第31卷 第8期 作 物 学 报 Vol.31, No.8
2005年8月 963~970页 ACTA AGRONOMICA SINICA pp. 963-970 Aug., 2005

Modified Backcross Pyramiding Breeding with Molecular Marker-Assisted Selection and Its Applications in Cotton

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Abstract: Using upland cotton (*G. hirsutum* L.) as research material, our objective was to describe and implement a modified backcrossing approach for introgressing transgenes or quantitative trait loci (QTLs) into target genetic backgrounds. Modified backcrossing is an improved breeding procedure combining inter-strain crossing and backcrossing methods to pyramid many traits. In the wake of availability of molecular marker technology for application to plant breeding, we propose a new backcrossing method called modified backcross pyramiding breeding (MBPB) combined with molecular marker-assisted selection (MAS). With MBPB, MAS was used in modified backcrossing and selection, *i.e.*, target genes or QTLs and the recurrent parental background was simultaneously selected by molecular markers. Henceforth, the breeding efficiency was significantly improved. By using this procedure, QTLs for stronger fiber strength and transgene *cryIA* have been rapidly pyramided in new cultivar NAU 85188 with Simian 3 background in cotton. The new cotton cultivar is characterized by elite fiber qualities, insect-resistance and high yield potential. The described breeding procedure can be used to simultaneously pyramid transgenes or QTLs in modern breeding programs.

Key words: Upland cotton; Fiber strength; Insect-resistance; MBPB population; QTL; MAS

分子标记辅助选择的修饰回交聚合育种方法及其在棉花上的应用

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摘 要: 修饰回交育种法是将杂种品系间杂交和回交方法结合起来,用于棉花多个优良性状聚合的育种改良方法。随着分子标记技术日益完善地用于育种的选择中,提出了分子标记辅助选择的修饰回交聚合育种方法。它以生产上推广或即将推广的品种为轮回亲本,将修饰回交育种法和分子标记辅助选择育种相结合,同时对轮回亲本的遗传背景和具有育种目标性状的基因或QTL进行选择,从而可显著提高育种效率。利用这一方法,以长江流域推广品种泗棉3号为轮回亲本,山西94-24和7235品系分别为抗虫基因和优质QTL的供体亲本,进行分子标记辅助的优质QTL系统选择和外源 *Bt* 基因的表型及分子选择。在(泗棉3号×7235)BC_1F_4中获得遗传背景与泗棉3号相近,株型稳定,且具有优质QTL的高强株系,在(泗棉3号×转 *Bt* 品系94-24)BC_4F_1中获得遗传背景与泗棉3号相近,抗棉铃虫效果明显的单株。进一步通过高世代优质和抗虫目标株系的互交,分子标记辅助目标性状选择,目标基因纯合及稳定性检测,使高强纤维QTL和 *Bt* 基因快速聚合,培育出了优质、高产的抗虫棉新品系南农85188。

关键词: 棉花;纤维强度;抗虫性;修饰回交育种群体;QTL;MAS

中图分类号: S562

Molecular marker technology is widely utilized in plant genetic research and markers associated with many crop agronomic genes and Quantitative trait loci (QTLs) have been successfully screened. However, only a few successful examples of molecular marker assisted-selection (MAS) have been reported thus far[1]. It has been verified that MAS is an efficient strategy to introgress qualitative traits into crops including wheat (*Triticum aestivum* L.)

Foundation items: International Atomic Energy Agency (12846), National Nature Science Foundation in China (30270806), and the National High-tech Program (2004AA211172).

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Received(收稿日期):2004-09-07, Accepted(接受日期):2005-01-16.

and rice (*Oryiza satva* L.) where multiple disease resistance genes have been pyramided by molecular marker technology, resulting in strains improved[2-5]. However, most traits in breeding programs are quantitatively inherited, complicating their manipulation through phenotypic or genomic approaches. How to make rapid selections for these target traits with molecular markers remains to the challenge for plant breeders. Advanced backcross QTL approaches[6] and single large-scale marker-assisted selection (SLS-MAS)[7] have been proposed to address the manipulation of quantitatively inherited traits. These new breeding methods present excellent considerations for the rapid realization of crop breeding goals. Presently, MAS breeding for quantitative traits is not widely practiced is possibly due to two reasons. Firstly, the genetic background and the environment easily influence the expression of most QTLs. And secondly, segregating populations used to detect QTLs for target traits and those used to develop new cultivars frequently differ in proportion of the genome derived from elite sources. The success of MAS breeding lies on heavily the genetic stability of QTLs in different environments and genetic backgrounds, QTL with prominent additive genetic control, and a set of sophisticated procedures to support molecular breeding. For those purposes, we propose the method of modified backcross pyramiding breeding (MBPB) combined with MAS to assemble multiple traits into an elite cultivar.

