2006 Beltwide Cotton Conferences, San Antonio, Texas - January 3 - 6, 2006

COTTON GERMPLASM ENHANCEMENT AND EVALUATION BY SSR MARKERS

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Abstract:

Plentiful cotton germplasm with variation traits including yield, fiber quality, and boll weight, lint percentage, resistant to disease, and insects, drought and salt ect. Were gained by use irradiation, transformation foreign DNAs and genes, multiple and interspecific crosses etc. Among them, 510 lines were identificated by using agronomic and economic trait and SSR molecular analysis. The results showed variation rate of different germplasm was significantly different, and diversity of different traits was also different. Multiple crosses combination with irradiation inducement, transformation foreign DNAs and genes etc. were best way to introduce external genetic resources, enhance and preserve the variation. Different exotic DNAs and genes were transferred into different Upland cotton by using pollen tube pathway transformation, and the transformation gene and DNA isogenic lines with significant variation of agronomic traits and molecular biological characters were obtained. Exotic genes and genetic diversity of SSR markers among 155 cotton introgressed lines from interspecific hybridization have been detected, and the results showed specific SSR loci were present among the interspecific lines with different exotic gene sources, and the more specific loci with exotic genes, the better the fiber quality, resistance to *Verticillium wilt*, tolerance of drought stress etc. This research also showed SSR molecular marker analysis techniques can be use for identification and evaluation of cotton germplasm effectively.

Key words: Cotton Germplasm, Enhancement, SSR Markers

Plant genetic resources are the basis for crop breeding and improving agricultural productivity. Mankind has gained great achievements in genetic improvement for crops by using the various germplasm in the natural world. On the other hand, those germplasm with high yield and good quality and better comprehensive characters mainly emphasized to be used as basic breeding materials in modern variety improvement, but the germplasm with broad genetic variation of yield quality and advise resistance etc were ignored, even lost. This results in rich germplasm with narrow genetic background. To increase the genetic diversity, utilization potential of present genetic resources should be paid attention, meanwhile variant resources should be enlarged. Germplasm enhancement mainly means create new types of varieties and materials by using various natural and artificial variations, and a series of novel

techniques and methods. Genetic enhancement is basic way to increase diversity of genetic resources, play a role of pre-breeding, and is a key to utilize germplasm effectively. In the past, a large quantity of germplasm were developed by employing the techniques of multiple crosses□convergent crosses□interspecific crosses□γ ray irradiation treatment□chemical inducement etc in our lab and other research institute. Meanwhile, the development of high technology of tissue□cell cultures□molecular marker□genetic engineering also provide more effective techniques for germplasm enhancement in recent years. In this paper, the germplasm enhanced by using biotechnology, transformation foreign DNA, irradiation, multiple and interspecific crosses etc. were evaluated by phenotype and molecular analysis in order to lay a foundation for enriching biodiversity of cotton in great extent.

1 Materials and methods

1.1 Materials

15 accessions key germplasm were chosen for germplasm enhancement after screening from larger quantity of germplasm resources. 1014 lines with variation agricultural traits and economic characters were obtained by using Gamma ray, Ion Beam, Laser inducement, back crosses, Multiple crosses, Gene and DNA transformation etc. In this research, The agricultural traits and molecular marker characters of 510 new lines such as isogenic lines□mutants etc. were identified and analysed□Table 1□.

Table 1 Overview of Germplasm enhancement techniques and the creation lines

Original germplasm	No.of new lines	No.of derived lines identifed	Enhancement techniques	Maim Characters
Acala927	20	5	Gamma ray inducement	Thick leaf, yield and fiber quality variation
Arcot-1	244	65	Gamma ray, ion Beam and laser inducement	Variation of Fiber length, boll weight disease resistance and maturation
CCRI12	6	6	nature mutant	High yield, resistant to disease□plant type
CCRI19	10	5	Gamma ray and laser inducement	Early maturation, less pubes
CCRI2220	84	10	Multiple crosses□DNA transfer	High yield, good quality□disease resistance
CCRI4612	40	12	DNA transformation ,Gamma ray inducement, Multiple crosses	Different mature, plant height and disease resistance
CCRIJ926	38	21	DNA transformation	Different maturation and fiber quality
Exotic *G.hirsutum*	208	155	Interspecific cross	variation of Fiber quality, boll weight, disease and abiotic stress resistance and maturation
J11	56	38	Gamma ray inducement and crosses	High yield, good quality□disease and insect resistance, big boll
K8	26	17	Gamma ray inducement, nature mutant ,Crosses and DNA transformation	Resistant to disease and insect, boll weight and yield variation
L115	25	12	Gamma ray and laser inducement	Compact plant type, high fiber quality, high plant

