New Trends in Cotton Biotechnology for Production Improvement

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Abstract

Genetic Engineering directed toward developing better cotton varieties is the most productive area in the market of transgenic plant production. It is not done to develop insect and herbicide resistance only, but also to improve yield and fiber quality and other adverse growing conditions. Biotechnology of cotton through genetic engineering has opened a new era of directed cotton breeding. If a specific desirable gene is identified in cotton or in other crops or organisms, genetic engineering provides the tool to isolate and introduce that gene(s) into cotton. In 1998/99, 2.63 million hectares or 8% of world cotton area was planted as transgenic varieties in Argentina, Australia, China (Mainland), Mexico, South Africa, and the USA. Many other countries are evaluating the performance of such cottons and some are even ready to plant them on a commercial scale. Genes resistant to a wide range of grasses and weeds and also the Bt gene, which provides resistance to lepidopteran insects in cottons, are utilized now but more can be identified and used for improvement of specific characters in cotton. This is a process towards development of genotypes of our own choice, which could have a feasible marketability for unique cotton traits. It is not only the insertion of desirable gene(s) but also the deletion of undesirable gene(s), a step toward pick and select genes for designing genotypes.

Molecular techniques including tissue culture, embryo rescue and marker assisted selection could be useful tools to increase the efficiency of conventional cotton breeding. In addition, they include transformation for the production of genetically modified cultivars via molecular genetic engineering. The benefits of recombinant DNA technologies to cotton breeding are well known because they have facilitated the transfer of useful genes from the wild relatives to cultivated cotton. This report is mainly concentrated on the benefits of using transformation or genetically modified cotton at the molecular level. Theoretically, one could support that genetically modified cotton could control effectively the pests and weeds and therefore reduce the chemicals used today in areas cultivated with cotton. In addition, an increase in yield could be accomplished given that annual losses due to poor conventional weed and pest control will be reduced. Genetically modified cotton varieties have been adopted rapidly since their introduction in the mid-1990s. By 1999, 60% of the total U.S. cotton acreage was planted to insect and herbicide resistant varieties: 32% Bt, 45% herbicide resistant and 16% "stacked" varieties with both insect and herbicide resistance. Understanding the reasons why growers are adopting these varieties is critical in an evaluation of the impact that the introduction of these technologies has had on agriculture. Here these technologies are viewed to better understand issues that have influenced grower decisions.

Confidence in Biotechnology

Genetically enhanced crop varieties grown worldwide have encountered problems in foreign markets as a result of both regulatory hurdles and lack of consumer acceptance. The key to addressing these problems is developing confidence in the safety of the technology and the effectiveness of the regulatory systems. Opposition to biotechnology forced a *de facto* moratorium on approval of new genetically enhanced crops. The public concern has spread to different countries, including the United States by some accounts, though nowhere has the reaction been as virulent as in Europe.

A Regulatory Problem and a Consumer Problem

When the first commercial crops of genetically enhanced cotton, corn, and soybeans were planted in 1996, the European Union (EU), Japan, and a handful of other countries required advance approval of the new varieties before they were permitted to be imported for planting for food or feed purposes. Today, roughly one fourth of the countries in the world have some type of regulation in place for agricultural products derived from biotechnology. Most regulatory regimes are concerned with either the release of genetically modified organisms (GMO) into the environment (e.g., seeds for planting) or the safety of an agricultural product derived from biotechnology which is to be used for food or feed. The list of countries requiring advance approvals for new varieties expands every year.

Transgenic Cotton Worldwide

Monsanto's Bollgard cotton is grown in Argentina, Australia, Mexico and China. In China alone, Bt cotton plantings are estimated to have been 81,000 ha in 1998. Bollgard cotton plantings are estimated at 101,000 ha in Australia and 25,000 ha in Mexico in 1999. South Africa's first commercially grown, genetically modified cotton and

corn crops were harvested this year. In 1996 and 1997, a cotton strain and two corn varieties were the first genetically engineered crops to be approved for commercial production by the country's official regulatory body, the South African Committee on Genetic Engineering. Many countries are in the process of establishing firm regulatory biosafety guidelines to adapt commercialization of genetically modified products. In Egypt, a National Biosafety Committee was established in 1993 to formulate the regulatory process and facilitate the introduction of GMO to Egypt. Farmers are convinced that returns from biotechnology far exceed their cost, and have moved quickly to plant these improved seeds where they were available (soybeans, cotton and corn and a few vegetable crops).

