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ORIGINAL PAPER

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Physical mapping of the *Rf*$_1$ fertility-restoring gene to a 100 kb region in cotton

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Abstract Cytoplasmic male sterility (CMS) plays an important role in crop heterosis exploitation. Determining one or more nuclear genes that can restore male fertility to CMS is essential for developing hybrid cultivars. Genetic and physical mapping is the standard technique required for isolating these restoration genes. By screening 2,250 simple sequence repeat (SSR) primer pairs in cotton (*Gossypium hirsutum* L.), we identified five new SSR markers that are closely linked to the *Rf*$_1$ gene, a fertility restorer gene of cotton for CMS-D2. Based on our previous fine mapping of the *Rf*$_1$ gene and assemblage of three published STS markers, we constructed a high-resolution genetic map of *Rf*$_1$ containing 13 markers in a genetic distance of 0.9 cM. The 13 molecular markers were used to screen a bacterial artificial chromosome (BAC) library from a restorer line 0-613-2R containing *Rf*$_1$ gene, which yielded 50 single positive clones. There was an average of 3.8 clones ranging from 1 to 12 BAC clones per PCR marker. These 50 clones produced an average insert size of 120 kb (ranging between 80 and 225 kb). Thirty-five primer pairs were designed based on 38 sequences of BAC ends, and two new STS markers tightly linked *to* *Rf*$_1$ gene have been tagged and integrated into this map. The physical map for the *Rf*$_1$ gene was constructed by fingerprinting the positive clones digested with the *Hin*dIII enzyme. We were able to delimit the possible location of the *Rf*$_1$ gene to a minimum of two BAC clones spanning an interval of approximately 100 kb between two clones designated 081-05K and 052-01N. Further work using these two BAC clones will lead to isolation of the *Rf*$_1$ gene in cotton.

Abbreviations CMS: Cytoplasmic male sterility · BAC: Bacterial artificial chromosome · STS: Sequence-tagged site · PCR: Polymerase chain reaction

Introduction

Cotton, one of the most economically important crops worldwide, is used to produce the most ubiquitous textile fiber and the second most valuable oil. Heterosis, a phenomenon also known as hybrid vigor, is critical to the increase of cotton productivity. Hybrid cotton, now roughly accounting for 20% of the total cotton growing area, is rapidly expanding in China following commercial exploitation of transgenic Bt cotton (Wang and Li 2000). However, hybrid seeds are still mainly produced by hand emasculation, genetic male sterility and pollination techniques (Zhang and Pan 1999). Primarily due to the tedium and expense of producing F_1 seeds, production of hybrid cotton has been restricted. Farmers in China had to purchase F_2 hybrid seeds, since heterosis is present in F_2 in cotton to some extent (Meredith 1990; Zhang and Pan 1999). India and China are currently the leading countries that plant and propagate hybrid cotton commercially on a large scale.

Cytoplasmic male sterility (CMS) plays an important role in heterosis usage in cotton. The combination of CMS and a nuclear gene that restores fertility (*Rf*) is essential for breeding hybrid varieties and for hybrid seed production. CMS eliminates the possibility of self-pollination, and is used commercially in the production of hybrid seeds for economically important plants (Newton 1998). Since 1965, CMS lines with cytoplasms from *Gossypium arboreum* L. (A_2), *G. anomalum* Wawr. & Peyr (B_1) (Meyer and Meyer 1965), *G. harknessii* Brandg. ($D_{2\text{-}2}$) (Meyer 1975), and *G. trilobum* (DC.)

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Skov. (D_8) (Stewart 1992) cytoplasm have been developed in USA. One new type of CMS, line 104-7A, selected from the progeny of a cross between *G. hirsutum* and *G. barbadense* (AD) has been developed in China (Jia 1990). Only *G. harknessii* CMS lines and 104-7A have been used to produce hybrid cotton in India (Basu 1993) and China, respectively. Two different dominant genes, Rf_1 and Rf_2, control the fertility restoration characteristic for two main CMS systems, CMS-D2-2 and CMS-D8. 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 male fertility to the CMS-D_8 lines (Zhang and Stewart 2001). So, Rf_1 possesses great potential for heterosis exploitation.