				and more boll setting
M8123	9	4	Gamma ray inducement	Disease resistance
MM-2	9	9	nature mutant and Crosses	Resistant to disease and insect, big boll , high yield fiber quality
Mtwin	10	4	Gamma ray inducement	Compact plant type, high fiber quality
R01	26	15	Gamma ray inducement, nature mutant and Crosses	Resistant to disease and insect, Brown fiber, big boll, high quality
S9108	91	85	Gamma ray, ion Beam and laser inducement, Crosses, nature mutation	Big boll and high fiber quality, resistant to disease and insects□naked seeds, brown fiber
TM-1	42	13	Hormone gene transformation	Different mature, fiber quality, resistant to disease and yield
Wild species	8	8	Interspecific cross	Resistant to disease and insect, high or dwarf plant, high quality, cone shape boll
Yumian17	62	26	DNA and gene transformation	Late mature, different fiber quality and boll size, resistant to insect and disease

Gene and DNA transformation lines: The leaf DNA of 15 foreign plant including Poplar, Bauhinia, Chinese rose, Purple amaranth, Sponge gourd, String bean Sunflower, *G.barbadense*, *G.hirsutum* with red leaf etc.were prepared. The genomic DNA from *Gossypium barbadense* was transformed to Gossypium hirsutum such as Yumian 17, Zhong 4612 etc. by using the pollen tube pathway technology and many DNA transformation lines were gained, and we also get transgenic lines with obvious variance in agricultural traits and molecule biologic characters by transforming different exogenous genes including hormone genes, disease and insect resistance genes. These materials are very useful for the research on genetic performance of exogenous genes under the same genetic background.

The germplasm with different sensitive to irradiation reaction have been selected and used to produce mutant pools. Gamma ray irradiation dose was 250Gy. The work rate density of Helium-Neon laser treatment was 2.39mW/ cm^2. N_2 Ion implantation was used the ion beam inducement. Now a lot of mutants and elite germplasm with different genetic background were gained by using gamma ray, ion beam and laser inducement.

Interspecific lines: Different kinds of interspecific lines were got after the incompatibility of interspecific hybridization was overcome in China. 155 domestic and overseas exotic hirsutum cotton lines have been collected in this research.

1.2 Agronomic trait evaluation

The observation and survey of the agronomy traits according to that of “Descriptors and Data Standard for Cotton Germplasm”which was ensured by the platform program of National Science and Technology. The traits observed were plant hight, boll number, fruit branch numbers, lint percentage, boll weight etc. Fiber qualities including fiber length, strength, and fineness were inspected by the Cotton Seed and Fiber Quality Inspection Center, Agriculture Ministry. Resistance of cotton bollworm resistance, Fusanrium wilt, Verticillium wilt etc. was carried out by the

Plant Protection Laboratory, Cotton Research Institute, CAAS. Salt and drought tolerance were detected by the Germplasm Identification Laboratory, Cotton Research Institute, CAAS.

1.3 SSR marker analysis

Cotton genomic DNA was isolated according to Paterson's method with some modification. PCR reaction was followed PCR amplification program of our lab. The reactions were completed by PTC—100TM thermocycler. The PCR product was run on the 8□ non-denature PAGE gel. The gel was dyed referred to Zhangjun's method, and then was photographed using SYNGENE gel system. 8 varieties were selected to survey the SSR primers of BNL, JESPRA, and TMB. The primers with polymorphism were used to make SSR diversity analysis in different population.

2 Results and analysis

After agronomic and economic traits were assessed among 510 new lines, the variation types with different boll types, boll size, plant height, plant type, early mature, resistance to disease and insect, naked seed, fiber color, fiber length, fiber strength etc. were found (Table 1). This is important for the genetics and molecular study.

