Theor Appl Genet (2005) 110: 237–243
DOI 10.1007/s00122-004-1817-3

ORIGINAL PAPER

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STS markers linked to the Rf_1 fertility restorer gene of cotton

Received: 14 March 2004 / Accepted: 1 September 2004 / Published online: 9 December 2004

Abstract Marker-assisted selection (MAS) can accelerate the process of plant breeding, and sequence-tagged site (STS) markers are highly specific for regions of DNA being used for MAS. The objective of this research was to develop STS markers tightly linked with Rf_1, the fertility restoring gene for cytoplasmic male sterility (CMS) in cotton (*Gossypium hirsutum* L.). Bulked segregant analysis was employed to screen for Rf_1-linked RAPD markers in a backcross population. Four RAPD markers were identified, three of which co-segregated with Rf_1 ($UBC147_{1400}$, $UBC607_{500}$, and $UBC679_{700}$). Another fragment, $UBC169_{800}$, co-segregated with the previously reported $UBC169_{700}$ in repulsion phase at a distance of 4.5 cM from Rf_1. A marker published by others ($UBC659_{1500}$) mapped to 2.7 cM from Rf_1 and 1.8 cM from $UBC169_{800}$. Four sets of STS primer pairs were designed based on the RAPD fragment sequences. The primer pairs from the $UBC147_{1400}$ and $UBC607_{500}$ fragments both amplified a single fragment specific to fertile plants. The $UBC679_{700}$ and $UBC659_{1500}$ STS primer pairs each amplified one fragment specific to fertile plants and a monomorphic fragment. These four Rf_1-linked STS markers were also present in the Rf_1 donor species *G. harknessii* ($D_{2\text{-}2}$). The three primer pairs that produced co-segregating STS markers also amplified fragments from *G. trilobum* (D_8). However, the D_8 fragment amplified by the $UBC147_{1400}$ STS primers was larger than that from $D_{2\text{-}2}$, and *G. trilobum* does not restore fertility to CMS-D2-2 lines. These STS markers will be useful in the development of restorer parental lines in cotton CMS breeding efforts.

Communicated by R. Bernardo

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Introduction

A cytoplasmic male sterility (CMS) system greatly simplifies the development of hybrid lines and production of hybrid seed. Two main CMS systems, i.e., CMS-$D_{2\text{-}2}$ and CMS-D_8, have been developed in cotton (*Gossypium hirsutum* L.) by transferring exotic cytoplasm from *G. harknessii* Brandegee ($D_{2\text{-}2}$) (Meyers 1975) and *G. trilobum* (DC.) Skov. (D_8) (Stewart 1992) into upland cotton nuclear backgrounds. Male fertility can be restored to CMS lines by their corresponding restorer genes transferred from the wild diploid nuclear genomes. One dominant gene was found to control fertility restoration in each CMS system (Weaver and Weaver 1977; Zhang and Stewart 2001a). The Rf_1 gene from $D_{2\text{-}2}$ can restore fertility to both CMS-$D_{2\text{-}2}$ and CMS-D_8 lines, but the Rf_2 gene from D_8 can only restore fertility to CMS-D_8 lines (Zhang and Stewart 2001b). Genetic analysis indicates that Rf_1 and Rf_2 are tightly linked with an average genetic distance of 0.93 cM (Zhang and Stewart 2001b). Although Rf_1 and Rf_2 are linked, the linkage is in repulsion. Zhang and Stewart (2004) report that the markers they identified are Rf allele-specific. Baral et al. (2004) found that in a $[(Rf_1 \times Rf_2)F_1 \times rf_1]$ population, two STS markers were associated with fertile plants containing the Rf_1 gene allele, but they were absent in sterile plants containing the Rf_2 allele.

The Rf_1 gene in cotton functions sporophytically, whereas Rf_2 functions gametophytically. However, restoration by Rf_1 is environmentally sensitive and often produces F_1 plants with poor pollen shed, leading to distorted segregating ratios in the F_2 (Zhang and Stewart 2001b; Liu et al. 2003). Pollen grains from F_1 plants restored by Rf_2 appear normal, but only 50% of the

pollen grains are functional (Zhang and Stewart 2001a). Presently, it is unknown whether F_1 plants pyramiding the two restorer genes would function sporophytically or gametophytically. Based on phenotype, it is not possible to differentiate plants containing Rf_1 and Rf_2 genes from plants with only the Rf_1 gene. Molecular markers tightly linked to these two *Rf* genes (in repulsion) will enable us to distinguish between plants with these two genotypes in a timely manner.