Both restorer genes Rf_1 and Rf_2 were fine-mapped in cotton. Guo et al. (1997) first reported a random amplified polymorphic DNA (RAPD) marker (OPV15-300) linked with Rf_1. Another RAPD marker, UBC659, 2.3 cM away from Rf_1 was tagged later and further converted into an RFLP marker (Lan et al. 1999). Liu et al. (2003) reported three SSRs and two RAPD markers that were linked to, but did not co-segregate with, Rf_1. Recently, additional RAPD markers tightly linked with Rf_1 were identified and converted into STS markers, which can be easily used in molecular marker assisted-selection (MAS) breeding techniques (Zhang and Stewart 2004; Feng et al.2005). A RAPD marker, $UBC188_{500}$, closely linked to Rf_2 with an average genetic distance of 2.9 cM was also reported recently (Zhang and Stewart 2004).

The *Rf* gene restores the self-fertilizing ability of a hybrid plant and is indispensable in crops in which seeds are harvested. Despite its importance in agriculture, the molecular mechanisms of CMS and its recovery are still unclear. To date based on transposon tagging and map-based cloning strategies several restorer genes have been cloned. The maize (*Zea mays* L.) Rf_2 gene encodes an aldehyde dehydrogenase (Cui et al. 1996; Liu et al. 2001). The petunia (*Petunia sp.) Rf* (Bentolila et al. 2002), radish (*Raphanus sativus* L.) *Rfk1* (*Rfo*) (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003) and rice *Rf-1* (Komori et al. 2004) encode a protein composed of 14 or 16 repeats of the 35-aa pentatricopeptide repeat (PPR) motif, respectively. However, no genes have been map-based cloned in cotton because an insufficient number of genetic markers are presently publicly available.

In the present study, we constructed a high-resolution genetic map for the Rf_1 locus comprising 15 SSR, RAPD and STS markers within 1 cM. An overlapping contig spanning the Rf_1 candidate region with bacterial artificial chromosome (BAC) clones was constructed based on BAC fingerprinting. The construction of the contig and the Rf_1 was localized to a region spanning roughly 100 kb in two BAC clones. This map provides a framework to cloning fertility restorer gene Rf_1 of cotton, and an important tool for studying male sterility and restoration mechanisms and MAS breeding.

Materials and methods

Genetic mapping

For the mapping procedures, we used three different BC_1 populations: Xiangyuan (XY) A × (XY-A × 0-613-2R), Zhongmiansuo (ZMS) 12A × (ZMS12A × 0-613-2R), and Sumian (SM) 16A × (SM16A × 0-613-2R). The restoring line 0-613-2R can completely restore the fertility of the CMS line in the *G. harknessii* cytoplasm. The XY-A line was kindly provided by Hunan Cotton Research Institute (Hunan, China). The ZMS12A and SM16A populations in Upland cotton have been developed through successive backcrossing to DES-HAMS277, with ZMS12 and SM16 as recurrent parents in our laboratory. Both ZMS12 and SM16 were widely grown cultivars in China. The crosses for genetic mapping were made in Nanjing, China, and the F_1 plants were backcrossed to the respective CMS lines to produce the BC_1s. The three mapping populations were planted at the Jiangpu Breeding Station, Nanjing in 2002. Fertility scoring was conducted as described previously (Liu et al. 2003).

For these studies we utilized 2,250 pairs of SSR primers to screen the polymorphism. Our criteria for determining valid SSR primers stipulated that they must detect the same polymorphism between the three CMS lines and the 0-613-2R fertility-restoring line. Qualifying primers were used subsequently to survey the linkage relationship to Rf_1 in these three BC_1 segregating populations containing 447 individual plants. The SSR primer sequences were obtained from the following sources: BNL primers from Research Genetics Co. (Huntsville, AL, USA, http://www.resgen.com); JESPR from Reddy et al. (2001); TM from Dr. John Yu, USDA-ARS, Crops Germplasm Research Unit, Texas, USA; EST from Dr. S. Saha, USDA-ARS, Crop Science Research Laboratory, Mississippi, USA, CIR from Nguyen et al. (2004) and NAU in our laboratory. All NAU-SSR primers derived from *G. arboreum* (Han et al. 2004) are publicly available now in two websites (http://www.algodon. tamu.edu/~mapbase/SSR-frame-page.htm and http://www.main-lab.clemson.edu/cmd/projects/nau) (Han et al. 2006).