1 MBPB with MAS and its genetic basis

Recurrent selection can be effective in breaking linkages resulting in undesired correlations between yield, pest (or disease) resistance and fiber quality. Wang & Pan[8] proposed the modified backcross method for pyramiding multiple traits because it combined intra-strain crossing and backcrossing. Their breeding method began with a desirable, widely grown cultivar followed by crossing with strains or germplasm lines possessing known target traits and then backcrossing with the recurrent parents respectively for five or more generations. In each backcrossing cycle, phenotypic selection was made for recurrent parent with target traits being introgressed. Finally, mutual intercrossing and selfing was done to pyramid the target traits for the ultimate goal of improving cotton yields, qualities, and resistance simultaneously (Fig. 1). The advantage of this breeding procedure was that most traits of the backcrossed progenies resembled those of the recurrent parents, while the frequency of undesirable genes from the donor parents was reduced[9]. Experiments in cotton revealed that the negative linkage between high yield potential, fiber quality traits, and early maturity might be circumvented by this procedure, providing substantial basis for breeding cultivars or strains with excellent comprehensive characters and resistance. However, due to the fact that many agronomic traits are quantitatively inherited, the shortcoming of this procedure results from the amount of crossing, complete reliance on the phenotype selection and a long breeding duration.

Following the advancement of molecular marker techniques, a new modified backcross pyramiding breeding system facilitated by molecular marker is proposed (Fig. 1). In this approach, selection occurs for both the recurrent parent background and genes desired to be introgressed from non-recurrent parent by MAS. While selecting the donor genes from the non-recurrent parent, the reminder of the donor genome should be replaced with the recurrent parent genome. Generally, most of the undesirable genes from the donor parent can be removed through six generations of backcrossing[10]. MAS can speed recovery of the recurrent parent genome, given sufficient polymorphism between donor and recurrent patents, plus adequate genome coverage to allow MAS to accumulate the recurrent genome. Moreover, the study completed has also showed, an introgressed segment could be reduced in two generations, by RFLP marker-assisted selection for the recurrent parent genotype, to a size which would require 100 generations of backcrossing without MAS[11]. Even if a chiasmate region analysis estimated by molecular mapping efforts showed that the AD cotton genome (*G. hirsutum* L.) would have at least 5 000 cM in genetic size, which a need for additional DNA markers was suggested to saturate the map with the ultimate objective of linking the map with 26 linkage groups corresponding to the 26 chromosomes of cotton[12,13]. The progress has been made in identification

Fig.1 Diagram of modified backcross pyramiding breeding (MBPB) procedure with MAS

of QTLs for fiber traits in upland cotton (*G. hirsutum* L.) and their allelic association with molecular markers will be useful in cotton MAS breeding[14,15]. There are a small number of QTL involved in MAS, their selection are similar to selecting for qualitative traits[16]. As the developing of cotton linkage groups and markers linked with genes controlling economically important traits, the availability of MAS in cotton breeding programs will be more distinct. For MAS for target characters, it is desirable to identify molecular markers that co-segregate with or are tightly linked with the target agronomic traits. Generally, there are three parameters related to successful MAS: marker-QTL distance, population sizes and total duration of the program. In practical situations, the most important parameter is the distance between the introgressed gene and the flanking markers, which should be chosen to be as closely linked as possible to the introgressed gene[17].

