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Molecular Marker Technology for Cotton Plant Improvement

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Cotton belongs to the genus *Gossypium* which contains about 50 species of which 44 are diploid species (2n=2x=26) and six are allotetraploid (2n=2x=52). The diploid species comprise genomic groups A, B, C, D, E, F, G and K and allotetraploid species are made up of two sub-genomic groups having an affinity with A and D genomes (Stewart, 1995). The cultivated cottons include *G. arboreum* L and *G. herbaceum* L, both diploid species with an A genome native to Southern Asia and Africa, and two allotetraploid species, *G. barbadense* L and *G. hirsutum* L, with an AD genome from Central, North and South America (Endrizzi et al., 1985).

During the past decade, the prospect for genetic engineering of cotton has soared with recent advances in the field of biotechnology. In 1996, the first two transgenic cotton cultivars, engineered for insect and herbicide resistance, were released for commercial production. The production of transgenic cotton exhibiting increased yields, enhanced fiber quality, and/or novel fiber properties holds significant potential for widespread impact on the global economy. Although small gains in yield and fiber quality continue to be made by conventional breeding programs, genetic improvement of agronomic traits is beginning to plateau as a result of an increasingly narrow germplasm

base for selection. To counteract this trend, the use of genetic engineering of cotton will become increasingly common as a means of bolstering breeding efforts.

Presently, plant breeders select desirable plants by looking at the phenotype. Most of the economically important plant traits are polygenic with complex non-allelic and environmental interactions. The biometrical genetic analysis has also been used for about half a century to gain an understanding of the genetic architecture of quantitative traits, which has helped to guide genetic improvement programs. Genetic variation between plants for a trait controlled by a single or a few genes inherited in a single, Mendelian fashion, is easy to manipulate in a breeding program. The biometrical genetic analysis determines the cumulative effects of all the genetic loci involved in a quantitative trait, but it is unable to identify the specific locus involved. If quantitative traits could be resolved into individual genetic components by finding DNA markers closely linked to each trait, it might be possible to manipulate them with efficiency for single gene traits. This would help the advancement of breeding material through consistent progress. DNA markers technology has provided plant breeders with a tool to select desirable plants directly on the basis of genotype instead of phenotype.

Conventional breeding of the cotton plant generally aims to improve agronomically relevant, or otherwise interesting traits, by combining characters present in different parental lines of cultivated species or their wild relatives. This has been achieved by generating F_2 populations and screening phenotypes of pooled or individual plants for the presence of desirable traits. Then, a time consuming and costly process of repeated back crosses, selfing and testing begins. All this depends upon accurate screening methods and the availability of lines with clear-cut phenotypic characters. Therefore, the combination of complex characters encoded by multiple genes with additive effects (quantitative trait loci), recessive genes, or accumulation (pyramiding of genes encoding the same trait) is difficult to achieve with classical methods. Molecular markers, however, facilitate all these processes, and can accelerate the generation of new varieties and allow connection of phenotypic characters with the genomic loci responsible for them. Both of these properties make molecular markers indispensable for cotton plant improvement.

Morphological and physiological features of plants have been used by plant scientists to understand genetic diversity. Some genes named by their physical appearance irrespective of their DNA nature and location on chromosomes were identified. Scorable morphological characters are very few in the cotton plant compared to biologically active genes. In closely related cotton plant varieties and species, there are very few morphological differences, which as a matter of fact do not represent the true genetic differences at the DNA level. Moreover, in most cases a plant genome has large amounts of repetitive DNA, which is not expressed and does not contribute to the physiological and morphological appearance of the plant. Therefore, there is a need to study polymorphism at the DNA level, which can be indicative of genetic diversity in cotton.

The number of polymorphic morphological markers is limited in the cotton plant, especially in intra-specific crosses, and their expression is influenced by the environment. Therefore, more reliable markers such as protein or, more specifically, allelic variants of several enzymes so called isozymes, and other biochemical characters such as lipid or sugars must be considered. Isozyme numbers are limited and their expression is often restricted to a specific developmental stage of tissues; and their presence can be determined by electrophoresis and specific staining. Unlike isozymes, the number of DNA markers is unlimited, their expression is not necessary for their detection, and all markers can be detected with a single technique. Polymorphism has been detected in restricted genomic DNA of plants, which paved the way for the development of molecular markers for cotton plant breeding. Today, various new marker techniques and breeding strategies, tailored for the inclusion of DNA markers, have been designed. The result of these efforts is an ever-increasing number of molecular markers of agronomically important traits, available for all crops. In the most advanced projects, this has already led to map-based cloning of the responsible genes.