In 1996, biotech crop area in the United States totaled just over 5 million hectares. By 1998, biotech area had grown nearly five fold to just under 30 million ha, 23% of all significant field crop area (including wheat, oats and tobacco, where biotech seed is not yet available). In 1999, biotech acreage has expanded still further, to over 36 million ha, 29% of total field crop acreage. Still, biotech plantings are dominated by relatively few crops – soybeans, corn and cotton, and adoption rates are substantially higher for those individual crops. In 1999, for example, 18 million ha were planted to biotech soybeans, a sustained increase over the 15 million ha planted in 1998, and a dramatic increase over levels seen in 1996 and 1997 (USDA 2,000). The data from 1996-99 indicated that the adoption of biotech varieties that reached 60% of total area in cotton in the United States. Varieties used were mainly Monsanto's Bollgard and Roundup Ready technologies. Transgenic procedures to develop new traits are coming to dominate genetic practices in the United States, and accounted for more than 70% of the total biotech area in the United States in 1998 (USDA, 2000).

Herbicide Tolerant Cotton

Herbicide tolerant crops were planted on 25 million ha in 1999, nearly double the combined area of insect-resistant and value enhanced crops. All herbicide-tolerant cotton varieties are transgenic. Roundup Ready cotton area almost reached 1 million ha in 1998 and increased to 1.2 million ha in 1999, about 20% of the U.S. crop. BXN cotton, sold by Monsanto's Stoneville subsidiary (in the process of being sold to the investment firm Hicks Muse), was planted on 0.35 million ha in 1998 and 0.4 million ha in 1999 (Penn, 2000).

Development of Insect Resistant Cotton

The biotechnology can help in the management of resistance in transgenic cotton, as the transformed varieties possess inherent ability to withstand lepidopteran pests. However, due to continuous exposure of the lepidopteran insect pests to Bt toxins and the experience gained with synthetic chemical insecticides, resistant crop varieties developed through conventional breeding suggest that insects will adapt themselves to transgenic plants within a short span (Fischhoff, 1996, Federici 1998, Shen et al. 1998). The risk of development of resistance by transgenic cultivation is probably greater than that for Bt formulations, due to continuous and extensive expression of Delta-endotoxin in plant tissues. The physiological mechanism of development of insect resistance to Bt Delta endotoxin includes a change in gut pH or in enzymes that would affect dissolution and activation of the proteinaceous crystal. The cross-resistance among toxins occurs in some insect species. The selection of a population of *H. virescens* for resistance to one type of toxin of cry IA(c) can lead to resistance to a broad range of unrelated toxins. These insects had developed resistance not only to cry IA(c) and closely related cryIA (a) and cryIA(b) toxins, but also to the more distant toxins cry IIA, cry IB and cry IC. Recently it has been reported that *Helicoverpa armigera* has developed resistance to Bt in the Yauggu and Xiuxiang provinces of China (Shen et al. 1998). Due to the development of resistance to Bt toxin, the average mortality of newly hatched larvae of *H. armigera* declined significantly (16-29%) as compared to the susceptible strain. It is difficult to predict the mechanisms by which insect develop resistance to various toxins. But in cases where resistance is attributed to modification in the binding sites, resistance seems to be inherited as a major recessive or partially recessive gene. In this case the level of resistance is high and cross-resistance is limited and involve toxins that share the same binding site. On the other hand, when it is due to other unknown modifications, resistance seems to be inherited in an additive way and the level of resistance is moderate. New strategies are being developed to maximize the durability and utility of transgenic cotton.

Bacillus thuringiensis (Bt) Gene

The *Bacillus thuringiensis* is a soil bacterium which produces parasporal crystals during spore formation (Koziel et al. 1993, 1997, and Mazier, 1997). These crystals consist of one or more proteins called Delta–endotoxins or insecticidal crystal proteins (cry). The Delta–endotoxin or Bt toxin is insect specific and is being used since 1930 as insecticidal spray (Aronson, et al., 1986) to control specific insects in biocontrol systems, due to its environmental safety and safety towards non-target insects. The endotoxin affects the membrane of gut epithelium of susceptible insect larvae and binds to the glycoproteins which results in lyses of membrane as a result of which insects stop feeding, get dehydrated and ultimately die.

Various insecticidal crystal proteins have been classified depending upon their nucleotide sequence homology and

their molecular weight (Hofte & Whiteley, 1989). The genes responsible for the synthesis of insecticidal crystal proteins are also called cry genes and are host specific. Cry I is lepidoptera specific, cryII genes are lepidoptera and diptera specific, CryIII genes are coleoptera specific and Cry IV genes are diptera specific. Within each major class, delta-endotoxins are grouped according to sequence homology. The CryI protein is further classified in to six groups i.e. CryIA (a), CryIA (b), CryIA(c), CryIB, CryIC and CryID. The CryIA(c) gene is active against all the lepidopteran pests of cotton. (Crickmore et al. 1995, Mazier et al, 1997).