Based on published data, four STS markers (Feng et al. 2005) were integrated into our genetic map. A linkage test was conducted with MAPMAKER/EXP (Version 3.0) software (Lander et al. 1987).

Construction of BAC library and pools

We constructed a 0-613-2R BAC library; with an average insert size of roughly 130 kb and covering 5.7 cotton genome equivalents for the map-based cloning of Rf_1 in our laboratory (Yin et al. 2005). Two levels of BAC pools (Super-pool and Column-pool) were prepared for analysis in two steps (Fig. 1). One Column-pool was mixed with each 384-well column, and each pool was

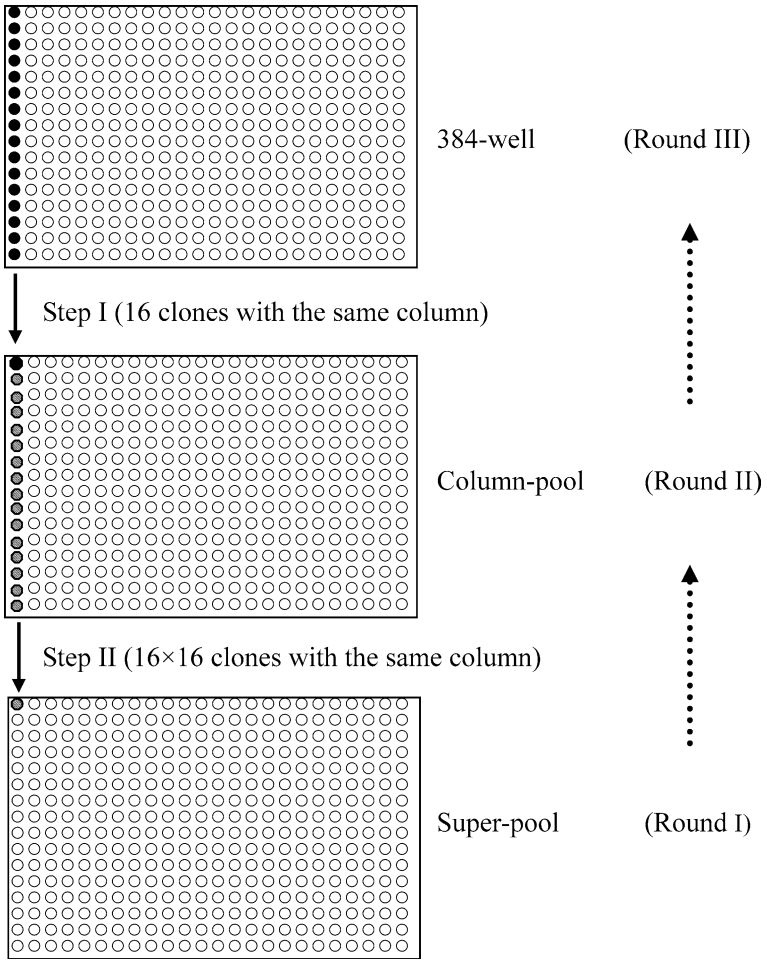

Fig. 1 The Schematic diagram of Column-pool and Super-pool construction and PCR screening procedure "*Arrow*" indicates the direction of Column-pool and Super-pool construction (two-step). One Column-pool was mixed with each 384-well columns and each pool comprising of all the clones in the same column contained 16 individual BAC clones. One Super-pool was mixed with each Column-pool (clones in the same column) and each Super-pool contained 256 (16×16) BAC clones. "*Dotted arrow*" indicates the direction of three rounds of PCR screening. DNA mixtures containing 256 independent BAC clones were used as PCR templates for first round PCR screening (Round I). The second round PCR screening was conducted for the DNA mixtures of the positive clones identified with Round I. Positive plates could be determined in this step. The third round screening was performed using plates with the same column. In this step, 16 PCR reactions per plate were required and individual positive clones were obtained

comprised of all of the clones in the same column, which contained 16 individual BAC clones. One Super-pool was mixed with each Column-pool. Each Super-pool contains 256 (16×16) individual BAC clones.