Techniques

The techniques for molecular markers include restriction fragment length polymorphism (RFLP) and polymerase chain reaction- (PCR) based random amplified polymorphic DNA (RAPD); amplified fragment length polymorphism (AFLP); and mini and micro-satellites. Each is described below.

Restriction Fragment Length Polymorphism (RFLP)

Restriction endonucleases cut genomic DNA at specific palindrome recognition sequences, generating thousands of fragments of defined length, the number of which depends on the number of recognition sequences in a given genome. If a recognition sequence is present at a distinct genome location in one individual but not in the other, the enzyme generates different sized restriction fragments of this locus. This length polymorphism is detected by a radioactively labeled complementary DNA probe derived from the same locus. Such RFLPs were the first DNA markers applied to genome mapping. In principle, RFLP probes are single copy markers that detect only one defined genomic fragment each, although multilocus probes such as repetitive DNA or cDNA, that detect several fragments at a time, are also used. RFLPs are co-dominant markers, and their coupling phase can be determined experimentally, because DNA fragments from all homologous chromosomes are detected. RFLPs are therefore very reliable markers in linkage analysis and breeding, particularly if a linked trait is present in a hetro- or homo-zygous state in an individual. This information is highly desirable for recessive traits. The suitability of RFLP for the elucidation of QTL has been documented by sev-

eral researchers. Despite their usefulness, the generation of RFLP markers and their application is time-consuming and expensive. First, only one out of several markers provides polymorphism. This problem is serious, especially in a cross between closely related cultivated breeding lines. Second, for every polymorphism locus tested in a cross, a single experiment has to be performed and this is a formidable task with saturated maps such as those of tomato or maize, with hundreds of markers. As the method also requires a large amount of DNA, the material from a F_2 generation is soon exhausted and this limits the number of markers that can be tested. To overcome these problems, the application of stable mapping populations has been suggested. RFLP markers can be used for the mapping of QTL, genes involved for resistance to diseases and the relationship between the components of tetraploid genome of *Gossypium hirsutum* and its ancestors.

Random Amplified Polymorphic DNA (RAPD)

PCR-based RAPD is much faster and cheaper than RFLP analysis and is used only in minute amounts of DNA. Instead of primers complementary to known sequences, as in normal PCR, randomly generated synthetic oligonucleotides of 9-12 bases are used as starting points for thermostable DNA polymerases (Welsh and McClelland, 1990: Williams et. al. 1990). This approach produces 1-10 fragments from a single primer PCR reaction, which are highly polymorphic and can be easily detected on ethedium bromide stained agrose gels. This approach has several modifications. For example, the use of shorter oligonucleotides, typically 5-15 nucleotides in combination with PAGE and highly sensitive silver staining, results in many more visible bands in DNA amplification fingerprinting (DAF). The high sensitivity of the method applied to pre-digested DNA permits the tagging of specific locus for some characters in nearly isogenic lines of cotton. RAPD markers can be used for map-based cloning of disease resistance genes in cotton. The random amplification process introduces some difficulties in reproducing RAPD patterns in different laboratories and in different thermocyclers. Recent reports claim that these problems arise from impurities and thus may be solved.

Minisatellites, Microsatellites and Dispersed Repetitive DNA

Although some RFLP markers, as well as many RAPD markers, recognize more than one locus in a given genome, the number of loci recognized is limited, as is their polymorphic information content. Markers derived from small, tandemly arranged repetitive elements offer a way around this limitation. Such markers are called micro- or mini-satellites or simple tandem repeats (STR), because their sequence organization resembles the tandem arrangement of classical satellite DNA. These sequences are arranged in the eukaryotic genome in many copies of varying repeat-unit numbers. Due to their varying repeat numbers at a given locus, the elements frequently change their length by slipped-strand mispairing and other, less understood, processes. The surrounding single copy sequences are normally not affected, and therefore provide a valuable source of polymorphism for many purposes, including linkage analysis (Nakamura et al., 1987), identification of species and cultivars and marker assisted breeding (Beckmann and Soller, 1990)

