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Contents

Introduction

Helicoverpa zea is a major pest on cotton in the USA and is popularly referred to as the bollworm. *H. zea* is also common on other crops where it is recognized under different names. Other bollworms/budworms also attack cotton in the USA. Since the adoption of biotech cotton in the USA, loses due to bud/bollworms have declined from 3.97% in 1995 to 1.2% in 2010. Losses due to other insects like *Lygus* and stinkbug that are not controlled by *Bt* toxin have remained almost at the same level over the last 15 years. The data show that despite the increase in insect pressure due to certain sucking insects and price hikes in insecticide products, the direct management cost of arthropods has not increased in the last 15 years. In 2010/11, the technology fee for insect resistant biotech cotton is estimated at about US$35/ha, which represents about 25% of the cost of arthropod management. The experience with dual gene technology in the USA has shown that cotton varieties with multiple *Bt* gene technologies may provide very good control of caterpillar pests, but they may not offer 100% control of the bollworm. Many researchers have observed that under extreme natural pressure from bollworms, insect resistant biotech varieties might display variable control of target bollworms and might require supplemental applications of insecticides to avoid yield losses due to injury from the species. Thus, there are many factors that could determine the need for additional sprays on dual gene varieties. The first article is about the "Need for Spraying on Dual Gene Insect Resistant Biotech Cotton in the US Production System."

The second article entitled "Cotton Color: Measurement and Discoloration", deals with latest developments in fiber color measurement and changes over time in color due to various reasons. The two independent parameters of cotton color are the degree of reflectance (Rd) and yellowness (+b). All open bolls in a field are not picked soon after opening. Seedcotton on the plant is subjected to weathering for many days. Delay in picking of cotton and continuous dew on open bolls also changes the natural color of cotton. Reports show that some cotton bales, especially those transported overseas, change color significantly, particularly +b, from their initial HVI measurement. Moisture and temperature are the two most important factors affecting color after picking and during storage. Yellowing may result in a slight weakening of the fabric, or even complete disintegration of the fabric. Color deterioration indicates reduced processing ability of the fiber, resulting in a lower market price. Color deterioration affects the ability of fibers to absorb dyes equivalent to non- color deteriorated fibers. Even if the color-deteriorated cotton is able to absorb dyes equal to non-color deteriorated cotton, the ability of discolored fibers to hold dyes is affected. Many more facts about cotton color are given in the second article.

The third article is from Dr. Sukumar Saha, ICAC Researcher of the Year 2011. Dr. Saha, who currently works for the US Department of Agriculture, was recognized at the 70th Plenary Meeting of the ICAC held in Argentina from September 4-10, 2011. Dr. Saha is an international authority in cotton genomics and cytogenetics. His research is used in the development of genetic and cytogenetic resources by researchers around the world. He has developed, evaluated and released backcrossed interspecific chromosome substitution lines from other tetraploid cotton species. This research opens new paradigms in cotton breeding and genetics studies, providing a tool to overcome the problems of interspecific introgression and in the discovery of some novel genes or traits. Dr. Saha also made a major contribution in developing PCR-based SSR markers, a critical first step for the use of PCR-based marker technologies in cotton breeding programs. Dr. Saha is one of eight founding scientists who initiated International Cotton Genome Initiative (ICGI) to facilitate collaborative research work on cotton genomics at the global level. Dr. Saha made a presentation at the Technical Seminar in Buenos Aires, September 2011. His paper, reproduced here, is focused on three specific areas on the role of biotechnology in cotton improvement: 1) use of transgenic technology in economically and environmentally sustainable cotton production, 2) marker assisted selection to expedite the cotton breeding programs, and 3) the future of cotton genome sequencing to unlock the secrets of genetics for the improvement of cotton.

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The Need for Insecticide Applications on Dual-gene Insect-resistant Biotech Cotton in the US Production System