PCR screening of the BAC library

The BAC clone DNA was isolated in 96 wells with a MultiProbe® II EX (Packard, Meriden, CT, USA) workstation (Wang et al. 2005). The screening was conducted in three rounds of PCR amplification on pooled BAC clones (Rounds I, II and III, Fig. 1). The first PCR round was performed on all of the Super-pools containing all BAC clones in the library using the SSR, RAPD and STS markers closely linked to Rf_1. The Round I PCR results were used to identify which Super-pool(s) contained the BAC clone(s) with the sequence of interest identifying more than one Super-pool. The second PCR round (Round II) was performed on the Column-pools for the specific Super-pool under investigation. We were able to identify the exact plate from the PCR Round II results, and the exact well position for one or more positive clones in the identified Column-pool from the final Round III PCR amplification.

BAC fingerprint and the construction of contigs

To prepare the BAC clone DNA for further analysis, 5 ml 2×TY chloramphenicol (12.5 μg/ml) cultures were grown from single colonies for 24 h. The BAC DNA was isolated using the alkaline-lysis protocol (Birnboim and Doly 1979) with some modifications. After the bacterial cells were resuspended in 300 μl ice-cold solution I containing 100 μg/ml RNase, 300 μl of freshly prepared lysis (solution II) and neutralization buffer (solution III) were added sequentially, and mixed thoroughly on ice for 15 min. Following centrifugation, a 0.6-fold vol of isopropanol was added to the supernatant and centrifuged at 12,000 rpm for 15 min to precipitate the DNA. The insert of the positive single clones was digested with the *Not*I enzyme at 37°C for 3 h and analyzed on 1.0% agarose gel by pulsed-field gel electrophoresis (PFGE) in 0.5× TBE buffer under the following conditions: 6.0 V/cm, 12.5°C, 80 (pump setting), 5–15 s switch time, 120 degree and 16 h (Zhang 2000).

Each BAC cloned DNA was individually digested with *Hin*dIII at 37°C for 6 h. After digestion, the samples were electrophoresed at 40 V for 16 h at room temperature in a 0.8% agarose gel, stained with ethidium bromide and photographed. The contig was made

based on the *Hin*dIII fingerprinting of positive clones with FPC (Version 7.2) software (http://www.sanger.ac.uk/Software/fpc).

BAC DNA end sequencing

Following contig determination, the BAC ends were directly sequenced using an automatic DNA Sequencer 377 (ABI, USA) in our laboratory. The BAC-end DNAs were isolated using the modified alkaline-lysis method described above. The sequence cycling reactions (20 μ) contained 0.1–1 μg BAC DNA, 3.2 pmoles of primer (pIB FP and pIB RP, Epicentre, USA), and 8 μL of Big Dye (ABI). Cycling conditions included a 95°C hold for 5 min, followed by 85 cycles of 95°C for 30 s, 50°C for 10 s and 60°C for 4 min. The large amount of dye in the reaction mixtures required us to precipitate the DNA in ethanol to remove residual dye-labeled dideoxynucleotides from the reaction before analysis on the ABI 377 Sequencer. All sequences were submitted to NCBI Genbank. Primers were designed for map-based cloning the *Rf1* gene according to the Primer 3 (http://www.genome.wi.mit.edu/genome_software/other/pdrimer3.html).