Minisatellites (repeat units of 9-20 nucleotides) can be hybridized to restricted and electrophoretically separated DNA blotted on to nylon membranes (Jeffreys et al., 1985). Microsatellites (repeat units of 1-5 nucleotides) can be hybridized to DNA in dried gels (Ali et al., 1986) or microsatellites can be cloned, sequenced, and amplification fragment length polymorphism detected by PCR, using oligonucleotides from the surrounding monmorphic DNA as primers. These sequenced tagged microsatellite sites (STMS; Beckman and Soller, 1990), like RFLP are co-dominant markers and therefore highly informative, which justifies the large amount of work needed for their generation. Microsatellites are present in different forms in plant genomes.

Amplified Fragment Length Polymorphism (AFLP)

This technique is based on the detection of genomic restriction fragments by PCR amplification. DNA is cut with restriction enzymes and double stranded adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. The sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments. Selective nucleotides are included at the 3' ends of the PCR primers, which therefore can only prime DNA synthesis from a subset of the restriction sites. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified.

AFLP produces a higher number of polymorphisms than RAPD or RFLP markers. It is a robust and highly reproductive technique.

Applications

Genetic Diversity

DNA polymorphisms are exploited in cotton plants by an ever-increasing number of molecular marker techniques for differentiation between individuals, accessions and species of plants, pathogens and pests. Their high resolution compared with all other markers makes them a valuable tool for varietal and parental identification for the protection of cotton breeders' rights. DNA markers further add to the repertoire of tools for the determination of the evolutionary relationship between *Gossypium* species and families. Molecular markers also allow an understanding of the relationship between chromosomes of the related *Gossypium* species. Cotton plant pathogens and pests also display genetic diversity, therefore, their distribution, variation and gene flow can be measured in time and space. Together with aggressivity tests, the study of genetic diversity provided the basis for the detection of annual changes in pathogen distri-

bution that can be used in forecasting and preventing future epidemics.

Marker-assisted Breeding

The use of molecular markers enables cotton breeders to connect the gene action underlying a specific phenotype with the distinct regions of the genome in which the gene resides, e.g., the phenotypic expression of fiber quality is confined to domesticated species. The genetic advances in fiber quality can be made indicative of the existence of genes that contribute to fiber quality in germplasm that does not express the phenotype. Molecular markers could provide the opportunity to use precision in identifying the phenotype of these traits. Molecular markers will allow direct selection for genotypes, thereby providing a more efficient means of selection for fiber properties. The genetic manipulation of cotton fiber properties using molecular strategies relies on the identification and isolation of genes that control fiber development and/or directly affect a particular structural property of fibers. Molecular markers provide an opportunity to identify and isolate the genes relating to fiber characters by map-based cloning. Once markers for an interesting trait are established, these should allow prediction of fiber characters, yield or resistance of individual offspring derived from a cross, solely by the distribution pattern of markers in the offspring genome. Besides the exploitation of genomic polymorphisms for germplasm utilization and protection of varieties, cotton breeders' interest in molecular markers currently focuses on three major issues.

- The acceleration of the introgression of single resistant genes for plant pathogens from wild species or cultivated donor lines into otherwise superior cultivars.
- The accumulation (pyramiding) of major and/or minor resistance genes into cultivars for generating multiple and more durable (horizontal) resistances against several pathotypes of the same pathogen.
- The improvement of the agronomic value of cotton by breeding for quantitatively inherited traits, such as yield, drought and cold tolerance.

Breeding for Resistance

In cotton, the main advantage of using molecular markers is introgression of resistance genes into the cultivars to save time. The use of DNA markers could speed this process by three plant generations, by allowing selection of resistant offspring that contain the lowest amount of the donor genome in every generation (Tanksley et al. 1989). Single gene resistances (vertical resistance) are often rapidly broken by pathogens, thus converting the previously resistant cultivar into a susceptible one. Breeders therefore aim to accumulate several minor and major resistance genes into one cultivar to achieve more durable resistances. With conventional breeding, it is nearly impossible to combine so many different genes. The action of different resistance genes cannot be distinguished even with a set of different pathotypes. However, many resistance loci from different sources can be determined, and their presence detected, by linked markers in parallel crosses.