2 Application of MBPB with MAS – practice in cotton

To overcome the negative correlation between high yield potential and desirable fiber quality, we focused our efforts on increasing yields and quality levels of cotton cultivars while simultaneously developing transgenic insect resistant cotton cultivars. Simian 3, a widely planted cotton cultivar in Yangtze River Cotton Growing Valley in China, was chosen as recurrent parent. This cultivar possesses high yield, resistance to *Fusarium* wilt disease caused by *F. oxysporum* f.sp. *vasinfectum*, wide

adaptation, and an ideal plant type, but with average fiber qualities, and is susceptible to bollworm larvae (*Helicoverpa armigera*). It has been planted more than 500 million hectares in China since it was released in 1993. It is still a check cultivar in formal trials at provincal and national level in this region. Both Shanxi 94-24 and 7235, used as trait donor parents, were as non-recurrent parents to develop new cultivars with high yields, insect resistance, and elite fiber qualities by our proposed MBPB with MAS (Fig.2). Shanxi 94-24 is a transgenic *Bt* cultivar resistant to bollworm pests developed by *Agrobacterium*-mediated transformation into Jinmian 7[18]. When *Bt* gene was introduced into Shanxi 94-24, a homozygous and stable strain was bred via pedigree selection breeding procedures and it was used as a donor of insect resistance gene. It was verified that one copy of *Bt* gene was inserted in the strain[19]. Superior quality fiber property germplasm line, 7235, was bred by the Industrial Crops Institute, Jiangsu Academy of Agricultural Sciences through crossing and backcrossing among such germplasm as *G. anomalum* L., Acala, Pee Dee, and other elite germplasm[20]. One major QTL for fiber strength from the strain has been identified and tagged with DNA markers[15].

