MOLECULAR AND MORPHOLOGICAL GENETICS OF ATRISPECIES F_2 POPULATION OF COTTON

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Abstract

A trispecific F_2 mapping population was developed by crossing *G. arboreum* (A_2) cv. Nanking with *G. trilobum* (D_8), resulting synthetic allotetraploid hybrid 2(A_2D_8). This hybrid was crossed with *G. hirsutum* (AD_1) cv. T-586 and the resulting hybrid self-pollinated to obtain the segregating F_2 population. The population was used to study inheritance patterns of segregating loci and to establish the linkage groups among the three genome species. A total of 216 markers (194 AFLP, 19 RAPD and 3 morphological markers) were scored, of which 85 markers showed normal Mendelian inheritance. A large number of markers showed distorted segregation perhaps due to a difference of three chromosome arm arrangements between A_2 and AD_1, divergence of the three genome species and areas of low recombination among the genomes. With 2-point linkage analysis, 11 linkage groups were identified that spanning 521.7 cM of the cotton genome with an average distance of 16.8 cM between markers. One morphological marker, pilose, which is located on chromosome 6 was found to be linked with molecular markers in linkage group T1. The addition of more markers will help to identify areas of low recombination among species genome and linkage groups will become more clear and informative.

Introduction

Cultivated species of the genus *Gossypium* provide the world's leading natural fiber, cotton, and are also a major oil seed crop. The annual rate of genetic improvement of yield for *G. hirsutum* as estimated to be about 0.75% per year by Meredith and Bridge (1984). The rate of progress in *Gossypium* from breeding and molecular genetics should increase if genetic knowledge of the germplasm is improved. Upland allotetraploid cotton has two subgenomes A and D [2 (AD)] which are homeologous with diploid A and D genomes of *Gossypium.* Introgression strategies based on trispecific hybrids involving allotetraploid species and two diploid species have been developed (Stewart, 1979, 1995; Stewart and Hsu, 1977, 1978a, 1978b). Genetic diversity created through interspecific introgression can be measured with morphological and molecular markers (Gepts, 1993). However, a large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner. This limits the use of morphological traits and isozymes, which are few or lack adequate level of polymorphism in *Gossypium* (Tatineni et al., 1996).

Many molecular marker systems including RFLP, AFLP, RAPD and SSR are in common use for genetic mapping. Random amplified polymorphic DNA (RAPD) markers are simpler to assay than RFLPs and can detect polymorphisms in both low-copy and repetitive DNA sequences (Williams et al., 1990). The first reports showed that RAPDs can efficiently generate markers that are randomly distributed throughout the genome or are linked to specific genes (Martin et al., 1991; Michelmore et al., 1991; Quiros and Morgan, 1991). RAPD analysis can reliably determine genetic relationships within a diverse array of *Gossypium* germplasm as shown by Tatineni et al., (1996).

Amplified fragment length polymorphism (AFLP) developed by Zabeau and Vos (1993) is an efficient PCR based technique used to generate a large number of polymorphic DNA fragments. The basic difference between RFLP and AFLP polymorphisms is that for RFLPs, "only the restriction sites determine polymorphisms, but in AFLP, the restriction sites plus the additional selective nucleotides determine polymorphisms". Therefore, AFLPs detect more point mutations per 100 nucleotides than RFLPs (Becker et al., 1995). Like RAPDs, most of the AFLP markers are dominant and show Mendelian inheritance (Meksem et al., 1995; Maughem et al., 1996). Most of the AFLP markers correspond to unique positons on the genome and can be utilized for genetic and physical mapping as each fragment is characterized by its size and primer combination required for amplification (Vos et al., 1995). Cotton molecular maps can provide opportunities for map-based gene cloning, for study of chromosome evolution, and to assist in efficient introgression of traits from *Gossypium* gene pools for cotton improvement (Reinisch et al., 1994).

A project is under way to develop linkage maps of *Gossypium arboreum* and *G. trilobum* relative to *G. hirsutum* via morphological and molecular markers, including RAPDs and AFLPs, on an F_2 population derived from a trispecies hybrid *G. arboreum* (A_2) x *G. trilobum* (D_8) x *G. hirsutum* (AD_1) cv T-586. The objectives of current studies are (a) to determine the genetic efficiency of the synthetic tetraploid bridge strategy; (b) to investigate the inheritance of morphological and molecular traits in the segregating trispecies F_2 population; and (c) to use these informative traits, both morphological and molecular, to establish linkage groups among the three genomes (A_2, D_8 and AD_1).

