Cotton biotechnology: Beyond Bt and herbicide tolerance

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ABSTRACT

Currently available cotton varieties with genetic traits derived from biotechnology have provided the adopting farmers and countries substantial benefit: increased profitability, risk reduction, labor savings, pesticide reduction, soil conservation, environmental and health benefits, and agricultural/rural sustainability. With the strong benefits of existing products, there is excitement for future cotton biotech traits. We can learn about near-term future traits by reviewing the cotton literature and regulatory releases, but a look further into the future requires analysis of research in other commercial crops, model crops, and the biomedical arena. A review of near-term cotton traits will be provided along with a glimpse into the future of potential trait and technologies that might be developed. There is no lack for potential benefit from near-term and long-term future traits. However, companies will weight the potential economic return from novel traits in elite germplasm against the significant costs to develop, register and market these traits. Institutes and companies developing novel traits will focus resources where farmers and governments accept new technology and a satisfactory return on investment can be achieved.

Introduction and history

Cotton biotechnology has already generated tremendous benefits for farmers, the environment and society at large due to the improved control over insect pests with substantially lower pesticides, expansion of reduced tillage cotton production and improved farm efficiency and profitability (James, 2002). These benefits have resulted from the earliest genetically modified commercial traits which incorporated bacterial proteins for lepidopteran control and glyphosate tolerance. As the research investment expands, so will the diversity and benefit from biotechnology in cotton. This paper focuses on the expansion of biotechnology beyond these early products and attempts to identify several exciting leads that may result in further benefit for cotton farmers and their customers.

This review will first review the history of biotechnology in cotton, since it is difficult to predict where we are going without understanding where we came from, then examine some of the new tools in cotton biotechnology and the fields of yield enhancement, abiotic stress tolerance, oil quality and insect tolerance. The important area of fiber quality will be addressed by Dr. Thea Wilkins, and thus not reviewed here.

Early history of cotton biotechnology

The genus *Gossypium* has a long history of successfully utilizing genetic modification (transgenic) to expand its adaptability and utility to man. The early history of cotton breeding is particularly impressive: with frequent transgenic exchange between prokaryotic organisms (Koonin *et al*., 2001) occurring prior to the transgenic insertion of approximately 6000 genes from an á-proteo bacteria to become later the modern day mitochondria 1-2 billion years ago and the transgenic insertion of ~3000 genes from a cyanobacteria to start the process of becoming our modern day chloroplast, 1 billion years ago (www.tolweb.org). These events generated the eukaryotic cell with intact specialized organelles some of which were optimized for continued genetic manipulation through various forms of recombination and duplication (the nucleus) while others were relegated to metabolic efficiency and clonal propagation.

Subsequent to the formation of the eukaryotic cell, numerous chromosomal rearrangements, transgenic exchanges, mutations, duplications occurred leading to the separation of the Malvaceae and the Brassicaceae ~85 million years ago (Bowers *et al*., 2003) and the evolution of the common progenitor of the *Gossypium* genus approximately 7.5 million years ago, the divergence and evolution of A genome and D genome cottons, and their eventual recombination to produce the AD tetraploid cotton approximately 1.5 million years ago (Cronn *et al*., 2002). This last large transgenic event inserted ~30,000 from an Eastern Hemisphere D genome species into a Western Hemisphere A genome recipient plant, doubling the number of chromosomes and genes (Senchina *et al*., 2003; Adams *et al*., 2003). Subsequent to the formation of the AD genome numerous *Gossypium* species evolved, most important to man were *G. barbadense* and *G. hirsutum* (Brubaker *et al*., 1999). These biotech events allowed the *Gossypium* genus to accumulate sufficient genetic diversity within and between genomes for man to identify variants of particular utility for both fiber and seed.

Recent cotton biotechnology history

Following on these impressive transgenic events, the modern history is less glamorous but equally as important for the utilization of fiber and seed by man. With the rediscovery of Mendel's laws of genetic inheritance approximately 100 years ago that science was applied to cotton breeding in the Mississippi Delta, starting 90 years ago, for the combined benefit of cotton planters and spinners. Over the next 40 years an impressive diversity of germplasm was developed through recombination and selection by an every widening array of public and private plant breeding stations.