Quantitative Trait Loci Analysis

Many agronomically interesting traits, such as yield, are controlled by polygenes, with every gene contributing only a small percent to the expression of the trait. Tagging of polygenes with molecular markers requires a saturated linkage map with a marker spacing of no more than 20 cM and at least 250 F_2 individuals from a cross between parental lines that differ markedly with respect to the trait in question (Tanksly, 1993). First the offspring is tested for the trait and its genotype determined for every marker locus. Then the likelihood that the observed data rely on the presence of QTL is calculated, against the likelihood that no QTL is present, using specially designed computer software such as MAPMAKER.

Population Development for DNA Markers

Population development is an important step in DNA marker studies. The appropriate design of a test population is a crucial step in the development of markers for agronomic traits. Several strategies for such a design, including different segregating populations and sampling regimes, have recently been developed. The following types of populations can be used for DNA marker studies.

Interspecific Crosses

In cotton, crosses of wild species with cultivated lines have generally been found useful for the generation of genetic maps, because of the relatively high degree of morphological isozyme and DNA polymorphisms in wild species. Such crosses helped in the generation of linkage maps and the identification of resistance loci. Once a map has been established and linkage markers found, commercially important accessions may also be included for linkage analysis using these markers.

F_2/F_3 Segregating Population

F_2 or F_3 populations can be used for DNA markers. While making crosses, the parents should be screened carefully. These parents should be widely different for the desirable trait. The segregating population should be phenotypically screened and the population to be screened should be large, normally about a population of 500 plants, depending upon the expected number of loci involved for the trait. It is more useful if after the screening of F_2 plants, the families of selected F_2 plants are raised and final selection of plants for DNA marker studies is based on the F_3 population. The progeny of the F_2 plants showing segregation should not be included. About 25 plants are finally selected for each contrasting trait. The presence of polymorphic DNA molecules present uniformly in one group of plants and absent in the other contrasting group of plants show its linkage with the trait of interest. A large F_2 population is most informative for genome mapping, especially if the map is at an early stage and only a few markers are mapped. However,

the F_2 selection has three major drawbacks for the development of markers for the agronomically interesting traits concerned.

- The same individual tested for the trait also has to be used for linkage analysis, e. g., some of the plants are too affected with pathogens and pests to provide enough DNA for linkage analysis.
- After completion of their life cycle, plants die and will no longer be available for backcrosses or further genetic analysis (especially the pheno- and geno-typically characterized individuals).
- Most multilocus markers, including RAPD and mini and micro satellites are dominant markers, whose homo- or hetrozygous state cannot be determined. The F_2 generation does not allow these two possibilities to be distinguished and much information is therefore lost.

Recombinant Lines

Advanced lines developed from the cross of two plants can also be used for DNA markers. A population of at least 500 recombinant lines is screened, and about 20 lines from each of the contrasting pairs are selected for DNA analysis. When using F_2/F_3 or recombinant lines, after DNA extraction of individual plants, bulked segregant analysis (Michellmore et al., 1991) can also be used instead of using DNA of individual plants in RAPD, RFLP, or AFLP, etc., analysis.

Isogenic Lines

Isogenic lines developed for a particular trait, using the recurrent back cross procedure, can also be used for DNA markers studies. The use of isogenic lines reduces the number of RAPD/RFLP/AFLP, etc., reaction as only two lines contrasting for the trait are used. However, it is a time-consuming and tedious procedure to develop isogenic lines. For more advanced studies with very tight linkage between markers and traits, which is a prerequisite for pyramiding of resistance genes or map-based cloning of genes, nearly isogenic lines (NILs), derived from several rounds of backcrossing of the recipient of an agronomically interesting gene with the recipient genotype, are used with high efficiency in several species. NIL contains normally only small amounts of donor DNA surrounding the gene of interest. To avoid the time consuming generation of NIL, specific genes can be tagged by bulking DNA from individual F_2 plants with identical performance in phenotypic (resistance) tests (bulked segregant analysis; Michelmore et. al. 1991). Individual DNA from existing mapping populations can also be grouped according to the distribution of RFLP markers and then RAPD analysis can be used to tag the region surrounding the gene(s).

DNA markers thus offer two advantages:

- Faster recovery of the recurrent genome.
- More efficient selection of genomes that have recombination events close to the target gene.