The development of Bt cotton expressing delta-endotoxin proteins of *B.thuringiensis* is a spectacular example of providing an environmentally benign alternative to chemical insecticides reducing thereby the input costs and ecological hazards. Various vector mediated and vectorless transformation methods have been exploited. The work on the applications of genetic engineering technology in the improvement of cotton cultivars has been recently reviewed by Wu et al., (1998). The Bt transgenic plants represent a novel means to control insect pests like cotton bollworms especially Helicoverpa and pink bollworm (Cousins et al. 1991, Bendict et al. 1993). The Bt toxin proteins are not harmful to beneficial insects due to their narrow host range and particular mode of delivery of toxin in transgenic plants and are effective in controlling insect pests that feed on plant parts and are difficult to control by conventional methods.

Low expression of Bt genes frequently observed in transgenic plants, has been attributed to mRNA instability, defective translation of mRNA, or hindrance of gene expression of transcriptional level (Perlak et al., 1993). The Delta-endotoxin coding sequence showed a number of motifs rarely present in plant genes and the overall G/C content of cry genes is much lower than the plant genes and there are numerous A/T rich stretches (Shaw et al. 1986). Motifs and numerous potential polyadenylation sites such as AATAAAT are responsible for the low toxin expression of Bt genes.

Methods for Improvement of Toxin Gene Expression

The toxin gene expression of Bt crystal proteins can be increased by any one of the following methods:

1. Modification of coding sequence: Improvements in the insecticide crystal protein have been accomplished by increasing the G/C content of their encoding genes and/or by using plant preferred codons. High G/C content does not necessarily result in an increase of gene expression unless improper splice sites and improper polyadenylation signals are removed during the re-synthesis of gene (Perlak et al. 1990).

2. Point Mutations: Point mutations in cry IA (c) and cry I (c) genes have increased their gene expression. The target area for point mutations includes regions with major AATAAA or AATAAAT or AACCAA sites (Vander et al.; 1994).

3. Synthetic Bt toxin genes: The improvements in gene expression due to the use of synthetic genes has made it possible to obtain numerous transgenic plants showing a high level of resistance The extensive changes and optimization of the coding sequence in synthetic genes have enhanced the level of toxin expression. Zhang et al.,(1998) transferred a synthetic Bt gene (GFM CryIA) into Chinese cotton varieties and recovered 29 transgenic plants. The high mortality (more than 80%) of *Helicoverpa armigera* larvae indicated that products of fully modified Bt gene had higher toxicity than original Bt protein. The larva death rate was more than 80% in variety Simia-3 (Ni et al., 1998).

Role in Alleviating Insecticide Resistance

The various biotechnological strategies involved in insect control and insecticide resistance management include accurate and quick identification of insect pests, identification of resistant genes and development of insect resistant transgenic plants. Accurate identification of insect pests is critical to insect pest control. If the visual identification is difficult or if the insect is very small, polymorphic or its symptoms are not easily interpreted, then biotechnological tools like RFLP, RAPD, SSR, Microsatellites and AFLPs can be used for its molecular characterization. The nucleotide sequence sites can be used for identification of different insect biotypes on the basis of DNA fingerprinting.

Antigenic characterization of proteins has been exploited for molecular characterization and antisera raised against proteins purified from positively identified specimens has provided the basis of many diagnostic tests. Enzyme-linked immunosorbant assay (ELISA) based kits that can identify a wide range of viruses can be developed at low price. The specificity of monoclonal antibodies allows differentiation between insect species, which cannot be separated by morphological criteria, for instance *Helicoverpa armigera* and *H. panctigera*.

All insect-resistant crops sold commercially to date contain genetic material from the *Bacillus thuringiensis* (Bt). Cotton and corn are the main Bt crops and the varieties currently available are effective against the European corn borer and the cotton bollworm and budworm. Monsanto's Bollgard cotton was introduced in 1996 (Penn, 2000). Bt cotton varieties offered an alternative to conventional insecticide spray programs. Insecticides were used on 75% of the total cotton area before the introduction of Bt varieties. The adoption of Bt varieties was extremely rapid in

some areas in the US and has been slower in others. Overall, Bt cotton was planted on 13% of the U.S. cotton area in 1996 (William et al 1998). Adoption has steadily increased, to 17% in 1997, 21% in 1998 and 32% in 1999 (William et. al. 1998, USDA 2000). After a year of very high budworm populations and damage in 1995, growers in Alabama adopted the new technology at an extremely rapid rate, planting 77% of total acreage to Bt varieties in 1996. In 1999, 75% of cotton acreage in Alabama was in Bt varieties. Florida and Mississippi also adopted over 30% in 1996. By 1999, South Carolina was a major adopter, at 84% of total acreage, followed by Alabama, Louisiana, Mississippi and Florida, all of which planted over 60% of total acreage to Bt varieties. The benefits of genetically modified cotton varieties include yield increases, lower costs and ease of management. Pesticide use has also decreased. Table 1 summarizes the impacts of the introduction of Bt and herbicide resistant cotton varieties. Cotton production is estimated to increase by over 800,000 tons. Insecticide and herbicide use has decreased by over 2 million pounds, the number of pesticide applications has decreased by an estimated 4 million ha-treatments. An increase in revenues is estimated at $177.5 (Penn, 2000).