2.1 Identification of irradiation induced genetic stocks

Among three irradiation induced populations of Arcot-1, SU9108 and J11, Variation of boll number was biggest, the second was boll weight, plant height, fruit branch numbers, while the fiber fineness had a significant variation, and the fiber strength and elongation also possessed a obviously difference (Table 2). The variation of these traits in the population of SU9108 was biggest, the variation of fiber quality was bigger in the population SU9108 and J11 than that in the population of Arcot-1.However, the genetic diversity of fiber quality in each induced population was not significant. Standard Normal z-value of fiber characters of different variation population indicated compared the induced lines of three population with that of their check varieties with no irradiation treatment, the fiber length and uniformity had significant variation in J11 population, the fiber uniformity in Arcot-1 population was also significant different, but the variation of fiber quality in SU9108 population was not significant. This illuminated the fiber quality of SU9108 change a little after it was treated by irradiation.

The genetic diversity of 3 colonies of M5 irradiated by γ-ray were studied by using 39 polymorphism SSR markers. The range of genetic similarity coefficient of SSR markers between the M5 population of Arcot-1, SU9108 and J11 and their check variety with no irradiation treatment were 0.6129□0.9848□0.6212□1.000□0.4857□0.9905 respectively, average 0.8637□0.9206□0.6818. This indicated that J11 can induce more variation than those of Arcot-1, SU9108 by γ-ray radiation. It matches the result of phenotype analysis.

SSR analysis for ZSR269□ZSR272□ZSR273 irradiated by γ-ray from Su9108 was carried out, and four pairs of polymorphism primers including BNL830□BNL1672□BNL1053□BNL4030 were screened. There were two

mutation loci at BNL 830and BNL 4030 among the mutants ZSR269□ZSR272□ZSR273, and one more mutation locus at BNL 1672 happen in the mutant ZSR273 but these kinds of mutation loci did not appear in other mutants of Su9108.

Table2 The variation of phenotypic characters of progeny M5 populations induced from three lines

Population	Items	Plant height (cm)	Boll number	No. of fruit branch	Lint percentage (%)	Boll weight (g)	2.5% fiber length (mm)	Fiber strength (cN/tex)	Micronaire	Fiber uniformity (%)	Fiber elongation (%)
Arcot-1	CK	74.87	15.40	10.30	39.03	5.29	30.97	29.73	4.50	84.20	6.70
	Average	74.69	14.06	9.75	39.21	5.28	30.85	28.91	4.35	85.27	6.79
	C.V.(%)	12.82	24.07	14.96	4.72	15.88	3.95	6.73	7.72	1.35	7.92
	H’	2.04	1.97	2.05	2.05	1.89	2.06	2.05	1.96	2.03	1.94
Su9108	CK	63.38	10.56	7.69	42.30	7.07	30.85	32.55	4.75	85.95	6.00
	Average	60.67	9.78	7.68	40.12	6.23	29.80	33.28	4.71	85.82	6.00
	C.V.(%)	14.60	33.49	14.41	7.58	15.75	5.28	6.57	17.39	1.09	7.55
	H’	1.91	1.90	2.02	1.63	2.06	1.84	2.00	1.89	1.98	1.98
J11	CK	64.12	13.83	9.11	39.05	5.26	30.70	29.70	5.45	86.45	6.70
	Average	68.14	11.52	9.27	40.71	5.51	30.78	28.24	4.91	85.63	6.94
	C.V.(%)	8.89	29.55	14.06	5.77	14.08	4.69	8.96	13.76	1.50	10.88
	H’	2.02	2.03	2.12	2.10	2.01	2.00	2.01	1.90	1.89	1.96

Note: C.V. =Coefficient of variation , H’= Shannon-weaver diversity index

2.2 Identification of DNA and gene transformation lines

Different exotic DNAs and genes were transferred into different Upland cotton by using pollen tube pathway transformation, and the transformation gene and DNA isogenic lines with significant variation of agronomic traits and molecular biological characters were acquired. These materials are helpful for research the trait performance of exotic genes in same genetic background.