Results and discussion

High-resolution mapping of fertility restoring gene *Rf1*

In the three BC_1 populations containing 447 individuals, 226 were fertile and 221 sterile. The fertility scoring and segregation is provided in Table 1. The ratio of segregation was 1:1 with chi-square test for goodness of fit, which confirmed its one-gene segregating inheritance ($\chi_c^2 = 0.0358$). Only the SSR primer pairs that all produced the same marker polymorphisms among all three CMS lines and the fertility-restoring line 0-613-2R were chosen for additional screening of individuals from the three BC_1 mapping populations. Among the 2,250 pairs of SSR primers used in the present research, we found five molecular markers that were closely linked to the *Rf1* gene. Additionally, four pairs of STS primers (Feng et al. 2005) were chosen to screen the parents and mapping populations, three of which produced polymorphisms and were linked to the fertility-restoring gene. Together with our two RAPD and three SSR markers published previously (Liu et al. 2003) and the three STS (Feng et al. 2005) integrated markers, we produced a high-resolution genetic map of *Rf1* containing 13 markers (Fig. 2). The total genetic distance was below 1.0 cM. Among the 13 markers, 2 RAPD (UBC169 and OPAE05) and four SSR markers (CIR222, CIR179, NAU441 and $BNL4047_{215}$) showed co-segregation. Three additional SSR markers (NAU1782, NAU790 and BNL3535) and three STS markers ($UBC679_{700}$STS, $UBC147_{1400}$STS and $UBC659_{1500}$STS) also co-segregated. The *Rf1* gene was mapped in a region of 0.7 cM flanked by the markers $UBC679_{700}$STS, including the other five markers and $BNL4047_{170}$, 0.5 and 0.2 cM away, respectively. Only did two and one crossovers occur respectively between the flanked marker(s) and *Rf1* gene. So, if we used the larger mapping population and recombination probability will be increased, these co-segregated markers might be separated. We found gene arrangements that differed to those described in previous reports (Liu et al. 2003; Feng et al. 2005). Small mapping populations documented in previous reports probably account for this difference.

As a common mapping population, we only used BC_1 mapping population for two purposes in present research. The BC_1 population has only two genotypes in

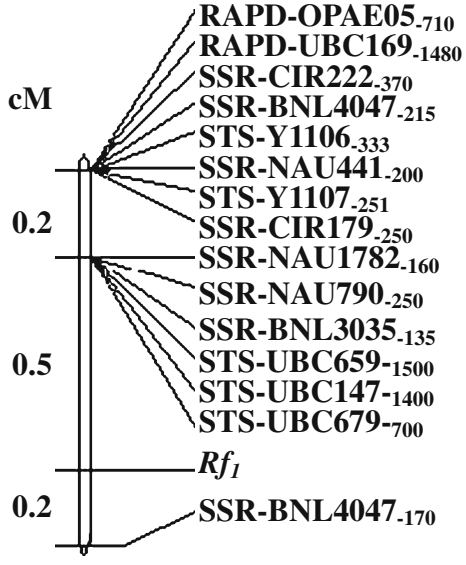

Fig. 2 High resolution mapping of *Rf1* gene in upland cotton. The genetic map contained 15 markers and the total genetic distance was below 1.0 cM. Based on fine mapping of *Rf1* published before (Liu et al. 2003), five new SSR markers in the present research as well as three STS markers (Feng et al. 2005) were integrated to produce this high-resolution map in cotton

Table 1 Segregation of fertile and sterile plants in backcross population

Crosses	No. fertile plants	No. sterile plants	χ_C^2 value (1:1)	Probability
XYA×(XYA×0-613-2R)	91	97	0.1330	0.750–0.500
ZMS12A×(ZMS12A-3×0-613-2R)	80	56	3.8897	0.050–0.025
SM 16A×(SM 16A×0-613-2R)	55	68	1.1707	0.500–0.250
Total	226	221	0.0358	0.900–0.750

the segregated gene localization, and directly reflects the segregation ratio of the F_1 gametes. Therefore, compared to other mapping populations, BC_1 should have a higher mapping efficiency than F_2, for example, for the dominant marker, considering the variability. And especially, in our previous report, we found that there exists a difference between the *Rf_1* and *rf_1* male and female gametes during the double fertilization (Liu et al. 2003), so distorted segregation often occurred in F_2. Theoretically, a larger mapping population begets a more accurate estimate of genetic distance. In this study, we utilized a larger BC_1 population containing 447 plants by combining the three different populations to map the *Rf_1* gene. We constructed a high-resolution map spanning the *Rf_1* gene, and consisting of 13 markers, with a distance below 1.0 cM.