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Materials and Methods

Plant Material

Three different genomic parents A_2, D_8 and AD_1 were used to obtain the interspecific hybrid population. First, *G. arboreum* (A_2) cv. "Nanking" was crossed with *G. trilobum* (D_8) to get an (A_2 x D_8) hybrid, and colchicine was used to double the chromosome number. This synthetic allotetraploid was crossed with the *G. hirsutum* multiple dominant marker line T-586 in which seven linkage groups are anchored on six chromosomes (Table 2). An F_2 population of 90 plants from a single trispecies F_1 hybrid was grown and maintained in the greenhouse to obtain DNA and morphological data. Self-pollinated progeny of five plants from each of the three parental genotypes, as well as the single F_1 plant, were used for preliminary survey of DNA polymorphisms.

Morphological Characteristics

Nine morphological traits (Table 2) were measured on each of the parental lines, the synthetic allotetraploid, the F_1 hybrid, and the 90 F_2 plants. Parental differences and F_1 performance (Table 3) for plant hair, leaf color and shape, petal color and spot, pollen and fiber color, and seed fuzz were observed. These characteristics are all highly heritable and stable traits. For those plants without flowers in the F_2 population, only plant hair and leaf traits were recorded.

DNA Extraction

DNA of five plants of each parental line was extracted and pooled in equal quantity. DNA from both F_1 hybrids (synthetic allotetraploid and trispecies hybrid), as well as from 90 F_2 plants, was also extracted for segregation analyses according to the protocol in Table 1. Young leaf tissue was collected from each plant and kept in the dark overnight at ambient temperature to metabolize starch. Completely restrictable DNA was obtained by this protocol.

RAPD Procedure

Total volume of amplification mixture was 25 µL with 2.5 µL 10X PCR-II buffer (50 mM Tris-pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Pharmacia Biotech), 0.2 µL oligonucleotide primer (University of British Columbia, Vancover), 0.5 Unit AmpliTaq DNA polymerase (The Perkin Elmer Corp.) and 10 ng of genomic DNA as template . Amplification reaction was programmed in a thermal cycler (Hybaid Omn-E-02HL) for 2 min at 94° C and 45 cycles of 15 s at 94° C, 30 s at 40° C, 90 s at 72° C, followed by 5 min at 72° C. Reaction products (12 µL) were resolved by adding 2 µL of loading buffer type II and applying the reaction mixture to (20 X 20 cm) 1.4 % agarose gels followed by electrophresis at 70 V for 5 to 6 h in 0.5 X TBE buffer. PCR products were visualized by UV light after ethidium bromide staining, and photographed with Poloroid film.

AFLP Procedure

AFLP detection was performed according to the protocol described in AFLP ™ Plant Mapping Kit for regular plant genomes (500-6000 Mb) Protocol (Perkin Elmer, 1995) and products were analyzed on an ABI Prism 377 DNA Sequencer, with Gene Scan and Geno Typer softwares.

Markers Selection Procedure

We considered both AFLP and RAPD primers to be informative only if unambiguous and qualitative (present or absent) fragments were obtained. Minor fragments, which seemed to possess the greatest lack of reproducibility or did not show up in either of the F_1's and F_2, were not considered as useful. Markers that were common in two of the three parental lines also were not considered useful. Criteria for the selection of markers were very stringent, as they not only represent polymorphism but also uniqueness to each genome.

Linkage Analysis

The segregations of the molecular and morphological loci were tested against 3:1 Mendelian segregation model using χ^2 with 1 degree of freedom for α=1%. Two point linkage analysis was performed with MAPMAKER v. 3.0 (Lander et. al., 1987) using the combined data of morphological, RAPD and AFLP markers. Linkage groups were established at a minimum LOD score of 3.0 and a maximum distance of 50 cM (Haldane function). With the groups defined, we used the "compare" and "try" commands to get the best sequence order of the loci within each linkage group. All map distances were calculated using the Haldane function.

Nomenclature

The first letter in each marker describes the genome (T for AD_1, A for A_2 and D for D_8). In morphological markers, the first letter is followed by the trait symbol. For RAPDs, the first letter is followed by a three digit primer number and then fragment size. For AFLP, the genome letter is followed by a three digit fragment size, and a letter and number representing the primer combination (one of 64 primer pairs). The linkage groups were assigned a letter representing the genome and numbered sequencially beginning with the group covering the greatest map distance.

Results and Discussion

Out of nine morpholgical markers (Table 3), we were able to score only three, including red leaf color (TR_1), pilose (TT_1) and seed fuzz (TN_2), in the F_2 population because other markers were common in two of the three parental genome species. These three morphological markers showed normal 3:1 segregation ratios. Out of 90 F_2 plants, 72 plants bloomed and 43 plants produced various numbers of mature bolls and seed under open pollinated conditions, indicating that most of the genetic recombination would be advanced into the next generation.