Stacked Traits

As efficacy of single traits shows, multiple benefits could also be offered in one type of seed. The first "stacked" trait variety that appeared in 1997 was Monsanto's Roundup Ready/Bollgard cotton, a variety now used more widely than standard Bollgard cotton. Stacked Roundup Ready/Bollgard cotton was used on almost one million ha in 1999 (Table 1). Monsanto also has sold a small amount of corn that combines Roundup herbicide tolerance and European corn borer resistance, but this product has been planted on a limited area.

Status of Bt Cotton

The transgenic cotton plants engineered for resistance to the lepidoptera group of insects are being commercially cultivated in the USA, Australia and Mexico and are at field testing stage in a number of other countries. During 1996, 0.7 million ha under Bt transgenic cotton was planted in the USA, 30,000 ha in Australia, 2,000 in Mexico, and in India transgenic cotton is at field testing stage. The era of transgenic cotton began when Perlak et al. 1990 introduced cry1A(b) and cry1A(c) genes into cotton plants and transformed plants showed a high level of resistance to Heliothis. In the beginning of 1996, Monsanto Company received authorization to launch a transgenic cotton known as Bollgard cotton. The transgenic cotton was tested in all the major cotton producing regions in the USA and plants were analyzed for insect control efficiency compared with traditional chemical insecticides. The transgenic cotton varieties have also passed the tests of the Environmental Protection Agency (EPA) of the USA. During the field and laboratory tests, it was demonstrated that transgenic cotton is highly effective against neonate larvae of *Heliothis virescense* (Tobacco budworm), *Helicoverpa zea* and *Pectinophora gossypiella*; the toxin gene delivers the Bt protein directly to the neonates immediately after their hatching when they are most susceptible. The Bt gene from the original mother plant can be transferred to the advanced cotton cultivars through backcrossing. It has facilitated the introduction of the Bt gene to cotton varieties that have good agronomic base and desirable fiber properties and are otherwise not responsive to regeneration in vitro. The control of insects in transgenic cotton was reported to be comparable with synthetic pyrethroid insecticides and their yield level was comparable to or higher than the same variety treated with traditional insecticides. Transgenic cotton requires less number of sprays and reduces the cost of cultivation. The in-planta delivery of crystal proteins does not have any undesirable effects on predators and parasites so transgenic plants can be effectively used in the IPM system (Fischhoff, 1996). Transgenic cotton has also been developed combining two Bts and the Bt and CpTI genes (Roush, et al. 1998, Zhao et al. 1997).

Other Insecticidal Proteins

Most of the transgenic plants are based on Bt Delta-endotoxins. The other insecticidal proteins of plant region, which interfere with the nutritional needs of insects, like polyphenol oxidases, proteinase inhibitors (Hilder et al, 1987), X-amylase inhibitors, deprive insects of nutrients by interfering with their digestive enzymes. Out of these, proteinase inhibitors are highly specific and act as insect growth retardants.

Table 1. Stacked Trait Crops

Company	Product	USDA Approval Date	Planted Area, Million hectares 1996 A	1997 A	1998 A	1999 E
Monsanto/ DEKALB	Roundup Ready & Bollgard cotton	N/A	N/C	<0.1	0.25	2
Monsanto/ DEKALB	BXN & Bollgard cotton	4/30/97	N/C	N/C	<0.1	0
Monsanto/ DEKALB	Roundup Ready & YieldGard corn	5/27/97	N/C	N/C	<0.1	N/A

Trypsin Inhibitor Genes

Proteinase inhibitors are highly effective to a particular class of digestive enzymes and act as growth retardants (Jongsma et

al, 1995). Trypsin inhibitase affect insects by reducing their capacity to assimilate plant proteins. Insects reduce feeding which leads to starvation but insects possessing proteinase inhibitors from transgenic switch their proteinase composition in their guts to overcome this effect (Jongsma et al. 1995). The cowpea trypsin inhibitor has been engineered into cotton and shown to have substantially reduced damage by lepidopterans (Zhao et al. 1997). Chitinases also have insecticidal properties and are presumed to target chitin structures such as peritrophic membranes which protect midgut cells in the insect gut lumen (Ding, 1995).

Lectin Genes

Lectins are carbohydrate-binding glycoproteins found in many plant species. Lectins possess a broad range of antimicrobial and insecticidal properties (Cavlieri, et al 1995). The insect toxicity of lectins relates to their ability to bind the midgut and impair the absorption of nutrients by insects, thereby inhibiting their growth. F_2 plants were developed from seed of individually regenerated plants of Coker 312 transformed with lectin genes which when fed to neonate larvae of *Heliothis virescence* inhibited larvae growth over controls (Rajgura at al., 1998).