In the United States, *Helicoverpa zea* is a major pest and is commonly known as the bollworm when it affects cotton. However, when *H. zea* attacks other crops, the caterpillar is often named after the host plant, e.g. corn earworm on corn, sorghum headworm on sorghum, soybean podworm on soybean, tomato fruitworm on tomato, and others. The wide range of hosts and the sequence of crops that the insect feeds on over a single growing season have a significant impact on its potential to develop resistance to the toxins expressed in Bt cotton and other biotech crops. This polyphagic quality gives rise to seasonal developmental scenarios where only a limited number of generations would not be exposed to the transgenic toxins, whether in Bollgard or Bollgard II. Furthermore, the use of similar Bt toxins in both Bt corn and Bt cotton may subject populations to multiple selection exposures within a single year. The commercialization of more biotech crops carrying the same Bt genes is going to increase the risk of developing resistance to the toxins. The tobacco budworm *Heliothis virescens* is a major pest and has always required a greater number of insecticide applications than *H. zea*. This situation changed, however, with the introduction of Bollgard cotton in 1996. Bollgard biotech cotton eliminated 100% of the applications for tobacco budworm, but supplemental control of the bollworm remained a routine practice until Bollgard II was adopted. Consequently, the threshold for bollworms was revised to control the escape population. Adoption of Bollgard II enhanced in-plant control of caterpillar pests, particularly the bollworm. Later, in 2005, Dow AgroSciences made available an alternate dual-gene technology, WideStrike®. While varieties with Bollgard II® or WideStrike® technology provided very good control of caterpillar pests, they do not offer 100% control of bollworms.

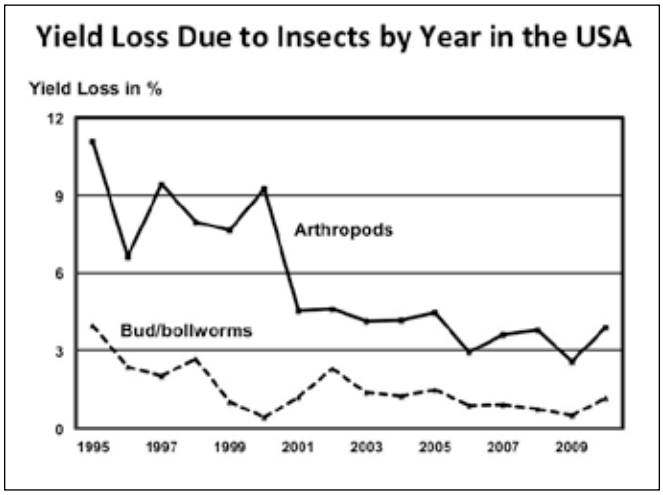
The situation with the fall armyworm *Spodoptera frugiperda* differs significantly from that of the tobacco budworm and the bollworm. The fall armyworm is an occasional-to-sporadic pest in many cotton areas around the world. It is also known to be a migratory pest in the USA. The pest attacks the bottom part of the plant, where it lives, thereby making it difficult to detect infestations using standard sampling protocols. Also, reactive insecticide sprays often yield inconsistent results. There have been questions concerning the performance of transgenic Bt cottons against this pest. WideStrike® provided better protection than either Bollard or Bollgard II cottons against the fall armyworm.

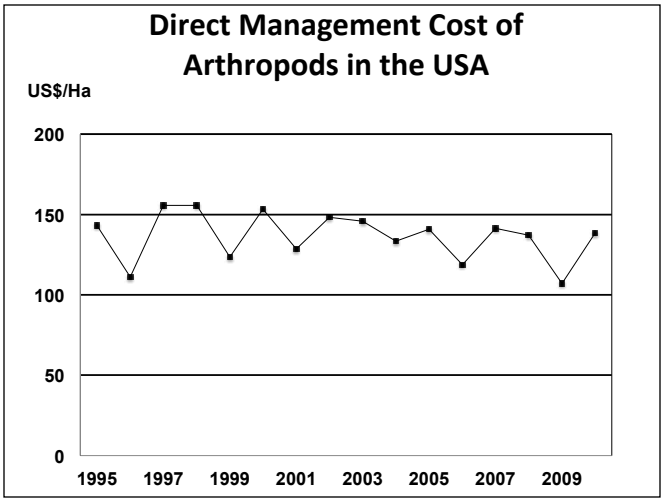
The other two-fruit damaging pests on cotton in the USA, where biotech varieties were adopted sooner than in any other country, are the pink bollworm *Pectinophora gossypiella*, and the cotton bollworm *Helicoverpa armigera*. The cotton bollworm, an American bollworm, is not a serious pest on cotton. The cotton bollworm is typically more difficult to control with Bt cotton than other targeted lepidopteran pests and often requires spraying with insecticides as a supplemental control measure. Protection against the pink bollworm improved with the introduction of dual-gene insect-resistant biotech cotton.

