. Individual F_2 progeny displaying both parental alleles were scored as heterozygous at that marker locus. The molecular weight of the *Xtxp18* allele was 225 bp (ATx623) or 218 bp (RTx432) while the molecular weight of the *Xtxp250* allele was 283 bp (ATx623) or 289 bp (RTx432). *Blank lanes* represent failed PCR reactions

et al. 1996). The use of *Xtxa2582* for marker-assisted selection, however, would require preparation of an AFLP template at considerable time and expense. The two SSRs, *Xtxp18* and *Xtxp250*, flanking the *rf1* locus should offer a more economical and robust screening tool compared to tightly linked AFLP markers. Two-point analyses of 373 F_2 individuals indicated that the recombination fraction between *rf1* and *Xtxp250* and *Xtxp18* was 6.5% and 6.8%, respectively. These SSRs are further from the *rf1* locus than *Xtxa2582* and would introduce a greater chance of error when used singularly (12.3%, *Xtxp250*; 13.13%, *Xtxp18*). However, when used in combination, the selection error rate of *Xtxp18* and *Xtxp250* would be approximately 1.6%. Hence, based on the high information content of SSRs (Taramino and Tingey 1996) and the ease of PCR-template preparation, *Xtxp18* and *Xtxp250* may provide an efficient alternative to traditional phenotypic evaluation for this character. We are presently examining the utility of molecular markers flanking the *rf1* locus for use in marker-assisted selection. Sets of R lines and B lines developed by sorghum breeders are being screened with *Xtxp18* and *Xtxp250* (along with AFLP markers flanking the *rf1* locus). As the recombination frequency between the *rf1* locus and molecular markers will differ between different genetic backgrounds (and with increasing generations of selfing/backcrossing), it is important to ascertain whether the markers accurately predict the allelic composition (i.e., *Rf1Rf1*, *Rf1rf1* or *rf1rf1*) in populations other than that used to generate the trait map.

The unified grass genome concept of Bennetzen and Freeling (1993) has received strong experimental support from recent comparative genome mapping studies of Gramineae species (for review see Gale and Devos 1998a, b). The fact that the content and linear order of genes in large chromosomal segments have been conserved for millions of years indicates that the map location of a gene in one species may, in some cases, be predicted based on its location in a related grass species. To this end, we attempted to compare the map location of *rf* loci in rice and maize to the first *rf* locus mapped in sorghum presented here. Schnable and Wise (1994) generated detailed genetic maps of the *rf1*- and *rf2*-containing regions of maize chromosomes 3 and 9, respectively. Peng et al. (1999) utilized sorghum, maize, oat, barley, and rice DNA clones to construct a consensus grass map that aligns the genomes of rice, sorghum and maize. From these studies it was ascertained that regions of LG H are homologous with chromosomes 3 and 10 of maize. Examination of the common markers present on the regional map of Schnable and Wise (1994) with that of Peng et al. (1999) indicate that the same general region of maize chromosome 3 that harbors *rf1* may correspond to *rf1*-containing regions of sorghum LG H. By contrast, homologous regions could not be identified between the genomic region encompassing sorghum *rf1* and regions proposed to harbor fertility restoration alleles in rice (Ichikawa et al. 1997; Yao et al. 1997). The present study and that of Schnable and Wise (1994) suggest that homologous regions of maize and sorghum may encode genes involved in pollen fertility restoration. Whether these regions of the genomes of maize and sorghum are collinear, and hence harbor homologues of fertility restoration genes, requires further investigation since the linkage information between different grass genomes is too sparse and inaccurate to pinpoint homologous genes except in a few cases (Bennetzen et al. 1998). Recent advances in sorghum genomics have permitted the gener-

ation of near-saturated recombination maps and the generation of integrated genetic and physical maps (Klein et al 2000). These advances in genomics provide the necessary tools to examine the micro-collinearity of grass genomes surrounding trait loci and permit the accurate identification of homologous genes between grass species.

A major goal of this project is to map-base clone the *rf1* gene of sorghum. To this end, an integrated genetic and physical genome map of sorghum is being assembled by this team of researchers. Methodologies developed (Klein et al. 2000) allow BAC clones encoding specific PCR-based markers (AFLP, SSR, STS) to be rapidly identified thereby linking the DNA-based physical map to the high-density genetic map. Using these methodologies, we have identified BAC clones containing the genetic markers flanking the *rf1* locus. At present, we are attempting to construct a contig of BAC clones that span the *rf1* locus. This information will provide a necessary starting point for identifying candidate genes and, hence for map-based isolation of the *rf1* allele.

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