Vegetative Insecticidal Proteins (Vips)

The clarified culture supernatant fluids collected during vegetative (log phase) growth of Bacillus are a rich source of insecticidal activities (Warren et al 1996, Estruch et al 1996). The genes Vip I and Vip 2 have insecticidal proteins associated with the binary system (Yu et al. 1997). The cloning and characterization of this supernatant fluid of certain *B. thuringiensis* cultures gave Vip 3A different proteins and is highly effective against lepidopterans. The toxic effect of Vip proteins is comparable with or is better than Delta-endotoxin. The molecular and biological properties of Vip proteins that are secreted and produced during vegetative stages make them distinct from the family of insecticidal proteins of delta endotoxins. Vip genes cause gut paralysis of insect, followed by complete lysis of gut epithelium cells, which resulst in larval death (Estruch et al.,1996).

Cholesterol Oxidases

The cholesterol oxidase (Co) protein is a member of acyl sterol oxidases which have been found to be highly effective against cotton boll weevil larvae; Co catalyzes the oxidation of cholesterol to produce ketosteroids and the hydrogen peroxide toxin, which affects the midgut epithelium (Greenplate et al., 1995).

Baculoviruses

Baculoviruses have significant potential for pest control because they don't affect predators and parasites, are safe to non-target insects, humans and the environment. Baculoviruses can be effective biocontrol agents to overcome resistance to insecticides (Bishop et al. 1988). Baculoviruses are complementary to Bt. Nuclear polyhedrosis virus (NPV) was the first baculovirus marketed in the USA to control the cotton bollworm Helicoverpa. The major limitation of baculoviruses is the slow rate of kill, that large-scale availability and distribution is limited, and that they are highly host specific. Biotechnology can help the development of recombinant baculoviruses that can kill a number of insects in 2-3 days. Insect specific genes into the baculoviruses genome can be inserted (Bonning and Hammock, 1992) using genetic transformation techniques, which can kill early and are more toxic (Corey, 1991).

Arthropods

Arthropods are natural enemies of a number of insect pests. But, until recently they have not been the subject of biotechnology. The new DNA based methods for monitoring genetic variation like PCR based RAPD markers can identify biotype of arthropod biological control agents. Biotechnology can also help in the development of cryobiological methods for preserving embryos of arthropod biological control agents. Biotechnology can also be used for genetic engineering of arthropods, i.e. development of transgenic arthropod biological control agents (Chambers, 1991).

New Trends in Cotton Biotechnology

Transgenic Cotton with Improved Fiber Qualities

Cool night temperatures tend to cause lower crop yield and production of immature fibers that have less value to the producer and the textile industry. These effects ultimately depend on hindered synthesis of cellulose when cotton is grown under cool nights. Analysis of the source (photosynthetic) and sink (fiber cellulose synthesis) metabolism both implicated one enzyme, sucrose phosphate synthase (SPS), as a likely candidate for beneficial change. Fiber quality attributes toward the premium range even when the plants are grown under a stressful cool night. One of three transgenic lines, carrying the spinach SPS gene tested, had higher seedcotton yield than either parental C312, a segregating transgenic line that was not expressing spinach SPS. Two of three transgenic lines had higher micronaire (4.6) than any of the other lines tested (averaging 4.0). These positive effects were observed in a relatively dry production field at the end of a hot summer. Over-expression of SPS helps stabilize or increase yield and fiber quality under various stress production conditions (Haigler et al., 2000).

Abiotic Tolerance Genes

Transgenic cotton tolerant to some abiotic stress factors has been developed (personal communication). Such factors represent harsh cultivated conditions such as heat, salt, and drought. Some single genes which are responsible for carbohydrate accumulation (mannitole dehydrogenase, levan sucrase, sucrose phosphate synthetase, etc), free amino acid accumulation (proline synthetase, proline reductase, etc.) and others have been tailored to be engineered in some cotton varieties to support their abiotic tolerance levels and help cultivation in more harsh conditions, some of these genes function to improve fiber quality.

Molecular Genome Mapping for Cotton Improvement

Modern cottons are grown as industrial raw materials for the textile and oil seed industries. Genetic improvement of the cotton plant has been a major activity to its continued productivity. Cotton has a gametic chromosome number of 26, more than any of the other major crops. While the application of DNA markers has offered a valuable tool for revealing the genetic basis of both simple and complex traits in crop plants, cotton genome mapping lags behind other major crops. A few research programs have been devoted to the molecular mapping of this large, complex genome. Molecular mapping and characterization of genes controlling fiber quality properties in both extra long staple (ELS) cotton (G. barbadense L.) and upland cotton (G. hirsutum L.) has been reported. QTLs for fiber quality properties were compared with those of ELS cotton, with regard to their location and gene effects. A total of thirteen QTLs have been identified, four for fiber strength, three for fiber length, and six for fiber fineness (Yu and Kohel, 1999). They are located on different chromosomes or linkage groups of molecular maps comprised of 355 DNA markers covering 4,766 cM of the cotton genome in 50 linkage groups. These QTLs explain 30% to 60% of phenotypic variance for each fiber quality property in the F_2 population. Both A and D sub-genomes contain fiber quality genes.