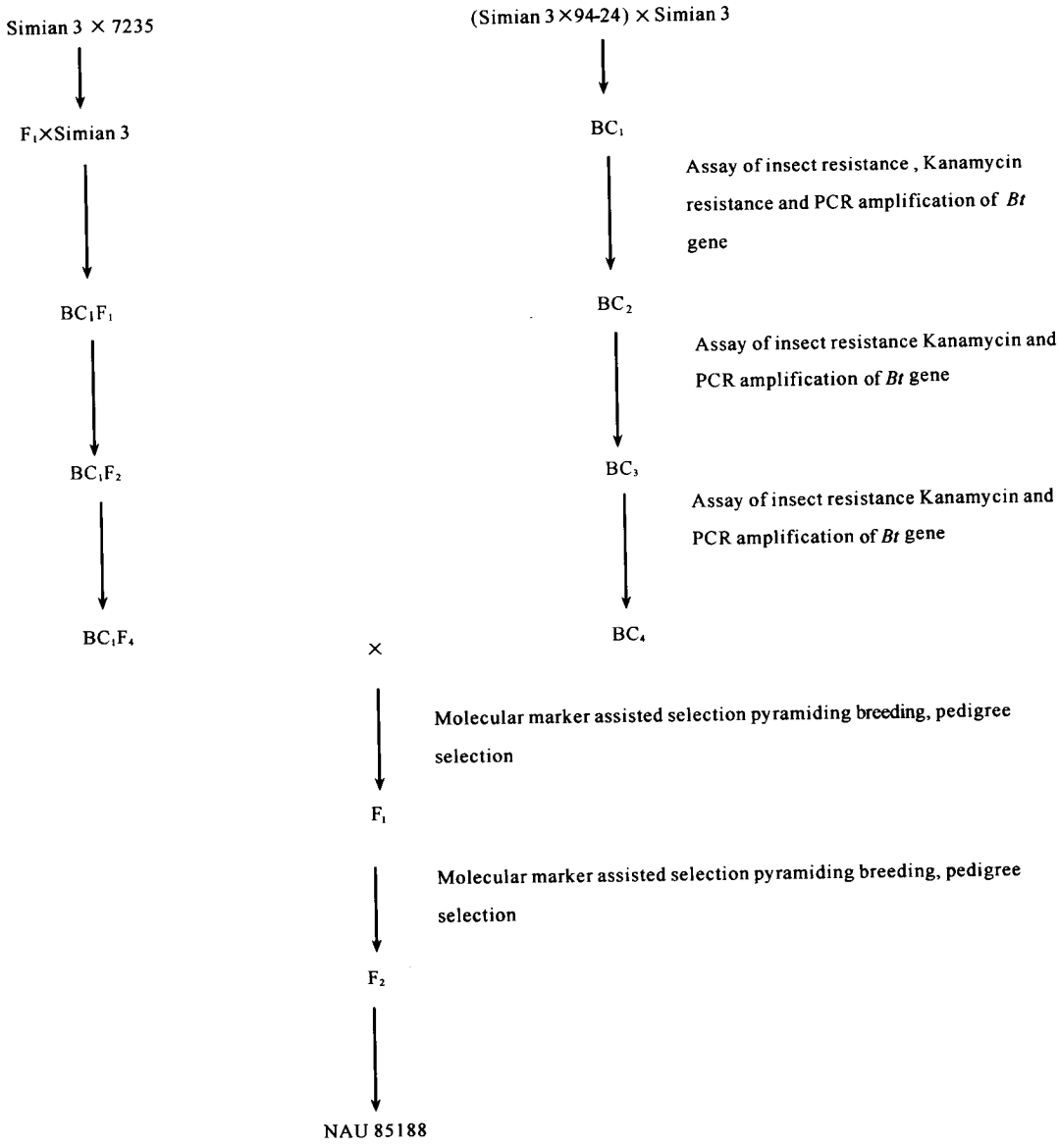

Fig.2 MAS pyramiding breeding for new cultivar that combines insect resistance and elite fiber quality cotton

Among these markers, two SSR (SSR1521, SSR1395) and one SCAR (SCAR431)[21] molecular markers associated with this QTL were used in the MBPB with MAS.

Shanxi 94-24 was crossed and then backcrossed four times with Simian 3 to transfer the *Bt* gene to Simian 3. Prior to each backcross, the product of the *Bt* gene was investigated from the phenotype of functional leaves to Kanamycin (Kan) at least twice according to the presence or absence of apparent yellow patches, since we found that the neomycin phosphotransferase Ⅱ (*NPT* Ⅱ) and *cryIA* genes were inserted the same cassette in Shanxi 94-24[22]. Plants resistant to Kan were bio-assayed for their resistance to bollworm in laboratory, and further tested by PCR amplification for the presence of *Bt* gene (Forward primer: 5′-GGGCCCGCTGAATCCAACTG GAGAGGC-3′ and Reversed one: 5′-CCATACAACTGCTTGAGTAACCC-AGAAGTTG-3′) in those insect and Kan resistant plants[23]. Simultaneously, Simian 3 was crossed with 7235 and backcrossed once with Simian 3, followed by pedigree selection for plant type, yield and specifically for fiber properties. Beginning with the BC_1F_4 generation, MAS was applied to select for a major QTL for fiber strength residing in 7235. Two strains with high strength marker and stable plant type were selected from the (Simian 3 × 7235) BC_1F_4 generation as parents and were crossed with (Simian 3 × Shanxi 94-24) BC_4 insect resistant strains, and were sent to Hainan Province for seed multiplication in 2000. Seeds were collected from each individual plant and 51 progeny-rows were seeded in 2001. Kanamycin assay of individual plant in functional leaves was conducted on these 51 rows. Among them, 28 rows produced resistant and susceptible segregation while 23 rows were susceptible to Kan. Furthermore, individual plants showing resistance to Kan were biologically assayed for resistance to bollworm. Resistant plants were screened by MAS for the QTL controlling high fiber strength, and were self-pollinated on Hainan Province in winter resulting in cotton strains selected for stable high yields, bollworm resistance, and desirable fiber qualities were bred. Selection criteria during line development included insect resistance, good fiber quality, bolls per plant, boll size, lint percentage, and fiber quality.

Through MBPB with MAS, a new cotton strain 85188 was developed with high yield potential comparable to Simian 3 and fiber quality potential comparable to 3-79, a genetic standard marker for Sea Island cotton (*G. barbadense*). Five rows of each tested lines (or cultivars) were separately planted in the same experimental condition; since the tested lines had been pure lines, 3 mature bolls from different individuals in each row were randomly selected for their fiber quality identification; yield components of different lines (cultivars) surveyed based on five rows plot of per materials; and five individuals per row were randomly selected for bio-assay of insect-resistance. Compared with Simian 3 and 3-79, the average boll weight of 85188 was 5.0 g, lint percentage 42.27%, 2.5% span fiber length 32.0 mm, fiber strength 35.3 cN/tex, fiber micronaire reading 4.4, which meets the elite cotton criteria, and high resistance to bollworm (Table 1 and 2). Strain 85188 has undergone the required bio-safety appraisal for commercialization of a cultivar with insect resistance. By MBPB, some new strains with pyramiding good fiber quality QTL and *cryIA* gene have also been created, these strains will be used to breed cotton hybrid with economically important traits (Table 3). In general, MBPB could be used to effectively break the negative correlation between high yielding capability and excellent fiber quality, while simultaneously introgressing resistance to key pest insects. This procedure can also be used to pyramid multiple genes. Therefore, novel cotton plant materials with multiple introgressed traits may be quickly bred with MBPB method in combination with MAS for insect resistance and elite fiber quality, as well as morphological recovery of the recurrent parent.