Losses due to Insects in the USA

In the USA, losses due to arthropods have been assessed since 1979. Damage is expressed in terms of loss in yield attributable to various individual pests. The necessary information is provided by county agents, extension specialists, private consultants and research entomologists. All data are averaged over the total area of each reporting unit. For example, if a unit report comprises 100 hectares and there has been an 8% loss on an area of 25 hectares, the average loss would appear in the data as a 2% loss. This averaging procedure is used on all reported data, including yields and control costs. Yield losses due to arthropod pests have diminished significantly in the last 15 years. Since the introduction of biotech cotton, loses due to budworms and bollworms have also declined, from 3.97% in 1995 to 1.2% in 2010 (Anonymous, 1996-2011). Losses due to certain other insects, such as *Lygus* and stink-bugs, have remained almost at the same level for the last 15 years. However, there have been high year-to-year fluctuations, which is also true for bud/bollworms. Yield losses due to bud/bollworms were only 0.5% in 2009/10. Based on the average yield data for 1995/96, it is estimated that 66.7 kilograms of lint per hectare were lost due to arthropods in that season, compared to 36.0 kilograms in the 2010/11 season. Thus, it may be inferred that the most recent pest control measures have not only saved on insecticide costs but have also contributed to lower losses and higher yields.

In the USA, data are also collected on direct management costs in connection with arthropods. The data appearing in the followng charts show that despite increased pressure from certain sucking insects and despite price hikes in insecticide products, the direct management cost of arthropods has not increased in the last 15 years (Anonymous, 1996-2011). In 2010/11, the cost of insect-resistant biotech cotton is estimated at about US$35/ha, which represents about 25% of the cost of arthropod management. There are many factors responsible for the lack of increases in the cost of arthropod control over the past 15 years. Biotech cotton is one of the major factors, but the boll weevil eradication program has also significantly





reduced boll weevil damage, particularly in Texas. Boll weevil infestation, which covered up to 0.9 million hectares in 2002, has been brought down to less than 50,000 ha in 2010.

The Resistance Issue and the Need to Spray

The year 2010 was the fifteenth anniversary of the introduction, adoption and large-scale commercial use of biotech cotton in the evolutionary quest to control insect damage in cotton. One of the most convincing lessons learned from the use of insecticides around the world as applied to biotech cotton was that the entire industry had to design resistance management programs for biotech cotton even before the first single-gene insect-resistant biotech cotton was commercialized in 1996. The dual-gene technology Bollgard II® (Cry1Ac + Cry2Ab) introduced by Monsanto in 2003 completed its first eight years of commercial planting in 2010. Two years later, another dual-gene, insect-resistant biotech cotton, WideStrike® (Cry1Ac + Cry1F), was introduced and commercialized by Dow AgroSciences and it completed its first six years of commercial use in 2010. Monsanto subsequently discontinued commercial production of Bollgard (Cry1Ac gene) cotton for fear that insects might develop resistance to a single–gene variety faster than to a duel–gene variety.

The experience with dual-gene technology in the USA has shown that cotton varieties with currently available multiple Bt gene technologies may provide very good control of caterpillar pests, but they may not offer 100% protection against the bollworm. Many researchers have observed that under extreme natural pressure from bollworms, insect-resistant biotech varieties might display inconsistent control of target bollworms and might require supplemental applications of insecticides to avoid yield losses due to bollworm damage. In 2010, Greene (2011) experienced the highest recorded pressure from *H. zea* in field trials run in South Carolina over the last five seasons. Trial fields of existing and promising biotech cotton technologies were inundated by natural infestations of bollworm, and variable results were observed. Greene (2011) noted peak boll damage levels approaching 20%, 60%, and 30% in unprotected varieties with Bollgard II®, WideStrike®, and TwinLink™ traits, respectively. The single-gene technology Bollgard® showed as high as 60% bollworm damage at the peak of bollworm pressure. He reported that research is underway to develop treatment thresholds designed specifically for multiple Bt gene technologies as they become available.

Jackson *et al.* (2011) observed that during the last few crop years, dual-gene insect-resistant biotech cottons (Bollgard II® and WideStrike®) have increasingly required greater numbers of insecticide sprays targeting the bollworm. Biotech cotton in Mississippi received an average of 1.7 applications per hectare in 2009 for supplemental control of bollworms; the figure was increased to 2.3 sprays per hectare in 2010. Pheromone trap captures of adult bollworms have also shown catch rates almost two times higher in 2010 than in the previous four years. The seasonal average number of bollworm moths captured per trap per week was <50 for 2006-2009 and >100 moths per trap per week in 2010. Researchers collected bollworms from resistant biotech cotton and corn (i.e. Bollgard II®, WideStrike®, and SmartStax®) and tested them for susceptibility to Cry1Ac and Cry2Ab through diet-incorporation, dose-mortality bioassays. A laboratory colony of the bollworm (LabZea) that is susceptible to various Bt toxins was assayed as a control line. Resistance ratios for Cry1Ac indicated that bollworm populations collected from pyramided-gene Bt crops were 3-8X less susceptible to Cry1Ac than the laboratory-susceptible colony. Susceptibility rates of these colonies to Cry1Ac was comparable to many of those found during 2002-2008 in Arkansas, where resistance ratios ranged from about 0.1 to >500. As with the Cry1Ac susceptibility estimates, the Cry2Ab resistance ratios ranged from 2-12.