Most of them are recessive in genetic background, making marker-assisted selection (MAS) more desirable. Molecular mapping of simple inherited traits includes Gle 2 for glandless cotton, Se for photoperiod sensitivity, im for immature fiber, Li for lintless cotton, and Lc for lint color. Among these major genes, Gle 2 is currently targeted for high-resolution mapping and positional cloning. An introgressed fragment of about 20 cM was estimated on chromosome 12. This fragment contains the Gle 2 gene, and two linked DNA markers. The closer one is about 5.6 cM away from the Gle 2 gene. Regional saturation of this locus is underway using a pair of NILs and bulks. Molecular characterization of Gossypium germplasm with DNA markers is another area of our genome programs. An initial set of 155 land races and cultivars have been examined with 60 DNA markers selected from different chromosomes or linkage groups of the cotton genome. Integrative physical mapping, and linkage between Arabidopsis and cotton genomes is a future target. Although cotton genome is large and complex, the physical size of a cM is only 50% larger than that of Arabidopsis. A high level of homology between Arabidopsis and Gossypium genomes and abundant polymorphism among Gossypium germplasm were detected using conserved cDNAs from the Arabidopsis genome. Integration of plant genes and DNA markers with large genomic clones such as BACs would move cotton genome mapping into a new phase of broader applications. Bioinformatics tools are being developed to interface with CottonDB (Yu and Kohel, 1999).

Marker Assisted Selection (MAS)

The molecular markers like restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), microsatellites, short sequence repeats (SSR) and amplified fragment length polymorphism (AFLPs) are being used for the enhancement of resistance gene deployment for stable crop protection through phylogenetic and population genetic analysis of insect pest and by gene mapping, gene tagging of genetic and analysis host plants (Andersen and Fairbanks, 1990). The molecular markers can be used in studying the genetics of resistance and through the marker-assisted selection (MAS) in the selection of resistant plants. Molecular markers can also be used for accurate identification of sources of resistance to insects at DNA level and in the accurate selection of pest strains for the identification of resistance genes. The identification and characterization of different insect biotypes takes more time by conventional means whereas molecular markers pest population characteristics can be understood and studied quickly. Development of a robust integrated framework map has greatly accelerated molecular genetic research in the model plant *Arabidopsis thaliana*. To efficiently develop a similar set of framework markers for cotton (with markers at approximately 20 cM intervals), multiple sources of DNA polymorphisms including microsatellites, single nucleotide polymorphism (SNPs) and amplified fragment length polymorphism (AFLPs) need to be taped. Methods for high-throughput retrieval of cotton sequences with simple sequence repeat motifs, primer design, and microsatellite detection (using economical agarose systems) were optimized. Out of 550 sequences captured using microsatellite repeat oligonucleotides, an initial set of 70 markers was tested in an interspecific F_2 mapping population (Randhawa, L.S. 1997a and b). In this analysis, 50% of the markers were polymorphic and segregated in a 1:2:1 mendelian ratio. 10 SNPs derived from 20 fiber-subtracted ESTs using the CAPs and dCAPs technologies were

mapped, thus demonstrating that existing EST sequences are a valuable source of polymorphisms for the development of informative DNA markers.

Hybrid Cotton Production

Hybrid cotton and heterotic response in cotton has been studied for several years. Although hybrid cottons are planted on large areas in India and China, no significant area is devoted to hybrid cotton in other cotton producing areas. Several studies on male sterile as well as female sterile transgenic parents of development for production of F_1 cotton hybrids were produced (Holland 1999). The objectives of these studies were to identify superior yielding hybrids and to study various factors involved in heterotic response via genetic engineering. The evaluated hybrids were developed by two independent programs and with the cytoplasmic male sterility system, which utilized the *Gossypium harknessii* Brandegee cytoplasm. Environmental effects on fertility restoration were noted and several proposed ideas for hybrid cottonseed production were evaluated. Results indicated that hybrids made with particular pedigrees produced significant yield advantage and fertility of the hybrids was not influenced significantly by environmental factors at the test locations.

Cytoplasmic Male Sterility in Cotton

Cytoplasmic male sterility (CMS), is a maternally inherited trait that inhibits production of viable pollen. It has been found in more than 150 plant species. CMS has been extensively utilized for hybrid production in major crops, e.g. maize, sorghum, rice, rapeseed, etc. However, even though the first CMS system, harknessii CMS (CMS-D2) was released in the early 1970s, no commercial cotton hybrids were produced by this CMS system in the U.S. Therefore, a new CMS system is needed.