Marker-assisted selection (MAS) can be used with high efficiency for indirect selection of both qualitative and quantitative traits by selection of molecular markers that are tightly linked with genes controlling the target traits (Mohan et al. 1997). In several crops, fertility restoration genes have been tagged with various types of molecular markers, such as RFLP (Jean et al. 1997, 1998; Kamps and Chase 1997; Delourme et al. 1998; Laporte et al. 1998; Tan et al. 1998;), AFLP (Bentolila et al. 1998), and RAPD (Jean et al. 1997, 1998; Zhang et al. 1997; Bentolila et al. 1998; Delourme et al. 1998; Laporte et al. 1998; Shen et al. 1998). In radish (Brown et al. 2003; Koizuka et al. 2003), petunia (Bentolila et al. 2002), and rice (Akagi et al. 2004; Komori et al. 2004), specific restorer genes have been identified and cloned. With this initial success, no doubt the restorer genes from additional species will soon be identified and cloned. However, for practical use the need remains for molecular markers, based either on the specific restorer gene or closely associated with it.

Techniques based on RFLP and AFLP are robust and generally repeatable, but they are often not suitable for MAS in plant breeding programs because of expensive and lack of technical expertise. RAPD techniques are fast and simple but may be unreliable or variable between laboratories. Sequence-tagged site (STS) markers have the advantages and few of the disadvantages of other marker types. STS markers have been used in comparative genetic mapping and to construct genetic maps, so it is feasible for them to be used in MAS (Ribaut et al. 1997). Because of this, RFLP, AFLP, and RAPD markers are often converted into STS markers for MAS (Bentolila et al. 1998; Klein et al. 2001; Matsui et al. 2001; Nakajuma et al. 1999). In cotton, several RAPD markers have been found linked with restorer genes Rf_1 and Rf_2. Zhang and Stewart (2004) found an RAPD marker, $UBC188_{500}$, linked to Rf_2, with an average genetic distance of 2.9 cM. Guo et al. (1998) reported an RAPD marker (OPV15-300) linked with Rf_1, with a recombination value of 13.0%, and Lan et al. (1999) identified one RAPD marker (UBC6592) that was 2.3 cM from Rf_1. This latter marker was cloned and partially sequenced, but the authors converted it into an RFLP marker that was subsequently mapped at 6 cM from Rf_1. Zhang and Stewart (2004) converted this marker, renamed as $UBC659_{1500}$, and another RAPD marker, $UBC169_{700}$, into STS markers. Recently, Liu et al. (2003) reported three SSR markers and two RAPD markers that were linked to, but not co-segregating with, Rf_1. However, these markers were coded without sequence information, and thus are unavailable to the cotton research community. Furthermore, the chromosomal localization assigned by these authors (A subgenome, chromosome 4) is questionable, since Rf_1 was transferred from the D-genome species, *G. harknessii* ($D_{2\text{-}2}$), most likely through homologous chromosome recombination with the D_h subgenome. Also, Zhang and Stewart (2001b) reported that Rf_2, transferred to cotton from the D-genome species, *G. trilobum*, was located less than 1 cM from Rf_1.

The objective of the present study was to identify additional RAPD markers tightly linked with the CMS fertility restorer gene Rf_1 of cotton, and to convert these into STS markers based on the DNA sequences for the benefit of the cotton research community.

Materials and methods

Plant materials

A backcross population was produced from a cross between B416R (Cook and Namkin 1995), a CMS fertility restorer line carrying the $D_{2\text{-}2}$ restorer gene Rf_1, and ARK8518, an experimental sterility maintainer line selected from an elite germplasm line developed at the University of Arkansas by Dr. Fred Bourland. The backcross population (B416R × Ark8518) × Ark8518 of 114 plants was grown at the Arkansas Agricultural Research and Extension Center, Fayetteville, AR, USA. At blooming, each plant was numbered and scored as male fertile or sterile.

In order to determine if sequences homologous to the STS markers associated with Rf_1 were present in other species, several diploid and tetraploid *Gossypium* species (Table 1) were screened with the STS primer pairs. A synthetic tetraploid hybrid $2(A_2D_8)$ was crossed with the CMS-$D_{2\text{-}2}$line to test for ability of the D_8 restorer gene to restore fertility. Two-hundred and fifty-six F_1 plants were evaluated for fertility under field conditions.