For 216 markers scored in the trispecific F_2 population, 131 (60.6%) showed significant deviation from the expected 3:1 dominant segregation ratio at P <0.01 (Table 4, Fig. 1). Distorted segregation has been reported in many interspecific crosses (Zamir et al., 1982; Wendel et al., 1987; Bonierbale et al., 1988; Paterson et al., 1988, 1991; Prince et al., 1992). Grandillo and Tanksley (1996) observed that when more distantly related wild species of tomato are used to generate segregating populations, more severely distorted segregation, ranging up to 80% of the markers, is often detected. Brubaker et al. (1997), while studying the comparison of segregation patterns of three F_2 *Gossypium* populations, found that the A and D subgenomes of tetraploid cotton derived from F_2 population of two tetraploid species, *G. hirsutum* (AD_1) X *G. barbadense* (AD_2), had 18% and 21% distorted segregation of loci, respectively. Thus, a total of 39% showed distortion from the AD_1, F_2 population. The A genome F_2 diploid population derived from *G. herbaceum* (A_1) X *G. arboreum* (A_2), had 8.7% distorted ratios. While the third F_2 diploid D genome population, *G. trilobum* (D_8) X *G. raimondii* (D_5), had 12.1 % distorted ratios. As expected, our trispecific F_2 population showed a higher distorted loci percentage as compared to bispecific populations, described above. Within each genome (A_2, D_8 and AD_1) , we found that 61.4%, 61.3%, and 59.1% showed distorted segregation ratios for each genome, respectively. At the chromosomal level, hybrids of *G. arboreum* lines form 13 pairs at meiosis. The chromosomes of the *G. hirsutum* A subgenome and *G. arboreum* differ by three reciprocal translocations involving chromosomes 1-5, thus one ring of four and one of six chromosomes occur frequently at meiosis in AD X A hybrids (Gerstel, 1953; Menzel et al., 1982) These three naturally occurring reciprocal translocations cause chromosomal duplications and deficiencies leading to pollen abortion that would result in skewed segregation of affected loci in the trispecific F_2 population. Other possible reasons for high abnormal segregation ratios could be evolutionary divergence of the three species that would result in areas of low recombinations between the genomes. Brubaker et al. (1997) suggested that allopolyploidy in *Gossypium* has been associated with increased chromosome structural divergence.

From the 216 markers, 85 (39.4%) showed normal 3:1 Mendelian segregation ratio and were used for linkage analysis. Thirty-one markers were linked in eleven linkage groups and 54 markers were unlinked. The resulting eleven linkage groups spanned 521.7 cM with an average interval length of 16.8 cM between markers (Table 5). A total distance of 184.4 cM was mapped for the A_2 genome that spanned over 4 linkage groups and 11 markers. The D_8 genome, which is physically shorter in length than the A_2 genome, mapped 140.9 cM over 4 linkage groups and 9 markers. AD_1 genome, which is twice the length of the A and D genomes, had relatively higher distance of 196.4 cM over 3 linkage groups and 11 markers (Table 5). Seven linkage groups had two markers each. Linkage group T1 had five markers with a total distance of 100 cM, including two RAPDs (T14714, T10614), two AFLPs (T328F5, T281F5) and the morphological marker pilose (TT1). Since TT1 is known to be on chromosome 6, the molecular markers linked to pilose can be anchored to this chromosome. Linkage group A1 also had five markers (all AFLPs) with a total distance of 108.5 cM ranging from 11.7 cM to 34.9 cM between markers.

The cotton map obtained from an F_2 population of *G. hirsutum* X *G. barbadense* has 705 RFLP loci with 41 linkage groups and 4675 cM. The cotton genome has 400 kb DNA per cM, and the genetic map of 5000 cM will require *ca.*3000 DNA markers to map at average 1 cM density (Reinisch et al., 1994). Thus, a large number of markers are required to have a densely saturated map of cotton.

Development of a genetic linkage map is the first step toward the detection of factors which control the expression of economically important traits. Much of the effort in constructing such a map is directed toward identifying useful polymorphic markers, and once identified, these markers can be used in numerous other pedigrees and related taxa. This project was initiated to develop maps of *G. arboreum* and *G. trilobum* relative to *G. hirsutum*. Markers derived from this project will be useful to identify allien introgressions and economically important traits in this and other cotton populations.

Conclusions

1. The trispecific F_2 population exhibited abundant phenotypic and genetic variation.
2. Molecular markers linked with pilose are located on chromosome 6.
3. Linkage groups will become more clear with additional markers.
4. A large percentage of markers did not segregate in Mendelian fashion\
   possibly due to:
   (a) three chromosome arm translocations which distinguish A_2 from the A subgenome of *G. hirsutum* and result in chromosomal deficiencies and duplications leading to selective pollen abortion;
   (b) divergence of the genomes of the three species leading to areas of low recombination among the genomes.
5. A large number of markers will help to identify areas of low recombination among species genomes.