The IAA and iPAs genes were transferred into genetic standard line of upland cotton TM-1 by pollen tube pathway transformation, and 42 progenies transferred with hormone genes were gained. Simple sequence repeat (SSR) was used to determine the DAN genetic relationships in TM-1 and their variation lines with the transferred hormone genes. The result indicated that the similarity coefficient between 13 lines transferred with hormone gene and TM-1 was below 0.5, which indicated that the 13 lines of T014□T039□T007□T044□T045□T048□T002□T034□T035□T049 □T001□T006□T038 maybe carried with hormone genes.

The DNAs of Gossypium barbadense were transferred into *Gossypium hirsutum* variety Yumian 17 by pollen tube pathway transformation, and a lot of variant lines were ob tained. There was significant difference of fiber length and strength, lint percentage, boll weight etc. among those lines compared with that of donor and receptor (Table 3). SSR marker analysis showed the four lines of HB1□HB2□HB3 and HB4, were the variant lines, which

DNA fragments of *G.barbadense* maybe been transfer into this Upland cotton Yumian 17. The results of SSR marker agreed with that of agronomic and economic traits analysis very well.

Table 3 Standard Normal z-value of fiber characters of different variation population

Population	Sample Size	Lint percentage	Boll weight	fiber length	Fiber strength	Micronaire	Fiber uniformity	Fiber elongation
Cri2220	59	0.85	1.66*	0.59	4.23***	1.82*	0.40	0.04
su9108	40	0.23	0.44	0.43	0.66	0.18	0.70	0.43
J11	23	0.43	0.42	1.60*	0.06	0.49	1.77*	1.17
Arcot-1	59	0.69		0.21	0.66	1.37	2.16*	0.39

* showed significance at 10%, *** showed significance at 1%

2.3 Genetic diversity of cotton introgressed G.hirsutum lines

The genetic diversity of 155 cotton introgressed *G.hirsutum* lines has been analyzed. The SSR marker analysis indicated that there were specific SSR loci among the different exotic G.hisutum. 45.2% of 155 interspecific lines possessed specific SSR loci. The percentage of the lines with exotic specific SSR loci among the exotic *G.hirsutum* which maybe transferred with the genes of *G. barbadense* and G. thurberi was highest, reached 62.1□ and 71.1□ respectively. The second was those with the specific SSR loci of *G. anomalum* and *G. bickii* etc., reached 57.1% and 42.9% respectively□Table 4□. Since the number of exotic genes transformation was different, the specific SSR loci detected were very different. The exotic G.hirsutum with G. barbadense genes possessed 1-4 specific SSR loci, and those with G. thurberi had 1-5 specific loci.

Table4 The specific SSR loci of introgression genes among some exotic *G. hirsutum*

Exotic *G. hirsutum*	No. of accession	No. of specific loci	Percentage of lines with specific loci(%)	Average specific loci	Range of specific loci
G. barbadense consanguinity	29	8	62.1	2.6	1□4
G. arboreum consanguinity	21	2	4.8	2.0	2
G. thurberi consanguinity	45	15	71.1	1.7	1□5
G. sturtianum consanguinity	12	2	25.0	2.0	2
G. bickii consanguinity	7	1	42.9	1.0	1
G. anomalum consanguinity	7	3	57.1	1.8	1□3
G. hirsutum race mexicanum consanguinity	5	1	20.0	1.0	1
G. raimondii consanguinity	5	2	40.0	1.0	1
Total introgression lines	155	25	45.2	1.8	1□5

Through analysis of the agricultural traits of the introgression lines with specific SSR loci, the correlation between specific SSR loci and the main elite traits of the introgression lines was found. The result showed that the more specific SSR loci of exotic species, the better the fiber length and strength, the more tolerance of drought stress etc., and the elite strains with resistance to *Verticillium wilt* maybe selected from the germplasm with specific SSR loci□Table 5□. This indicated there were desirable genes with high fiber quality, tolerance to draught, resistance to *Verticillium wilt* etc. among these exotic *G.hirsutum* lines. For example, *G.hirsutum* variety Shenmian 5 with exotic genes of *G. barbadense* had 5 specific SSR loci. This caused its high fiber quality with length 34.1mm and strength

24.3Cn/tex, which indicated the high fiber quality of *G. barbadense* maybe been transferred into this exotic line. Similarly, the character of resistance to Verticillium wilt of *G.hirsutum* variety Shenmian 718 maybe resulted from *G. barbadense* since it had 2 specific SSR loci of *G. barbadense.* The high fiber quality traits of *G.hirsutum* variety Acala SJC-1,FJA and J line with consanguinity of *G. thurberi* maybe also resulted from potential high quality genes of *G. thurberi*. Moreover, the similar molecular and agronomic characters of exotic *G.hirsutum* of *G. barbadense* and *G. thurberi* indicated *G.hirsutum* maybe originated from *G. thurberi*.