Table 1 *Gossypium* species screened for sequences with possible homology to sequence-tagged site (STS) sequences linked to Rf_1

Species or hybrid	Genome	Species or hybrid	Genome
G. herbaceum	A_1	*G. turneri*	D_{10}
G. arboreum	A_2	*G. schwendimanii*	D_{11}
G. anomalum	B_1	*G. stocksii*	E_1
G. capitis-viridis	B_3	*G. somalense*	E_2
G. sturtianum	C_1	*G. areysianum*	E_3
G. thurberi	D_1	*G. longicalyx*	F_1
G. armourianum	$D_{2\text{-}1}$	*G. nelsonii*	G
G. harknessii	$D_{2\text{-}2}$	*G. bickii*	G_1
G. davidsonii	$D_{3\text{-}d}$	*G. nobile*	K
G. aridum	D_4	*G. pulchellum*	K
G. raimondii	D_5	*G. hirsutum* (TM-1)	AD_1
G. gossypioides	D_6	*G. barbadense* (57-4)	AD_2
G. lobatum	D_7	*G. tomentosum*	AD_3
G. trilobum	D_8	*G. mustelinum*	AD_4
G. laxum	D_9	*G. darwinii*	AD_5

Genomic DNA extraction and polymerase chain reaction

Several unfolded young leaves were taken from the terminals of each plant in the segregating population and from plants of the various species, and DNA was extracted by the miniprep method of Zhang and Stewart (2000). Bulked segregant analysis (Michaelmore et al. 1991) was conducted to screen for RAPD markers linked with the male fertility restorer gene Rf_1. Equivalent amounts of DNA from ten fertile plants or ten sterile plants were used to construct fertile and sterile DNA bulks, respectively. Three hundred random oligonucleotide (10-mers) primers from the University of British Columbia (UBC primer sets 1, 2 and 6) were used to screen for RAPD markers linked with Rf_1. RAPD reaction mixtures were 25 µl, containing 20 ng DNA, 2.5 µl 10× *Taq* polymerase reaction buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTPs (2.5 mM each, Promega), 0.33 µl 15 µM primer, and 0.5 U *Taq* polymerase (PerkinElmer). The reactions were at 94°C 2 min, then 40 cycles of 94°C 30 s, 40°C 40 s, and 72°C 90 s, followed by 72°C 5 min. Polymerase chain reaction (PCR) products were separated by electrophoresis through 1% agarose gel. Subsequent STS reactions were the same as RAPD reactions, except the annealing temperature was between 55–65°C based on primer sequence.

Linkage analysis

MAPMAKER/Exp, version 3.0, software (Lander et al. 1987) was employed to calculate the genetic distance (Haldane function) between Rf_1 and associated RAPD and STS markers and to draw the linkage map.

DNA fragments cloning, sequencing, and characterization

Specific PCR-generated fragments were sliced from the agarose gel and purified with a QIAquick Gel Extraction Kit. The retrieved DNA fragments were inserted into the pGEM-T vector, according manufacturer's (Promega) technical instructions. Recombined plasmids were cloned in *Escherichia coli* strain JM109, and bacterial colonies screened on Amp^+/X-gal/IPTG LB solid media plates. White colonies were selected and grown overnight in LB liquid media. Plasmid DNA was extracted from *E. coli*, according to Sambrook et al. (1989). Sequences of the cloned DNA fragments were obtained with an ABI Prism 377 instrument (PerkinElmer) in the Core Molecular Biology Laboratory, Dale Bumpers College of Agricultural, Food and Life Sciences, University of Arkansas. A search for sequences homologous with the RAPD (and STS) fragments was conducted in GenBank (http://www.ncbi.nlm.nih.gov/BLAST), and Clustal W (http://www.ebi.ac.uk/clustalW) was used to compare marker sequences for homology.

STS primer design

STS primers were designed with Stanford Web Primer software (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer). The lengths of the STS primers were 18–21 bases, GC contents ranged from 45% to 61%, and T_m ranged between 60°C and 68°C. All STS primers were synthesized by Sigma-Genosis, Tex., USA.