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Table 1: DNA Extraction protocol

1.	Grind tissue in liquid nitrogen and mix with extraction buffer*, 3 mLg^{-1} tissue.
2.	Incubate at 70ºC for 1 h and let cool.
	Extract two times with an equal volume of chloroform: isoamyl alcohol (24:1) and centrifuge at 16, 000 g for 10 min. Keep the supernatant.
4.	Precipitate with an equal volume of isopropanol and keep at -80ºC for 1 h.
	Spool out DNA pellet with a small glass hook.
6.	Wash with 80% EtOH+15 mM ammonium acetate for 20 min with gentle shaking.
7.	Wash with 100% EtOH for 20 minutes with gentle shaking.
	Air dry the pellet and dissolve in 5 mL of high salt TE (10 mM Tris, 1 mM EDTA-both pH 8.0, and 1 M NaCl) by incubating at 60ºC for 1 to 2 h.
9.	Add 5 ml of extraction buffer without β-mercaptoethanol and keep on shaker for a minimum of 2 h, to dissolve the remaining pellet.
	Repeat steps 4 to 7.
11.	Air dry the pellet and dissolve in 100 to 200 µL of normal TE.
12.	Add 2 µL of RNase A (10 mg/mL) per 100 µL of DNA solution.

*Extraction Buffer: 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1 M NaCl, 2% CTAB, 2% PVP-40, 1 mM phenanthroline and 0.2% β-mercaptoethanol .

Table 2: Dominant mutants carried in the multiple dominant marker line of *G. hirsutum* cv. T-586

Symbol	Characteristic	Chromosome Number	Genetic Linkage Group
Y1	Yellow Petal	A Subgenome	12
P1	Yellow Pollen	5	11
T1	Pilose	6	4
R2	Petal Spot	7	1
Lc1	Brown Fiber	7	1
N1	Naked Seed	12	13 (5)
L2	Okra Leaf	15	2
Lg	Green Fiber	15	2
R1	Red Plant	16	3

Table 3: Parental differences and F1 performance

Trait	A2	D8	A2D8	T-586	(A2D8)xT-586
leaf color	green	green	green	red	red
leaf shape	ovate lobes	narrow lobes	sub-okra	okra	okra
plant hair	pubrule-nt	glabrous	pubrule-nt	pilose	pilose
petal spot	present	present	present	present	present
petal color	yellow	pale yellow	yellow	yellow	yellow
pollen color	yellow	yellow	yellow	yellow	yellow
fiber color	white	no fiber	white	brown	brown
seed fuz	present	absent	present	absent	absent

Table 4: Morphological and molecular markers scored

Morphological	RAPD	AFLP	Total	Fit to 3:1 Ratio
8 (3)	19	194	216	85

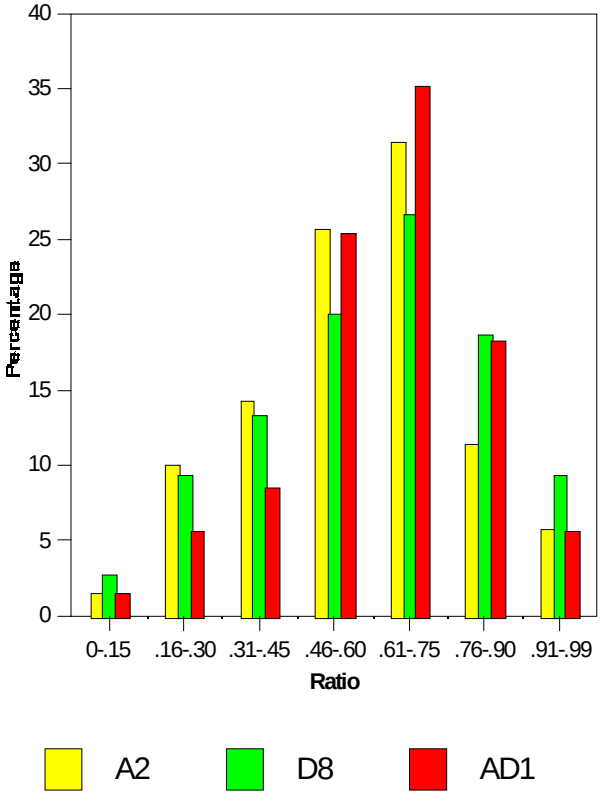

Fig. 1: Marker percentage distribution with F_2 population.

Table 5: Distribution of linked markers among the three genomes.

Genome	L. Group #	# of L. markers	Distance cM	Unlinked Markers
A2	A1, A2, A3, A4	11	184.4	16
D8	D1, D2, D3, D4	9	140.9	20
AD1	T1, T2, T3	11	196.4	18
Total	**11**	**31**	**521.7**	**54**