Table 5 Correlation coefficient between SSR loci and fiber traits and stress resistant traits

	Drought tolerance (%)	Salt tolerance (%)	Verticillium wilt tolerance(%)	Fusanrium wilt resistance(%)	Bollworm resistance(%)
specific SSR loci	0.521**	-0.051	-0.100	-0.068	0.026
percentage of lines with specific loci(%)	-0.029	-0.439*	0.475*	-0.288	-0.494**

	Length (mm)	Fiber uniformity (%)	Fiber strength (cN/tex)	Elongation rate (%)	Micronaire
specific SSR loci	0.185	0.020	0.085	-0.009	-0.139
percentage of lines with specific loci(%)	0.215	0.192	0.438*	0.319	0.100

* showed significance at 5%, ** showed significance at 1%

3. Discusses

3.1 The techniques for germplasm enhancement

The purpose for germplasm enhancement is to acquire abundant variation. There are many ways to produce variation. Here we discuss the main characters of different technology for creating germplasm.

Comparison of 3 types of irradiation inducement indicated that mutation frequency in progeny induced by Gamma ray is higher than that of Ion Beam and Laser; the beneficial mutation frequency induced by Ion Beam was higher than those of Laser and Gamma ray; genetically stability of the descendants in the population induced from was better than those of Laser and Gamma ray. However, The biological effects and genetic mechanism of Ion beam implantation and laser induced remains to be further studied. After the principles of irradiation inducement are discovered more clearly, the physical irradiation methods for germplasm enhancement, breeding and gene engineering will be widely used. The genetic and physiological variation should be effectively identified in the procession of screening the irradiation variation. Genetic variation mainly results from the mutation of chromosome number□chromosome structure and gene, which show chromosome deficiency□duplication□translocation□abnormal meiosis□gene arrangement etc, and produce new characters or sterile plants. Physiological variation can not inherit stably. The variation of first generation of induced plants belongs to physiological one. So this variation should select when the mutation characters are stable instead of screening in the first generation of mutation.

The variation directions are fluctuant and the beneficial variation directly used was fewer among the irradiation variation even though they may produce abundant variation. The variation directions are easy to control for the gene and DNA transformation but their variation types are fewer. The interspecific crosses can introduce new gene types and enrich the germplasm at quality. More and effective variations may be obtained by using multiple crosses combination with irradiation and gene transformation techniques.

Wild species and race have abundant genetic diversity due to their various environmental distribution and long-term natural selection. Therefore, they possess lots of excellent genes that can be used to exploit potential traits, such as drought resistance, disease and insect resistance, cold resistance, male sterility, fine and strong fiber quality etc. (Liang,1999, 2002, Hu1994). Both domestic and foreign cotton breeders have widely carried out interspecific cross to utilize the genes from wild species and races , and lots of exotic *G.hirsutum* with good quality and stable traits including high-quality fiber, disease resistance, pest resistance, high lint percentage, etc. have been selected (Zhou 2003, Du 2000). They broaden the genetic basis of the present upland cotton resources and break the bottleneck of cotton breeding, so their practical value is very huge.