Results

Fertility segregation analysis

Plants of the backcross population [(B416R×Ark8518) × Ark8518] were unambiguously distinguishable as male fertile or sterile. Sixty-two plants were fertile and 52 were sterile. The segregation ratio fits a monogenic Mendelian inheritance model of 1 fertile:1 sterile ($\chi^2_c = 0.886$, $P > 0.95$) in this mapping population, confirming that fertility restoration is conditioned by one dominant restorer gene, Rf_1.

Identification and linkage analysis of RAPD markers associated with Rf_1

Five primers were identified that amplified polymorphic bands between the Rf_1 and rf_1 DNA bulks and between individual fertile and sterile plants comprised in the bulks (Fig. 1). When tested against all plants in the segregating backcross population, three polymorphic fragments ($UBC147_{1500}$, $UBC607_{500}$, and $UBC679_{700}$) were present in all 62 fertile plants, but absent in all 52 sterile plants; that is, they co-segregated with Rf_1 in this population. Primer UBC169 amplified two polymorphic fragments. One, identified previously (Zhang and Stewart 2004), was approximately 700 bp in size and was present in most fertile plants, while the second fragment of approximately 800 bp was present in most sterile plants. Among five recombinant plants identified in the population, $UBC169_{700}$ was absent from two fertile plants and present in three sterile plants, while the reverse was true for $UBC169_{800}$, in that it was present in the two fertile plants and absent in the three sterile plants. Thus, these two fragments co-segregated in repulsion phase. RAPD marker UBC6592 ($UBC659_{1500}$), previously reported by Lan et al. (1999), was also polymorphic in our population. This fragment was present in 59 fertile plants and one sterile plant but absent in three fertile and 50 sterile plants, or 3.5% recombinants. All RAPD loci segregated in a ratio of 1 dominant:1 recessive in the population.

Genetic linkage analysis of the distance between Rf_1 and associated molecular markers indicated $UBC147_{1400}$, $UBC607_{500}$, and $UBC679_{700}$ were contiguous with the Rf_1 gene, while $UBC169_{700}$ and $UBC169_{800}$ were mapped at 4.5 cM from Rf_1. The genetic distance between Rf_1 and $UBC659_{1500}$, which we

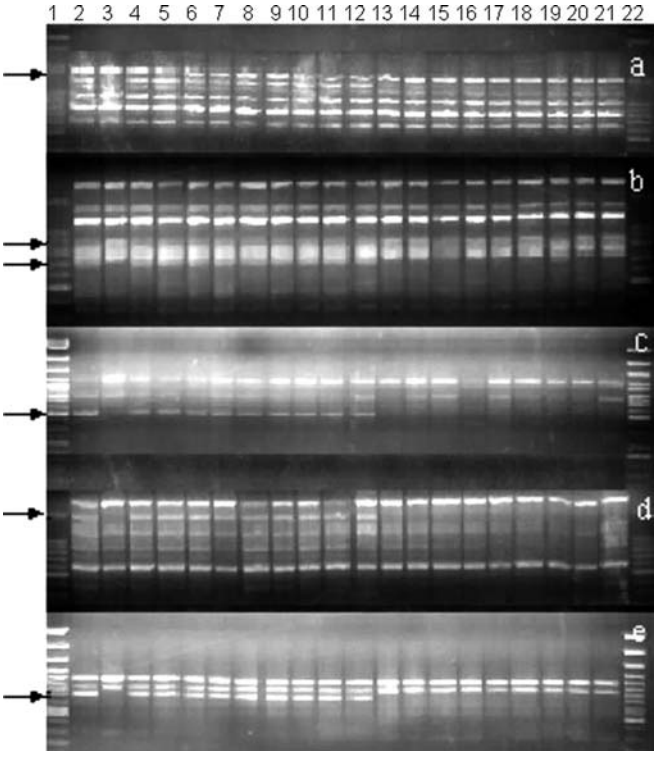

Fig. 1 RAPD markers linked with cotton cytoplasmic male sterility restorer gene Rf_1. **a** UBC147. **b** UBC169. **c** UBC607. **d** UBC659. **e** UBC679. *Lanes 1 and 22* 100-bp molecular-weight marker (Promega), *lane 2* Rf_1 bulked, *lane 3* rf_1 bulk, *lanes 4–12* fertile plants, *lanes 13–21* sterile plants