The BAC library screening by PCR

A 0-613-2R BAC library was constructed to isolate the *Rf_1* gene in our lab. The library contains 97,825 clones; the average insert size is approximately 130 kb and roughly 5.7× haploid genome equivalents (Yin et al. 2006). The 13 markers closely linked with *Rf_1* included eight SSRs, two RAPDs and three STSs, which were all used to screen the library via PCR technology. The positive clones identified with the three different representative marker types, such as SSR-NAU441, RAPD-OPAE05 and STS- $UBC147_{1400}$, are illustrated in Fig. 3. In all, we obtained 50 single positive clones. The average insert sizes were roughly 120 kb, ranging from 80 to 225 kb, as determined by *Not*I digestion (Table 2). An average of 3.8 clones ranged from one to 12 BACs for each PCR marker, although theoretically six BAC clones should be detected for each marker on average. We found that eight positive clones were tagged at the same time by the markers.

There were 25 positive clones screened with eight SSR markers, and on average 3.1 BAC clones per SSR marker. Three positive clones, 014-06I, 014-06J and 018-06A were tagged by both CIR179 and NAU441, and the 018-23 N clone by both CIR179 and NAU1782. Using *Not*I and *Hind*III digestion and BAC ends sequencing, we determined that two clones, 012-24E and 012-24F, are identical. There were 11 positive clones screened with 2 RAPD markers, or an average of 5.5 clones per one RAPD marker. We found 14 positive clones by screening with three STS markers, or an average of 4.7 clones per STS marker. Four positive clones, 052-24M, 077-04C, 086-12F and 007-21J were identified by both $UBC147_{1400}$STS and $UBC679_{700}$STS (Table 2).

Using BAC end sequencing of 38 positive single clones, we designed 35 primer pairs. Two new STS markers,Y1106 (amplified by primers F: 5′-TCCACTGGTAAAACATGTGC and R: 5′-TACT-TGTTGGGGTCATTCCT) and Y1107 (amplified by primers F: 5′-TGCTTAGAAGGGTTTTCGAC and R: 5′-GCAACAGATATGGGTTAGGG), from 027-18L and 058-05D clones, respectively, identified to be tightly link with *Rf_1*, were integrated into high-resolution map and were found to co-segregate with the targeted markers ($BNL4047_{215}$ and NAU441). These two clones were screened with the $BNL4047_{215}$ and NAU441, respectively (Fig. 2, Table 2). Other primer pairs could not amplify polymorphism between the R and A lines perhaps due to too short sequences available from BAC end sequencing.

Screening a BAC library is the first and most vital step in contig construction and DNA marker development. Screening BACs by restriction fragment length polymorphisms (RFLP) provides the specificity and efficiency required for BAC screening. However, RFLP requires costly equipment and training, and the use of radioactive materials, thus necessitating the development of a simplified BAC screening system. The three-round PCR screening method that we propose herein is particularly powerful since it allows for isolation of a desired BAC clone(s) within approximately two days using a MultiProbe® II EX (Packard, Meriden, CT, USA). The creation of BAC DNA pools and Super-pools for PCR screening successfully reduced the time and number of experiments required to identify the candidate clones. After three rounds of screening with 13 markers, 50 positive clones were identified from a total number of 97,825 BAC clones. The result revealed that the PCR screening method in three steps from the Super-pool to the single clone was highly feasible and effective.