Fifty years ago the era of directed biotechnologi-

cal manipulation was launched with the elucidation of the DNA structure (Watson and Crick, 1953), followed by a string of biotechnological successes in cotton. In 1973 Beasley and Ting reported on the first in vitro tissue culture of cotton ovules. By manipulating the plant growth substance levels in tissue culture media, cotton ovules were induced to initiate and grow fiber. In 1983 Horsch and Chilton reported on the first successful transformation and regeneration of tobacco using *Agrobacterium tumefasciens*. Shortly after this breakthrough the regeneration of cotton plants from callus tissue was reported by Davidonis and Hamilton, 1984. Three years later the transformation of cotton using *Agrobacterium tumefasciens* was reported by Umbeck leading to the first field release of transgenic cotton by Agracetus in 1989. An alternative transformation method, biolistics particle bombardment, was first demonstrated in cotton by Finer and McMullen in 1990. This foundation of research over a 45 year period permitted the first commercial sale of elite cotton varieties containing transgenic material in the US and Australia in 1996.

After the commercial approval of cotton varieties incorporating transgenic material, local farmer experience and success with these varieties has resulted in their rapid adoption, exceeding 50% farmer adoption within three years after approval and exceeding 80% farmer adoption after six years (US, Australia, South Africa, Mexico, China and Argentina; farmer adoption is calculated from the area approved for planting to transgenic cotton divided into the area actually planted, James 2002). As additional farmers gain experience with cotton varieties containing transgenic material, their own experiences will continue to hasten the global benefit derived from the available biotech traits. Concomitant with the farmer adoption of biotech cotton, has been the expanded research investment in new methodologies and traits for cotton. These new traits will continue to add further benefits for the cotton farmers of countries that permit their use.

New methodologies in cotton biotechnology

The recent expansion of investment by the private and public sector into cotton biotechnology has generated new tools that will multiply the benefits that can be derived from biotechnology beyond the initial transgenic traits.

Agrobacterium *transformation*

Methods to tranform cotton were introduced in 1987 (Umbeck *et al*., 1987) following previous discoveries in cotton issue culture (Beasley and Ting, 1974; Davidonis and Hamilton, 1984) Since that time numerous reports of cotton transformation with diverse strategies and plant tissues have been made (Burke *et al*.., 1999; Kosegi *et al*., 2002, Finer and McMullen, 1990; Sunilkumar and Rathore, 2001)

Marker aided selection

The plant breeding tool, Marker Aided Selection, derives from the expanding field of genomics, transcriptomics, proteomics, and metabolomics. These genomics based fields utilize sophisticated computer applications combined with the ability to evaluate and manipulate very large genetic experiments. Historically crop improvement has been divided into two separate fields based on the number of genetic elements that are manipulated. Traditional plant breeding included gross manipulation of whole plants and their very large gene sets while traditional biotechnological research included the fine manipulation of usually two or three genes. The genomics based fields extends the evaluation and fine manipulation to very large sets of genes or alleles, leading to breeding methods that operate at both the whole plant and gene level simultaneously, commonly referred to as marker aided selection.

Marker aided selection relies upon laboratory and field components to identify and validate close associations between genetic sequences (markers) and useful agronomic traits, such as disease resistance. Various genetic sequences are used as sets of markers in cotton (ALFP, SSR, SNP) that can be rapidly screened using PCR or microarray techniques (Paterson *et al*., 2003; Reddy *et al*., 2001; Koebner and Summers, 2003). To develop the close association between the markers and the trait requires exposing segregating populations for the trait of interest to the field conditions that generate clear trait expression followed by determination of linkage relationships with the marker set. Once the associations and markers have been developed, then breeders can select for plants that contain markers associated with positive traits and discard plants that contain markers associated with undesirable traits. This laboratory screening allows breeders to handle larger populations of more divergent genotypes and focus their efforts on germplasm with a higher probability of generating transgressive gene combinations (Morandini and Salamani, 2003).