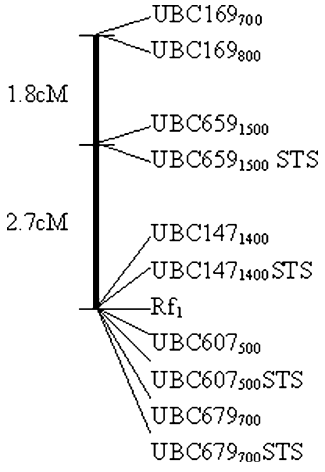

Fig. 2 Genetic linkage map of cotton cytoplasmic male sterility (CMS) restorer gene Rf_1 and associated RAPD/sequence-tagged site (*STS*) markers

assume corresponds to UBC6592 of Lan et al. (1999), was 2.7 cM (Fig. 2).

Sequence analysis and development of STS markers

The sequences of the cloned RAPD fragments, as expected, contained the primer sequences at the ends of the fragments. The exact sizes of the cloned fragments were 1,346 bp for $UBC147_{1400}$, 730 bp for $UBC169_{700}$, 807 bp for $UBC169_{800}$, 500 bp for $UBC607_{500}$, 726 bp for $UBC679_{700}$, and 1,426 bp for $UBC659_{1500}$. These sequences are registered as accessions AY570287 through AY570292 in GenBank.

Genetically, $UBC169_{700}$ and $UBC169_{800}$ appear to be allelic; however, alignment and comparison of their sequences reveal that nucleotide identity is only about 40%, and no conserved clusters were present, i.e., identity of nucleotides appears to be randomly distributed throughout the fragment. Because of their dissimilarity and distance from Rf_1, specific primers were not designed for these fragments, but for the three co-segregating RAPD fragments and the $UBC659_{1500}$ fragment, STS primers were designed with the Stanford Web Primer program. $UBC147_{1400}$ STS forward and reverse primers contained the last 8 bp and 9 bp of the UBC147 primer, respectively. $UBC607_{500}$ STS primers did not include any of the UBC607 primer sequence, whereas $UBC679_{700}$ STS forward and reverse primers contained 5 bp and 6 bp of the UBC679 primer sequence, respectively. $UBC659_{1500}$ STS primers contained UBC659 primer sequences. The complete STS primer sequences and relative parameters are given in Table 2.

When the STS primer pairs were tested on the original segregating backcross population, both $UBC147_{1400}$ and $UBC607_{500}$ STS primers amplified a specific fragment from the restorer DNA bulk and from each fertile plant. No product was obtained from the sterile bulk or from any sterile plant. The $UBC679_{700}$ STS primer pair

Table 2 Sequences and relative parameters of STS primers

Marker ID	STS primer	Sequence	Length (bp)	T_m (°C)	GC (%)	STS length (bp)
$UBC147_{1400}$	Forward	GCGTCCTCATATAAACTACGG	21	60	47	1,343
	Reverse	TGCGTCCTCCAATGTCTTTT	20	65	45	
$UBC607_{500}$	Forward	GGTGGGCAGGGTTGTTGT	18	66	61	475
	Reverse	GGTAGCGCTTGAAAACGACAT	21	65	47	
$UBC679_{700}$	Forward	GGTGGGGGAATGATAATTG	19	61	47	717
	Reverse	GGGTGGCAAGGCATATTTTA	20	63	45	
$UBC659_{1500}$	Forward	CGGTTTCGTATATGATTACG	20	57	40	1,476
	Reverse	CGGTTTCGTAGGGAGCCGCT	20	71	65	

amplified one fragment from each sterile plant and the same fragment plus a second one of different length from each fertile plant. Thus, the second fragment was fertility-specific while the first was monomorphic. Similarly, $UBC659_{1500}$ STS primers amplified a weak 2-kb monomorphic band and a specific band that was present only in the fertile bulk and fertile plants (Fig 3). Sequence analysis also confirmed that the specific bands amplified by the four pairs of STS primers were part of the original RAPD markers. The STS markers mapped to the same positions as the original RAPD markers (Fig 2). This confirmed the reliability of the RAPD markers developed in our system.