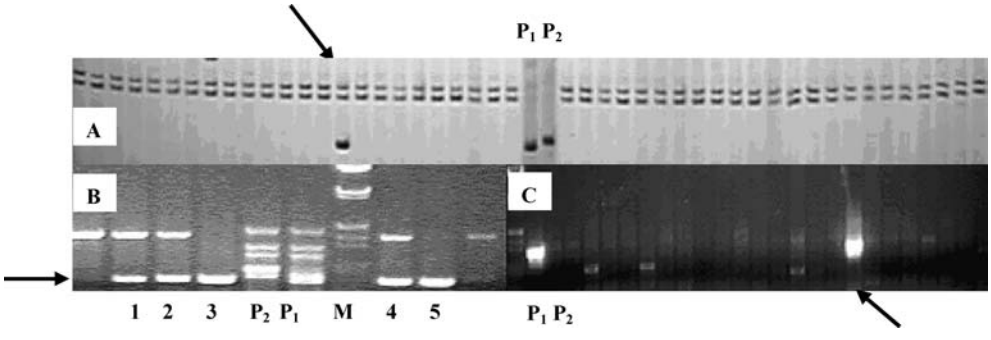

Fig. 3 Positive single clones screening by PCR. **a** Screening with SSR-NAU441 primer set, **b** screening with RAPD-OPAE05 primer, **c** screening by $UBC147_{1400}$STS. An *arrow* indicates a positive single clone; 1, 2, 3, 4 and 5 are positive single clones. *M* Molecular marker ladder, *P_1* R line, *P_2* A line

Table 2 The size of positive single clones

Primer set	Clone ID	Size (kb)	Primer set	Clones ID	Size (kb)
CIR179	012-24E	170	UBC169	078-16E	100
CIR179	012-24F	170	UBC169	078-16F	110
CIR179	012-24G	97	NAU790	033-10M	120
CIR179/NAU441	014-06I	100	BNL3535	097-23H	125
CIR179/NAU441	014-06J	103	$UBC147_{1400}$STS	254-15D	220
CIR179/NAU441	018-06A	225	$UBC147_{1400}$STS	254-15E	105
CIR179/1782	018-23N	130	$UBC147_{1400}$STS	254-15G	100
NAU1782	027-08E	115	$UBC147_{1400}$STS	254-15L	150
NAU441	058-05D	115	$UBC147_{1400}$STS	254-15N	140
$BNL4047_{215}$	027-08M	130	$UBC147_{1400}$STS	254-15O	105
$BNL4047_{215}$	073-07H	105	$UBC147_{1400}$STS	058-23B	110
$BNL4047_{215}$	027-18L	115	$UBC147_{1400}$STS	058-23F	115
$BNL4047_{215}$	033-10A	110	$UBC147_{1400/679_{700}}$STS	052-24M	105
$BNL4047_{215}$	033-10B	130	$UBC147_{1400/679_{700}}$STS	077-04C	105
CIR222	009-23L	110	$UBC147_{1400/679_{700}}$STS	086-12F	135
CIR222	087-23D	125	$UBC147_{1400/679_{700}}$STS	007-21J	105
OPAE05	044-15D	95	$UBC679_{700}$STS	052-01N	110
OPAE05	015-24F	100	$UBC659_{1500}$STS	044-02M	130
OPAE05	063-24M	90	$BNL4047_{-170}$	033-10C	120
OPAE05	063-24N	100	$BNL4047_{-170}$	033-10D	120
OPAE05	058-23G	100	$BNL4047_{-170}$	087-08I	105
UBC169	255-04A	115	$BNL4047_{-170}$	073-07M	130
UBC169	083-03E	125	$BNL4047_{-170}$	081-05K	80
UBC169	056-13G	115	$BNL4047_{-170}$	081-07E	125
UBC169	064-18N	130	$BNL4047_{-170}$	081-08J	100

The assembly of the contig and physical mapping of Rf_1 gene

The contig was constructed through fingerprinting of the positive clones digested with *Hind*III and using FPC software (Version 7.2), with a value of tolerance of 10 and a cutoff of 1e-10. Excluding the buried clones, we selected 16 representative BAC clones spanning the Rf_1 region for the physical map construction (Fig. 4). The genetic map, including 13 markers closely linked with the Rf_1 gene, was consistent with the physical map. Based on the genetic and