Markers are already in use by cotton breeders when they use PCR and ELISA techniques to identify transgenic traits, instead of selecting for the presence of a transgene based on the plant response to either a herbicide or insect. Due to the difficulty of screening large populations for certain diseases and nematodes, the application of marker aided selection in this field should speed up the development of agronomically superior cotton varieties containing multiple disease resistances. Some of the disease resistance alleles will be found in primitive race stocks of cotton. During the domestication of crops, many potentially beneficial alleles were not carried through to selected cultivars, and traditional backcross incorporation methods of alleles in primitive material is very time consuming. Marker aided selection will facilitate and accelerate the utilization of these alleles in modern plant breeding thereby expanding the genetic diversity upon which breeders

can select form.

Inducible gene regulation

Current transgenic traits in cotton are expressed constitutively, throughout the tissues and life of the plant, without the targeting of the transgene expression to specific plant tissues and tissue ages or generations. Constitutive expression, while satisfactory for the current transgenic traits, may be replaced by regulated expression for other transgenic traits. Methods of regulated expression include: tissue specific, temporal specific, and generation specific.

Tissue specific transgene expression derives from the use of tissue specific promoters that allow gene expression in specific tissues. These will be highly useful with output traits such as fiber, oil or protein modification that improve the value of the harvested product, since gene expression in non-seed tissue would be superfluous and possible deleterious.

Temporal specific transgene expression derives from the use of promoters and are regulated by either endogenous or exogenous conditions. Many of the proposed mechanisms of drought tolerance could generate undesirable results in certain environments, thus drought tolerance may require inducible gene regulation under water stress to avoid metabolic costs or disruption in tissue or to insure the trait is only turned on when needed. Gene switches that turn genes on or off, or excise genes, have been proposed to control the expression of transgenes. The diversity of gene triggers is broad and includes: seed treatments, foliar chemicals, heat, light, reduced water content, among others (Oliver *et al*., 1998; Lefebvre and Malboobi, 2001; Zuo *et al*., 2001).

Future of cotton biotechnology

The future of cotton biotechnology can be learned from several sources. From the announcements made by technology providers we can learn of the near-term introductions. However, for a longer-term view of potential products, a review of research literature in cotton or in other plant species is necessary to see a broader range of potential technologies that may become available to cotton farmers. Whether these technologies do become useful field production tools will be limited by economic, social and political factors.

Near-term products

Since the first commercial introduction of transgenic cotton in elite germplasm in 1996, a limited number of announcements regarding additional products have been made. This limited number of new technology announcements does not reflect the level of research into new products, rather it reflects the industries reluctance to generate farmer enthusiasm for products when commercial approval is years away.

Beyond the initial insect control products derived from single Bt genes, expanded insect efficacy has already been introduced with Monsanto release in 2002 of the dual Bt gene cotton product (Bollgard II™) in Australia and the USA. This product contains the Bollgard Cry1Ac and a Cry2Ab gene (www.agbios.com). Additional planned introductions have been announced for 2004. Dow AgriSciences has announced their dual Bt gene product (WideStrike) should be commercially available in the USA. WideStrike contains a Cry1Ac and a Cry1F (Pello *et al*., 2002). For that same year, Syngenta has announced the VIP, vegetative toxin gene from Bt, should be commercially available in the USA (Shotkoski *et al*., 2003)

Expanded tolerance to herbicides is also anticipated in the near-term. For 2003 to 2004, Bayer CropScience has announced that their Liberty Link cotton may be commercially available in the US. Around the year 2006, Monsanto has announced that their new Roundup Ready Flex technology may be commercially available in the USA (www.agbios.com).

Long-term products

The range is very broad of potential cotton technologies that have not reached the stage where a commercial introduction is anticipated. For this review, I will only focus on a few areas: oil quality, drought tolerance, cold tolerance, salt tolerance, yield enhancement, non-Bt insect control. Fiber quality will be covered by Dr. Thea Wilkins in her presentation.