Table 1 Performance of fiber qualities of 85188 strain and its control

Strain and cultivar	Fiber length (mm)	Fiber strength (cN/tex)	Fiber micronaire
3-79 (*G. barbadense* L.)	38.50 A	42.90 A	4.30 Bb
NAU85188	32.00 B	35.30 B	4.40 Bb
Simian 3	29.70 C	29.80 C	5.10 Aa

Notes: The same alphabet represents no significant difference; the different alphabet indicates significant (small letter) or highly significant change (capital letter).

Table 2 Insect resistance and yield performance of NAU 85188 and its control

Strain or cultivar	Insect resistance in seedling		Seed cotton yield (kg/ha)	Lint yield (kg/ha)	Lint percent (%)
	Mortality %	Leaf damage index			
NAU85188	100.00 ± 0.00**	0.67 ± 0.58**	3 305.7	1 411.50	42.27
Simian 3	13.33 ± 16.33	3.46 ± 0.51	3 339.0	1 452.75	44.06

Notes: ** shows significance at the 1% probability levels.

Table 3 A series of superior fiber quality lines produced by MAS breeding

Line	Fiber length (mm)	Fiber strength (cN/tex)	Micronaire
3-79	38.50 Aa	42.90 Aa	4.30 Cc
85195-2	35.70 Bb	39.10 Cc	4.40 Cc
85226-1	34.50 Cc	40.80 Bb	4.60 Bb
85194-13	34.50 Cc	38.00 Cd	4.70 Bb
85195-5	34.00 Cd	38.20 Cd	4.00 Dd
Simian 3	29.70 De	29.80 De	5.10 Aa

Notes: The same alphabet represents no significant difference; the different alphabet indicates significant (small letter) or highly significant change (capital letter).

3 Problems and prospects

3.1 Pyramiding breeding and multi-line cultivars

The concept of developing multiline cultivars was successfully applied in crop disease resistance breeding[24,25], in reference to utilizing an excellent cultivar as a recurrent parent that is crossed with cultivars or germplasm containing different vertical resistance genes respectively. Backcrossing and selection for resistance genes were carried out repeatedly, and finally sets of near isogenic lines with elite agronomic traits of the recurrent parent with different resistance genes respectively were bred. Then, according to the changes of pathogenic physiological form (strains) and biotypes of harmful insects, different near isogenic lines were mixed in a certain ratio as cultivars. The objective of breeding multiline cultivar is to multiply and sustain disease (or insects) resistance in crop plants. However, among different resistance management strategies, a pyramiding line with two or more genes for disease resistance could be an effective mean to counter the biotypic variability and also to provide greater durability. Gould *et al*.[26,27] indicated through a computer simulation that under most conditions, a cultivar with two major genes for resistance would provide more years of plant protection than sequential release of two genes in separate cultivars, or a seed mixture of two cultivars. Especially, if economically important traits such as yield, quality and resistance are expectedly improved simultaneously, only effective pyramiding breeding method can realize the goal while multiline breeding one could not accomplish. On the basis of pyramiding breeding, we propose MBPB method, which begins by transferring the target genes followed by backcrossing to recover the recurrent parent genome, and then conducting pyramiding crosses to combine target traits. This method is very effective, particularly for stacking quantitatively inherited traits. Moreover, application of MAS can accelerate stacking different target genes.

3.2 To pyramid crop target agronomical QTLs with definite objectives

Molecular markers linked with target agronomical QTLs may essentially be divided into two categories, based on the difficulty of their manipulation. The first category is molecular markers closely linked with QTLs of major effect, which contribute additive effects and are little affected by the environment or genetic background. The MAS result for such QTLs is similar to that from MAS for simply inherited characters. The MAS for the QTL with major effects on fiber strength discussed earlier represents an easily manipulated QTL.