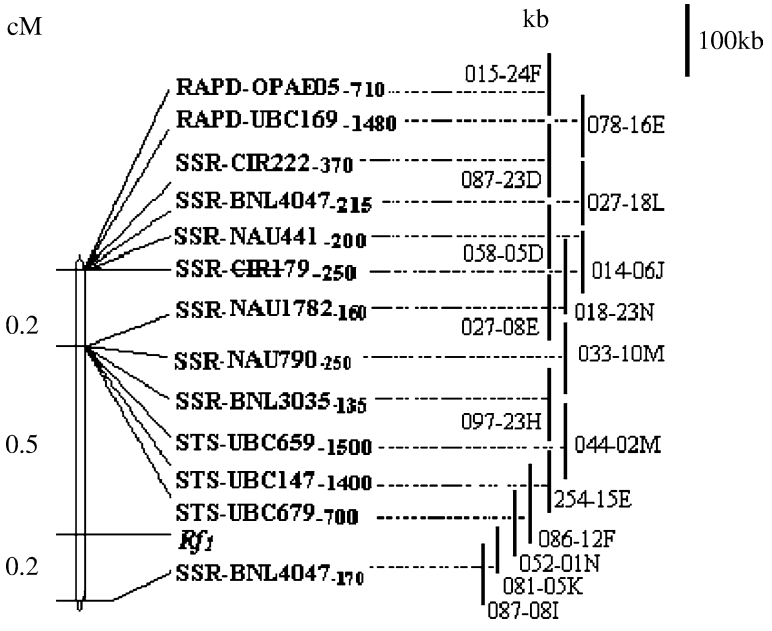

Fig. 4 Physical map of CMS fertility restorer gene Rf_1 On the right, the physical localization of the markers in the BAC contig is shown, and their localization on the genetic map is shown on the left. The clones, 087-08I and 081-05K screened by SSR-$BNL4047_{-170}$; 052-01N screened by STS-$UBC679_{700}$, 086-12F screened by STS-$UBC679_{700}$ and STS-$UBC147_{1400}$, 254-15E, 044-02M, 097-23H, 033-10M and 027-08E screened by STS-$UBC147_{1400}$, STS-$UBC659_{1500}$, SSR-BNL3535, SSR-NAU790 and SSR-NAU1782, respectively; 018-23N screened by SSR-NAU1782 and SSR-CIR179, 014-06J screened by SSR-CIR179 and SSR-NAU441, 058-05D, 027-18L, 087-23D, 078-16E and 015-24F screened by SSR-NAU441, SSR-$BNL4047_{-215}$, SSR-CIR222, UBC169 and OPAE05, respectively

physical maps, preliminarily we located the Rf_1 gene to a minimum region of two BAC clones, 081-05K and 052-01N. The size of 081-05K was about 80 kb, and that of 052-01N was about 110 kb. The overlap of the two clones was about 90 kb. Thus, we were able to restrict the possible location of the Rf_1 gene to a minimum region of two BAC clones spanning an interval of approximately 100 kb. Theoretically, 1 cM of genetic distance in Upland cotton may have 400 kb in physical distance on average reference. But in the present research, 100 kb spanned by two BAC clones identified by markers are 0.7 cM apart. We observed that only two and one crossovers occur respectively between the flanked marker(s) and Rf_1 gene, especially two crossovers occur almost at the same and/or very close position, but in two different BC_1 mapping populations used in the present research. Such crossover will not likely happen in one mapping population. It may be an advantage to use three and more mapping populations in gene mapping.

Map-based cloning has been successfully used to isolate several restoring genes in various plant species (Cui et al. 1996; Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003; Komori et al. 2004). This cloning method consists of several essential steps, including precisely mapping the gene region, constructing a large insert library, screening the library with the closest linked flanking markers for chromosome walking and contig assembly and identifying the target gene from the selected fragments by transformation. In this study, we present a localized linkage map covering the Rf_1 gene region and a BAC library from one parent of the BC_1 population. A contig spanning the Rf_1 gene region was constructed. According to the genetic and physical mapping results, the Rf_1 gene is located along a region of 100 kb regions between clones 081-05K and 052-01N. With our present findings, we can sequence the BAC ends and attain the new STS markers that link to or co-segregate more closely with the Rf_1 gene. Furthermore, we can construct BAC sub-clone libraries to isolate the putative restoration gene. Sequencing of 081-05K and 052-01N BAC DNA and identification of candidate sequences are presently underway in our laboratory. The results of our research contribute significantly toward the understanding of the molecular mechanism governing cotton CMS, and establishing an efficient foundation for studying and exploiting cotton heterosis.

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