Most CMS types have occurred naturally or in intraspecific crosses, while other CMS systems have also been induced by interspecific cytoplasm transfer. CMS-D8 was developed by introducing the cytoplasm from *G. trilobum* (D8 genome) into cotton. D8 was used as female to cross with cotton, and then the hybrid chromosome number doubled with colchicine. The resulting hexaploid line was recurrently backcrossed as female with cotton. From a segregating BC_5 population sterile plants were isolated as the CMS line and the restorer line were derived from fertile plants. Compared with the fertile flowers of normal cotton, CMS-D8 sterile flowers are smaller with shorter filament, no pollen, and much smaller anther. When CMS-D8 is crossed with its D8 restorer line, F_1 plants are fertile with normal flower phenotype. But, the heterozygous F_1 plants produce two types of pollen grains in roughly equal numbers: one type stains with I2 -KI and the other does not stain, indicating that the latter has no starch accumulation, and is thus sterile. Therefore, the restoration of fertility is apparently a gametophytic system. The segregation ratio of the two pollen types also confirmed that the restoration to CMS-D8 by the D8 restorer is controlled by one gene (rf2). In order to obtain insight into the mechanisms of CMS-D8 male sterility and its restoration, differential gene expressions in anther tissues between a heterozygous D8 restorer line (8518R) and its isogenic non-restoring line (ARK 8518) were compared by using mRNA differential display techniques. Approximately 3000 cDNA fragments were assayed that represented ca. 10-20% of the genes expressed in the tissues. Among 100 differentially displayed cDNA bands, 38 were cloned, sequenced, and differential expression confirmed by reverse Northern blot analysis. In the heterozygous D8 restored line, five up-regulated genes and 12 down-regulated genes were detected. The DNA sequences of the up-regulated genes did not show high homology to any known sequences in the GenBank. The down-regulated genes that were highly homologous to known sequences were: phosphoribosylanthranilate transferase for tryptophan synthesis, starch synthase for starch synthesis, calnexin for protein maturation, polyubiquitin for protein targeting for degradation, and ascorbate oxidase for pollen germination. Based on the above results, a picture regarding the D8 CMS and its restoration can be drawn as follows. In the heterozygous restored F_1 plants, the expression of the restorer gene (rf2) suppresses the D8 cytoplasm effect, most likely its CMS-related gene expression, so that normal microsporogenesis and microspore development occurs. However, during microspore maturation, the microspores with the Rf2 gene go through the first mitosis and starch accumulation, and develop into fertile pollen grains. On the contrary, the genes for amino acid, protein and starch synthesis, protein maturation and targeting for degradation, and pollen maturation are suppressed in the microspores with the non-restoring allele (rf2). Consequently, the rf2 pollen has no starch deposition and is sterile.

Producing higher yielding varieties is a major goal of plant breeders. This is most effectively accomplished by the use of F_1 hybrids, which are estimated to yield 20-25% more seeds and are more uniform than the best open-pollinated varieties. Many plant varieties (e.g. rapseed, grasses, cotton, etc.) are capable of both self-pollination (70%) and cross-pollination (30%), thus control of pollination is required to produce 100% F_1 hybrid seeds. Cytoplasmic male sterility is currently used to produce hybrid seed. However, the pol cytoplasm, the most common male-sterility inducing cytoplasm used throughout the world, is subject to high temperature reversion, and 100% hybrid seed is difficult to obtain (Pinnisch and McVetty, 1994).

Genetic Engineering Technology Application May Override This Problem

The subjects of plant transformation events were genetically engineered to express genes for male sterility (MS),

restoration of male fertility (RF), to enable the production of pure hybrid varieties by the use of a new type of pollination control system. Male fertile RF plants can be used in control pollination of male sterile MS plants to produce pure hybrid progeny with restored male fertility. The pollination control traits in MS and RF are linked to other benefit traits as the glufosinate herbicide tolerance trait. This trait allows for selection of plants during breeding that carries the linked pollination control genes and provides tolerance to glufosinate herbicides, which could be used to control weeds. Weed management is critical to maximize crop yield and obtain high-quality seed harvest free of weed seeds; but it is expensive and labor intensive. Glufosinate-tolerant plants offer farmers an additional option for post-emergent weed control. Often farmers use pre-emergent herbicides that will stop weeds seeds from germinating. However, this assumes that weeds will always be a problem in all the fields. A diversity of plant transformation systems was used to transfer the new genetic material into the parental variety to produce plant transformation events MS and RF (De Block et al., 1989). Sequences necessary for the expression of the desired trait were introduced to create chimeric plasmid vectors to directly integrate in different plant genomes. MS and RF plants contain the following sequences necessary for pollination control, MS contains the coding region of the barnase gene from *Bacillus amyloliquefaciens* (Hartley, 1988) and the 3'untranslated region downstream from this gene. The barnase gene encodes a specific ribonuclease enzyme which when expressed in the tapetal cell layer of anthers, blocks pollen development and results in male sterility (Hartley, 1989; Mariani et al., 1990; De Block and De Brouwer, 1992). RF plants contain the coding region of the barstar gene, also derived from *B. amyloliquefaciens* (Hartley, 1988), and the 3'untranslated region downstream from this gene. The barstar gene encodes a specific protein inhibitor of the Barnase ribonuclease protein (Hartley, 1989). Co-expression of both barnase and barstar in anthers prevents male sterility caused by the barnase gene (Mariani et al., 1992). Anther-specific expression of both the barnase and barstar gene are controlled by the promoter region of the TA29 gene from tobacco (Nicotiana tabacum) (Seurinck et al., 1990). Sequences necessary for polyadenylation of mRNA for the inserted barnase and barstar genes are provided by the 3' untranslated sequence from the nopaline synthase gene (3'nos) from A. tumefaciens. The hybrids exhibited similar agronomic behavior as control plants regarding seed germination rates, plant stand, plant vigor, flowering times, deleterious effects, and disease and pest resistance or susceptibility.