The second category is molecular markers linked to multiple QTLs affecting a given character, while displaying epistasis and interaction with background genotype and the environment. The presence of epistasis among QTLs and QTL × environment interaction reduces selection efficiency under different environments and backgrounds. Kandemir *et al*.[28] utilized a set of wheat populations from a "Steptoe"/"Morex" cross and identified three QTLs relevant to grain yield. They transferred QTL-3 and QTL-5L from Steptoe into Morex, while simultaneously introgressing QTL-2S from Morex into Steptoe, and bred near isogenic lines, respectively. Results indicated that the near isogenic lines with the high yielding QTL fragment transferred from Steptoe did not increase the yield of Morex, whereas these

isogenic lines acquired the characteristics of Steptoe such as reduced height, lodging, and head shattering. The yield of Steptoe isogenic lines, with the Morex QTL-2S region, was not altered. On the contrary, the presence of short photoperiod QTL on the chromosome 2S influenced the tillering synchrony, hence affecting yield. From this point of view, a successful MAS for such QTLs relies on the best combination of multiple loci, but not the accumulation of all the beneficial QTLs.

3.3 Selection for target gene and genetic background in MBPB with MAS

The key points of the successful application of MAS in backcrossing breeding are whether the population size for selection or the number of backcross generations can be reduced, reducing linkage drag and hastening the recovery of the recurrent parent. Hence, the distance between the marker and the QTL is the key element avoiding the linkage drag, while the drawing of crop linkage group is the premise for the execution of background selection. Frisch[29] examined corn (*Zea mays* L.) linkage map composed of 80 markers as a reference point to simulate transferring a single gene by backcrossing. He found that backcrosses might be reduced to 2 – 4 generations by means of MAS when a single gene is transferred, in accordance with the results of Tanksley *et al*.[30] and Hospital *et al*.[31] With MAS, the recovery of the recurrent parent genome as early as the BC_3 generation may be attained through MAS while four additional backcrosses would be done through conventional backcrossing. To study the potential of MAS in selection for a target gene and the genetic background of the recurrent parent, Hospital *et al*.[32] simulated backcross breeding with MAS by a computer program. The results indicated that to select for a target gene, at least three markers should be used for each QTL. It is best for them to flank markers most preferably on each side of the target gene. If there are markers with given locations, in a population with several hundred individuals, it may be effective to manage as many as four unlinked QTLs. Similarly, recovery of the recurrent parent genome by MAS can be very effective. To improve the efficiency of background selection, the number of markers to be used in background selection and the decision of the genetic distance between different markers are the factors that must be taken into consideration. This also applies to the saturation level (marker density is less than 10 cM, 1 cM is the best) in constructing the genetic map of the cross to be studied.

Due to the fact that a saturated (average marker spacing of 1 cM) genetic map had been constructed in tomato, Tanksley *et al*.[33] proposed combining introgression from exotic germplasm with cultivar development by use of MAS backcrossing. On the basis of genotypic selection in the backcrossing progeny, not only can the target trait molecular marker be acquired, beneficial genes can also further be transferred into elite crop cultivars by utilizing MAS, to speed genetic improvement. Simultaneously, several sets of QTL-NIL (near isogenic lines) and breeding strains can be acquired, to conduct map-based cloning studies toward the target QTL subsequently. However, dense maps remain unavailable for many crops such as cotton, wheat, based on low mapping density or not utilizing markers in breeding. Therefore, it's more important in genetic and breeding significance to select accurately parents with target traits and accommodation with breeding targets, further to screen molecular markers tightly linked with these traits, and apply them in MAS lastly, to gain breeding superior materials in the segregating progenies.

Based on limited polymorphism in upland cotton for all marker types developed to date and limited application of markers to cotton improvement, good MAS breeding strategy is important so that limited markers tightly linked with QTLs of agronomic traits are successfully used to breeding improvement. In this study, the target characters-insect resistance and elite fiber qualities not present in the recurrent parent Simian 3 pyramided by us were controlled by major effect QTLs or *Bt* gene with qualitative inherited character. Therefore, by use of MBPB, a new cultivar in the genetic background of Simian 3 with insect resistance and excellent fiber quality traits was created. However, phenotypic selection is certainly with experience at any rate. Therefore, MAS has big potential in cotton breeding, even if there lays a limitation that markers exhibit little polymorphism in cotton. Based on this, MAS for new target traits must be

based on such conditions as: one of the parents should be the elite commercial cultivar; while target trait molecular marker is acquired, this molecular marker should be utilized together with other molecular markers polymorphic between parental genetic backgrounds. Molecular marker-assisted selection should be conducted simultaneously on target traits and genetic background so as to acquire target new cultivar. This platform of molecular breeding is being further improved through our explorative breeding efforts in cotton.

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