Marker- assisted selection is still limited by three main factors:

- The number of samples that can be analyzed.
- The number of lines that can be improved within a given time.
- The belief that QTL identification is required whenever additional germplasm is used.

PCR based markers have also opened new doors for genome manipulation, since their use allow:

- Earlier sampling, because of the small amount of tissue required.
- Faster DNA preparation, because of the small amount of template DNA required.
- More efficient handling of large sample sizes, because of the efficiency of PCR technology.

Now is the time to consider the development of new breeding strategies that take into account genetic characteristics, such as the complexity of the genome; the nature and the number of the molecular markers available; the complexity of the traits to be improved; the number of plants that can be screened at each selection step; and the number of populations that can be concurrently manipulated. It is also crucial to explore the complementarity between marker-assisted selection and conventional breeding, and to develop overall strategies that tightly and interactively integrate the two approaches.

Marker-assisted selection for polygenic trait improvement is an important transition phase, and the field is on the verge of producing convincing results. Recent efforts in comparative genetic analysis allow identification across different plant species of gene sequences involved in the expression of target traits. The superior alleles identified among genomes in those target genes can be used as DNA markers to develop efficient screening techniques. Finally, technological developments, including automation, allele-specific diagnostics and DNA chips, will make marker-assisted selection approaches based on large scale screening much more powerful and effective.

Map-based Cloning

The detection and cloning of distinct genes in cotton of unknown sequence and function, when only their involvement in specific traits and their chromosomal location is known, has been termed reverse genetics. In contrast, the conventional approaches, where a gene is cloned on the basis of a known product or sequence and then localized to a chromosomal region starts with the localization of a gene on a specific chromosomal region by determining the linkage of the phenotype it specifies to a set of flanking molecular markers. These linked markers are then used as starting points for physically mapping the gene-flanking region with pulsed field gel electrophoresis and rare-cutting restriction enzymes. Large-scale restriction-site mapping is necessary because physical and genetic distances between markers may vary over several orders of magnitude.

Physical maps are especially useful in polyploid crops such as cotton where duplicated sequences could prevent the assignment of markers to a single distinct location.

The construction and screening of yeast artificial chromosome (YAC) libraries is a next step towards isolation of desired genes. Clones containing at least one of the markers are selected. End fragments of these YAC are then used to construct contigs of the genomic region defined by the markers, to identify cDNA derived from that location. The cDNA are sequenced and candidate sequences retransformed into suitable hetrologus and homologues hosts for proving the gene identity by complementation in transgenic plants, using different transformation techniques.

An absolute prerequisite for map-based cloning of genes in cotton is the availability of tightly linked markers flanking the locus of interest. These may be found by chance but are normally the product of a systematic effort to saturate a genome with polymorphic DNA markers.

Due to the economic dependence of many countries on cotton production, it is necessary to focus on reducing the cost of production, increasing yield and quality, and diversifying the product spectrum of fiber. Another objective for cotton research is to enable farming practices, as well as processing of fiber, to become more environmentally friendly. Molecular markers technology has made it possible to identify genotypes carrying desired characters efficiently and correctly. This may help in the selection of genotypes and boost conventional breeding programs.

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~~Fiber Strength~~

~~Cotton is grown for lint and unlike other commodities it is traded based on quality parameters rather than just weight. Fiber quality affects the end products made of cotton. If fiber quality is low, spinning performance and efficiency will be affected negatively. Weak fibers give rise to a number of problems in today's high speed spinning industry, and to yarn breakage during processing. Weak fibers contribute to weak yarn and consequently to low quality fabric. Strong fibers can be spun at higher speeds thus improving the economics of yarn formation. Therefore, stronger fibers are needed to offset the resistance losses of yarn obtained by the new high speed spinning processes.~~

~~Fiber strength started to be routinely measured only when the Pressley strength tester was developed about 60 years ago. When the Pressley tester became popular, most varieties did not measure stronger than 75,000 pounds per square inch (PSI). At present, most varieties in the world measure over 90,000 PSI.~~

~~Strength is not the only criteria that determine the performance of a given cotton. Other important characteristics include fiber length, micronaire and length uniformity. Based on changes in the spinning industry toward high speed machinery, requirements for raw mate-~~