STS markers in *Gossypium* species

The $UBC147_{1400}$ STS primer pair amplified a single product from each of the D-genome species, $D_{2\text{-}1}$, $D_{2\text{-}2}$, D_4, D_5, D_6, D_9, and D_{11}, that corresponded in size to the fragment obtained from the restorer line; however, a larger fragment was obtained from the two sister species, D_1 and D_8. No product was obtained from other *Gossypium* species in other genomic groups. The $UBC607_{500}$ STS primers amplified a fragment of expected size from D_1, $D_{2\text{-}1}$, $D_{2\text{-}2}$, D_5, D_8, D_9, and D_{10}, but D_4 yielded a larger fragment. Other species did not give a PCR product with these primers. The $UBC679_{700}$ STS primers amplified a fragment of the expected length from C_1, $D_{2\text{-}2}$, and D_8, and a smaller fragment from A_1, A_2, B_1, $D_{2\text{-}1}$, D_5, D_7, D_9, D_{11}, AD_1, and AD_2. $UBC659_{1500}$ STS primers amplified a monomorphic band from each species and a second band of expected length from $D_{2\text{-}1}$, $D_{2\text{-}2}$, and D_{10}.

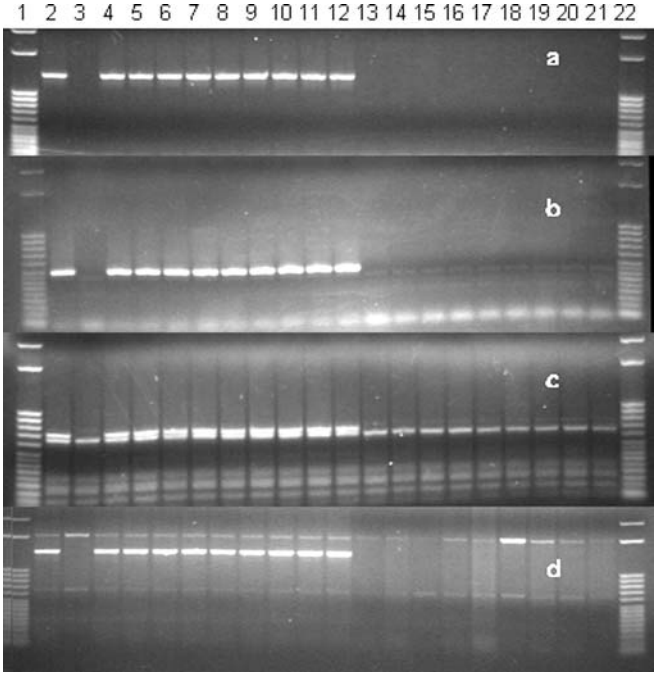

Fig. 3 Polymerase chain reaction profiles of STS markers associated with cotton CMS restorer gene Rf_1. **a** $UBC147_{1400}$. **b** $UBC607_{500}$. **c** $UBC679_{700}$. **d** $UBC659_{1500}$. *Lanes 1 and 22* 100-bp molecular-weight marker (Promega), *lane 2* Rf_1 bulk, *lane 3* rf_1 bulk, *lanes 4–12* fertile plants, *lane 13–21* sterile plants

Among the species, only *G. harknessii* contained all four STS markers found in the fertile plants in the population. *G. trilobum* yielded three STS fragments, two of which corresponded to those from the fertility restored plants, but the fragment amplified by $UBC147_{1400}$ STS primers was larger than that from fertile plants. Three fragments that were the same size as those of $D_{2\text{-}2}$ were also present in $D_{2\text{-}1}$. Two bands or less were generated from other species with these STS primer pairs.

Test of ability of *G. trilobum* to restore fertility to CMS-$D_{2\text{-}2}$

The D_8 genome contained two STS fragments that were the same size and one larger than the corresponding fragments obtained from Rf_1 fertile plants. It is known that CMS-D_8 restorer lines (D8R) with Rf_2 introgressed from *G. trilobum* cannot restore fertility to CMS-$D_{2\text{-}2}$ lines (Zhang and Stewart 2001b); it is not known if the wild species *G. trilobum* itself could restore fertility to CMS-$D_{2\text{-}2}$. When a synthesized tetraploid 2(A_2D_8) was used as pollen source to cross with CMS-$D_{2\text{-}2}$ all progeny were sterile. This determined that the inability of the D8R lines to restore CMS-$D_{2\text{-}2}$ was not due to loss of the restorer gene during the development of D8R lines, but because *G. trilobum* did not contain a restorer gene that could restore the fertility of CMS-$D_{2\text{-}2}$ lines. The two restorer systems derived from the two D-genome species are different.