3.2.The advantage of transformation with pollen tube pathway

Recently, there are many transformation techniques including Agrobacteria mediated, particle gun bombardment micro injection pollen tube pathway laser micro bean immerse embryo method etc. Germplasm enhancement should choice different gene transformation technologies according to the different characteristics of exogenous genes and DNA. The main purpose of germplasm enhancement is to produce broad variation. So the simplest technology of gene transformation with no vector mediated such as bombardment pollen tube pathway immerse embryo method etc. should be considered for germlasm enhancement in order to transform more materials. Especially some transformation techniques such as the pollen tube path way and immerse embryo method can be used for transformation of exogenous DNA segment, and it need not separate clone or synthesize any gene, and reduce a lots of heavy jobs. Moreover, these methods can also transform the DNA of different family and Genus, even animal DNA(Zhou guangyu at.al 1986, Fu jun at al. 1990, Zhang xuewen 1994, Kohel et al. 2001). It is clear this transformation technology can develop new germplasm and species in large quantity. Since 1978, Introduction of exogenous DNA into cotton embryos has been done in some crops. However, the biological effects and proves were disputed. It was doubt that exogenous DNA transfer to plants by using the non-vector mediated ways in the past. However, lots of experiments have proved these methods and they were considered as an important way of plant genetic improvement in recent years(Wong jian at al. 1984). For instance, by using the transformation techniques of pollen tube path way and immerse employ method, the DNA of wild rice for medicine using black glutinous rice purple rice maize sorghum millet oats soybean buffalo pancreases were transferred to cultivated rice plants, and the target characters inherit and express in the offspring(Duan xiaofen at al. 1985, Li zhongxian, 1993); The exogenous DNA of the grass family maize sorghum barley etc. were transferred to the wheat plants; The DNA of G.barbedense G.arboreum castor bluish dogbane etc. were transferred to upland cotton(Du 2005); The DNA of wild semi-wild soybean peanut pea etc. were transferred to cultivated soybean plants, and new strains with different protein fatty acid contents were bred. Meanwhile, these transformations of exogenous DNA were also successful in some vegetable plants such as cucumber eggplant tomato etc(Li zhongxian, 1993). In this experiment, various foreign plants DNA successfully mediated the obvious phenotype variations. Therefore, we believe this

transformation method is efficiency for cotton germplasm enhancement.

3.3 DAN molecular maker techniques and germplasm enhancement

The effective used crop germplasm only occupy 0.3% among 355,000 accessions of total crop germplasm in China. This may resulted from following main reasons:

a. The characteristics of germplasm resistant to disease and insect, resistant to adverse environment were not identified and evaluated sufficiently, and their genetic background remains unclear.

b. There are some obstacles for the exchanges of germplasm and information.

c. There is a linkage of advantageous and disadvantageous genes in wild species□relative Genus plants and the germplasm with specific characters□so those genetic resources were difficulty to be used .

These resulted in that many germplasm cannot be used in modern variety improvement and the breeder had to do crosses among the modern elite varieties. However, the development of new biotechnology of DNA molecular markers provides advantage condition for releasing and using the potential beneficial genes in the crop germplasm.

Morphological markers used for identifying and evaluating germplasm in the past were simple with limited markers and affected by environment easily. The diversity of the cell markers mainly with karyotype analyses of chromosomes and isozyme markers is also limited. However, Molecular markers are large amount□high polymorphism and not affected by the growing season environment□ the difference of plant tissues and organ etc. It is very important to identify□evaluate□classify the germplasm effectively(Zhang zhuxin at.al. 1993, Zhu 2002). Moreover, DNA molecular markers can screen beneficial genes effectively, and decrease the linkage of these genes with the disadvantageous genes at large extent. This may lay a base for germplasm enhancement by using the wild species and particular genetic materials. The crosses between species with long distance relatives usually possess problem of genetic balance. However, the molecular marker can determine the homologous among different germplasm, and this can guide the hybridization breeding among different crops for developing new species.

Liu et al. (2000) and Nie al. (2000) used RAPD technique to analyse some interspecific hybrids. The showed that the specific bands of wild parent species appeared in the interspecific hybrids. SSR distributes wildly in the cotton genome. SSR marker analysis is more powerful to detect the genetic diversity of cotton germplasm resources (Kohel et. al. 2002). In this research, SSR technology was employed to detect genetic components of wild cotton among the introgression lines and also to identify the mutants and transformation of genes and DNA with satisfactory results. This not only enables us to evaluate the effects of germplasm enhancement, explore the molecular mechanisms of different breeding techniques, but also to detect the genetic components of wild cotton species in the introgression lines and screen specific germplasm in the offspring of exotic *G.hirsutum*, irradiation induced mutants, and gene and DNA transferred population.

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