The male sterility and male fertility restoration traits engineered into MS and RF plants would not be expected to increase the weediness potential of transgenic plants. In fact, male sterility alone would provide a significant disadvantage to seed production and thus persistence of MS plants in natural habitats where pollen from other sources may be limiting. Male sterility in MS plants is unlikely to increase the weediness potential anymore so than would cytoplasmic-male sterility used for the production of hybrid cultivars. Fertility of RF plants was reported to be similar to the nontransformed parent, and these plants will not affect the male fertility of plants that lack the barnase gene (Bing, 1991; Jrrgensen and Anderson, 1994).

Germplasm Engineering in Cotton

DNA markers have been applied sparingly in cotton improvement. Their use is limited to areas of proprietary protection, transgene conversion and tagging simple qualitative traits. This is due, in part, to the paucity of intraspecific polymorphism for most available DNA markers. An immediate and appropriate use of DNA marker technology is introgressive breeding or germplasm engineering. This involves molecular characterization of the genetic diversity of cotton germplasm resources. Careful discovery of novel quantitative trait loci (QTL) alleles are undertaken at the same time they are being introgressed into an elite genetic background. This can be used to exploit the secondary Gossypium gene pools, such as the *G. hirsutum* primitive race stocks. Microsatellite or simple sequence repeats (SSR) markers are very suitable for introgressive breeding. They can be modified to high-throughput systems essential to efficiency of germplasm engineering. Advanced backcross QTL strategies, such as those successfully applied in rice and tomato, should be appropriate to germplasm engineering in cotton. The strategic use of DNA markers can laminate the novel QTL alleles from exotic germplasm for genetic enhancement of the elite primary gene pool. This is a more appropriate use of DNA markers in cotton than trying to use marker-assisted selection within intra-specific elite populations for QTLs.

Genetic Diversity in MAR Cotton Germplasm

The importance of genetically distant parents to cultivar improvement has been established. Genetic diversity and variation between parents is important in creating new superior cultivars with unique and new gene combinations. Extensive use of closely related cultivars by growers could result in vulnerability to insects, pathogens, and abiotic stresses. Recent reports indicate a decline or slow down in the rate of yield increase, which is attributed to a decrease in useful genetic variability within breeding programs. Several researchers concluded in recent studies that the genetic base of modern upland cotton cultivars is not particularly narrow and continue to offer opportunities for cultivar development. Their studies highlighted the contribution of several individual breeding programs to genetic diversity of cotton. Both adapted and

exotic cotton lines are useful for introgressing into germplasm. The MAR germplasm began with a diverse gene pool which included strains with genes for bacterial blight resistance (B genes) transferred from *G. barbadense* and *G. hirsutum* to upland cotton. Currently, the MAR program introgresses only adapted germplasm. Germplasm introductions contributed to fiber quality and resistance to Verticillium wilt, PD to fiber quality and the Shepherd germplasm to root-knot nematode resistance. Before parental material is intogressed into the MAR germplasm, it is screened using the MAR procedures. However, selection pressure is not as stringent as with the MAR germplasm. New selected parental strains are crossed to the most advanced MAR germplasm. After crossing, selected strains become part of the main MAR gene pool. The established MAR techniques and procedures utilize seed, seedling, and plant selection in the laboratory and greenhouse, followed by extensive field testing and evaluation. These procedures make it possible to identify superior cotton strains with genetic gains to many important traits. The new elite MAR germplasm releases will combine high yield potential and early maturity, improved fiber and seed quality, drought tolerance, higher levels of resistance to insects and pathogens, and stability over diverse environments benefitting cotton growers and industry, (El-Zik and Thaxton 1999, Thaxton, 1999).

Future Expectation

While the pace of adoption of biotech products has been spectacular, the review of products still being tested indicates that current developments are the tip of the iceberg, with many more, different products yet to emerge. And, it suggests that future developments could come even more rapidly in the next few years.

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