Discussion

While transferring exotic cytoplasm into *G. hirsutum*, the donor species was used as female to cross with *G. hirsutum*, and *G. hirsutum* lines were used as the recurrent male parent for a minimum of eight backcrosses to generate the restorer lines. Theoretically, the content of the donor genome is reduced by one half that of the previous generation. Fertile plants are selected in each generation, so the restorer gene and its genomic vicinity are maintained during the development of the restorer lines. At the molecular level, this donor DNA contributes to the polymorphism between CMS and restored isolines. Theoretically, remaining donor DNA fragments without the restorer gene will be distributed randomly in the genome of backcross progenies and, thus, will normally give monomorphic fragments when screened by the BSA method. Only those that differ in the target locus and its adjacent region will exhibit polymorphism.

In the segregating backcross population, $UBC147_{1400}$, $UBC607_{500}$, and $UBC679_{700}$ fragments are derived from *G. harknessii*-donated DNA and tightly

linked with the Rf_1 restorer gene, so these occur as co-segregating, dominant markers. The $UBC659_{1500}$ and $UBC169_{700/800}$ fragments are farther from Rf_1, so several recombinant plants were found in this population. The $UBC659_{1500}$ fragment may also originate from *G. harknessii*. However, the PCR products obtained with 10-mer primer UBC169 reflect a length difference between two primer binding sites. The $UBC169_{700}$ fragment may be derived from the restorer gene donor, *G. harknessii*, but the $UBC169_{800}$ fragment is from the *G. hirsutum* genome, since it was only amplified from upland cotton. Although these two RAPD fragments co-segregated in repulsion, they are probably not allelic, because there is limited sequence similarity between the two fragments. A rather large genomic region of foreign DNA probably has been retained in the Rf_1 lines of cotton because of low recombination related to low homology in the region. Low recombination would make the markers genetically closed to Rf_1 than a physical map would indicate. STS markers, which are based on the PCR technique, can be either dominant or co-dominant. Because the RAPD fragments of $UBC147_{1400}$, $UBC607_{500}$, $UBC\ 679_{700}$, and $UBC659_{1500}$ are derived from DNA introgressed from *G. harknessii*, and are absent in wild-type *G. hirsutum*, it is understandable that the STS markers developed from these fragments are dominant rather than co-dominant.

The genetic distance between markers may vary among populations. Based on RAPD markers, Lan et al. (1999) calculated the genetic distance between UBC6592 and Rf_1 to be 2.3 cM, compared to 2.7 cM in our population. Theoretically, the larger the population, the more accurate the estimation of genetic distance. The genetic distance between Rf_1 and these markers should be tested in a larger population to refine the area map.

Conventional breeding for restorer lines is laborious and time-consuming, since selected plants must be tested for their ability to restore fertility. The STS markers reported here can be used to select fertile plants at the seedling stage long before the plants flower. Restorer plants with molecular markers can be used as one parent to cross with a recurrent parent without having to test for restoration ability in each generation. These markers will be useful to accelerate transfer and maintenance of the Rf_1 restorer gene in elite parental lines. Zhang and Stewart (2004) found an RAPD marker that was 2.8 cM from the Rf_2 male fertility restorer gene. So, possibly the two restorer genes Rf_1 and Rf_2 could be pyramided into a single elite parental line through indirect selection of these molecular markers; however, markers more tightly linked with Rf_2 are needed for high-efficiency MAS. Moreover, these fragments could be used as landmarks to screen a *G. harknessii* BAC library to identify clones that may contain the specific Rf_1 gene. Possibly the full-length Rf_1 gene could be obtained through the technique of chromosome walking.

The PCR profiles of STS fragments in different species that are homologous to the Rf_1-linked fragments provide clues about additional species that may contain a restorer gene. Among the profiles of STS fragments from different species, *G. trilobum* has the highest similarity with the Rf_1 gene donor, *G. harknessii*, but it does not contain a restorer gene that can restore CMS-$D_{2\text{-}2}$ lines. *G. armourianum* ($D_{2\text{-}1}$) yielded three STS fragments and should be tested to determine if it can restore CMS-$D_{2\text{-}2}$. $D_{2\text{-}1}$, as well as *G. turneri* (D_{10}), is closely related to *G. harknessii*, so the probability is high that these species contain a restorer gene for CMS-$D_{2\text{-}2}$ or CMS-D_8.

Acknowledgements This research was supported in part by a grant from Monsanto Company, St. Louis, Mo., USA.

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