Cotton research has been boosted by the close phylogenetic relationships between cotton and the lead model plant, *Arabidopsis thaliana*, a member of the Brassica family. Of the non-Brassica crops, *Gossypium* is the closest genus, phylogenetically, to *Arabidopsis* and thus many genetic elements, such as promoters, are cross functional (Sunilkumar *et al*., 2002). Considering the difficulty of transforming cotton compared with *Arabidopsis*, extrapolating from *Arabidopsis* research to cotton is a logical first step in estimating long-term product potential in cotton.

Drought tolerance

Cotton is the most drought-tolerant major crop when measured in economic output per mm of water (US $3 per hectare-mm at US $1 per kilogram of fiber for water supplied from 0 to 60 days after planting; the response to water applied from day 60 through day 120 is approximately double this amount (Morrow and Krieg, 1990). With current cultivars and farming technology, if the available water falls below ~350 mm of water, even cotton production may not be profitable. Thus enhancing drought tolerance of cotton would sustain farming in areas otherwise abandoned, and allow the use of both water and land resources that could not otherwise be utilized.

Several strategies are being considered for en-

hancing drought tolerance of crops: osmotic regulation (Quisenberry *et al*., 1983; Serraj and Sinclair, 2002), stomatal regulation (Mustilli *et al*., 2002), stress response signaling (Hsieh *et al*., 2002), heat shock proteins (Burke *et al*., 2001), cell cycle regulation (Inze *et al*., 2002), and production of compatible solutes such as trehalose (Garg *et al*., 2002), mannitol (Abebe *et al*., 2003), choline (McNeil *et al*., 2001), proline (Maggio *et al*., 2002; McNeil *et al*., 2001), and glycine-betaine (Adams *et al*., 2001). Regardless of the strategy used to obtain drought tolerance, due to the variable nature of drought conditions, field testing and a systems approach to drought tolerance will be required to quantify the stability of a trait's performance (Sinclair and Muchow, 2001).

Cold tolerance

Cotton's origin as a subtropical plant results in significant chilling injury to plant tissues (Brubaker *et al*., 1999). Although all stages are sensitive to chilling, the most economically damaging period is the seedling phase. Sensitivity to chilling injury has previously been associated with the various lipid constituents in cell membranes. Membranes with a higher ratio of saturated and monounsaturated fatty acids have been identified as being more susceptible to a phase shift at higher temperatures leading to membrane disruption and thus greater chilling injury sensitive (Buchanan *et al*., 2000). The association between chilling injury and fatty acid unsaturation has been questioned in cotton (Speed *et al*., 1996; Lauterback *et al*., 1999). Recently the role of signal transduction pathways leading to specific transcription factors have been investigated as potential targets for development of improved chilling injury tolerance (Sung *et al*., 2003). Due to the multiple genes involved and their complex interactions, commercially successful transgenic improvement of cold tolerance using signal tranduction pathway modification may require a large (~100) number of genes to be manipulated (Sung *et al*., 2003). In addition to direct membrane injury during chilling injury, photosynthesis is severely inhibited by suboptimal temperatures of 3-5 °C in cotton. Conditions of high light and low temperature generate reactive oxygen intermediates in the sunlit plant tissues, that damage cell membranes. The use of biotechnology to increase antioxidant enzyme activity in chloroplasts holds some promise for improving the chilling tolerance of cotton (Payton *et al*. 2001).

Salt tolerance

In addition to cotton's relatively high level of drought tolerance, cotton is one of the more salt tolerance annual crops (Ayers and Wescot, 1985). Thus, expanded salt tolerance in cotton would allow use of saltier soils and water resources, than currently utilized. Recent research to improve salt tolerance of crop plants has focused on compatable solutes (Ferjani *et al*., 2003), partitioning sodium into the vacuolar space (Zhang and Blumwald, 2001; Zhang *et al*., 2001), and signal transduction pathway modification (Kreps *et al*., 2002).

Oil quality

Cottonseed oil represents an additional source of income and nutrition for cotton farmers. However to use cottonseed oil for high temperature cooking and solid foods (margarine, ice cream) it is necessary to convert the polyunsaturated lipids to monounsaturated and saturated lipids. The commercial process to achieve this, hydrogenation, can create trans (double bond) fatty acids from the normal cis double bonds in addition to the intended outcome of converting cis double bonds to single bonds. The resulting trace levels of trans fatty acids are considered less healthy than cis fatty acids. In addition lipids with one or more cis double bonds are considered more healthy than fully saturated (hydrogenated) short chain lipids such as palmitic acid with regards to the formation of low density blood lipo-proteins and serum cholesterol. Since multiple double bonds both reduce the melting point of lipids and present sites for high temperature oxidation the conversion of double bonds to single bonds makes cottonseed oil more valuable for frying and in solid foods. Thus for different cooking and nutritional uses, there are opportunities to improve cottonseed oil by altering the ratio of fatty acids with regards to the number of double bonds.

The fully hydrogenated carbon backbone of plant lipids is first constructed by adding 2-carbon residues at a time and then once full length is obtained, the double bonds are inserted by specific desaturase enzymes. These desaturase enzymes have been the targets for successful down regulation using antisense and hairpin RNA complementary to plant desaturases (Chapman *et al*., 2001; Liu *et al*., 2002a; Liu *et al*., 2002b; Green *et al*., 2002). The resulting cotton plants can be combined to generate a wide range of oil qualities.

Yield enhancement

The recent domestication of cotton as an annual plant from the commercially harvested perennial shrub creates multiple opportunities to enhance yield using biotechnology. Based on comparison with other crops and physiological studies, many of cotton's morphological and metabolic characteristics appear to be non-optimal for production under the short growing season (Wullschleger and Oosterhuis, 1990a, 1990b), that farmers prefer for reduced production cost and risk. Although large genetic gains in yield have been obtained using traditional plant breeding (Wells and Meredith, 1984; Bassett and Kerby, 1996) the range and speed of improvements is limited by the diversity of favorable alleles with the *Gossypium* genus, and the tools of traditional plant breeding. Various strategies are being employed to enhance yield in crop plants using biotechnology: photosynthetic efficiency (Barry *et al*., 2002a; Barry *et al*., 2002b; Doerner and Lamb, 2001; Gelvin, 2003; Horton, 2000; Jenner, 2003; Mar-

tin and Herrmann, 1998; Martin *et al*., 1998; Martin *et al*., 2002; Miyagawa *et al*., 2001; Regierer *et al*., 2002; Smidansky *et al*., 2002), delayed leaf senescence (Amasino *et al*., 2002; McCabe *et al*., 2001; Ori *et al*., 1999) and altering plant hormones (Chory and Li, 2001).

Novel insect control products

Although the research and commercialization of transgenic insect control strategies using diverse proteins derived from *Bacillus thuringiensis* are well advanced (Liao *et al*., 2002), the use of other natural proteins and plant secondary metabolites is in the early stages of development. Specific proteins and compounds reported to have a potential role as plant incorporated protectants include: canatoxin (Carlini and Grossi-de-Sa, 2002), chaperonins (Yosida *et al*., 2001), cyanogenic glucosides (Tattersall *et al*., 2001), cyclotides (Jennings *et al*., 2001), cytochrome P450 (Daborn *et al*., 2002; Li *et al*., 2002), endosymbiont proteins from parasitic nematodes (Karmer *et al*., 2002), glucosinolates (Chen *et al*., 2001), lectins (Carlini and Grossi-de-Sa, 2002), protease inhibitors (Anderson *et al*., 2002; Burgess *et al*., 2002; Chen *et al*., 2002; Lawrence and Koundal, 2002; Xuanjun, 2001), monoterpenes (Meyer and Yalpani, 2001), pentin-1 (Cigan *et al*., 2002), peroxidase (Lagrimini and Desai, 2002), and polyketides (Betlach *et al*., 2001).

In 2001, the global expenditure on crop biotechnology research by the both the private and public sectors was US $4,400,000,000 (James, 2002). Considering this tremendous investment and the diversity of projects under way (only a few of which have been highlighted here) the future direct and indirect benefits of plant biotechnology on the lives of cotton farmers and their families will grow even more significant that it currently is (Falck-Zepeda *et al*., 2000; Huang, 2002).

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