Mortality ratios generated from the % mortality of a colony subjected to discriminating doses of either 100 µg/ml of diet (Cry1Ac) or 150 µg/ml (Cry2Ab) showed that Cry1Ac-susceptibility remained unchanged from 2002-2008. However,

the mortality ratio for Cry2Ab in 2010 was 0.4, which was lower than the range of 0.6-1.0 between 2002 and 2008 (in the state of Arkansas). These data suggest that, whatever reduction in susceptibility is being observed, it is most likely due to reduced susceptibility to the Cry2Ab protein. This, however, would not explain increased survival of bollworms on WideStrike cotton varieties, which produce both the Cry1Ac and the Cry1F Bt proteins. Jackson *et al.* (2011) have also pointed toward another factor that could be responsible for higher survival on dual-gene biotech cotton. They suggest that greater population densities of bollworms during the season could be the cause of the increased number of survivors observed in certain fields planted to biotech varieties.

Siebert *et al.* (2011) of Dow AgroSciences LLC concluded that their WideStrike varieties, as opposed to non-Bt varieties, showed a multi-year mean reduction in boll damage using WideStrike at 82% at each location. They saw a difference in the damage done by bollworms on insect-resistant biotech cotton, but no one claims complete control of all bollworms. The study of reference showed an 82% reduction in bollworm damage, but that only means that there was 18% damage that might be avoided either through more perfect biotoxins or by the application of insecticides. Some other studies found slightly higher mortality rates, i.e. 85.4%, against the bollworm complex, including: beet army worm *Spodoptera exigua*, cabbage looper *Trichoplusia ni*, bollworm and fall armyworm *Spodoptera frugiperda*. The single-gene Bt cotton produced a mortality rate of 45.3%. Siebert *et al.* (2011) also found no trends toward a change in efficacy over a given time span. According to Siebert *et al.* (2011), the contributing factors explaining the levels of boll damage in a WideStrike variety in the absence of supplemental foliar sprays may include: 1) intensity and duration of bollworm infestations; and 2) Cry protein expression patterns during periods of bollworm pressure linked to soil moisture and daytime and nighttime temperatures. The Dow AgroSciences LLC team found no evidence that decreased susceptibility to Cry1Ac or Cry1F over time is a factor leading to greater plant damage. Researchers concede that supplemental sprays targeting bollworm have always been occasionally necessary in WideStrike cotton, particularly against high densities and/or sustained infestations.

Transgenic cotton with Bt genes has reduced the need for conventional insecticides, while providing benefits for human health and the environment. For example, in U.S. cotton, the average number of insecticide applications used against the tobacco budworm and the bollworm complex decreased from 5.6 in 1990-1995 to 0.63 in 2005-2009 (Williams, 2008-2010). It is advised that varieties containing WideStrike should continue to be scouted for the bollworm. When supplemental insecticide treatments are warranted, appropriate insecticides and application rates should be selected and timed appropriately to manage infestations.

Endotoxin Expression

Genes determine all physiological and morphological traits and their ultimate impact on living organisms. A gene is a basic unit that determines variation/diversity and similarities/heredity and is defined as a DNA segment containing a specific sequence of nucleotides. All genes in all living organisms express themselves through proteins or enzymes. The expression of a gene varies according to the sequence of nucleotides, the nature of their promoter, their insertion site in the modified plant, the plant's internal environment and the different sources of modification (biotic and abiotic). Transgenes are able to fully express more perfectly when conditions are optimal. Thus, there are many factors that might determine the need for additional sprays on dual-gene varieties. These factors directly and indirectly influence the amount of endotoxin expressed in each biotech cotton, as well as the insects' reaction in terms of sensitivity and/or tolerance to the toxins produced within the plant. Some of these factors are discussed below:

- Different lepidopteran species vary in their susceptibility to endotoxin proteins. Some larvae will continue to live for two to three days after feeding has stopped. Individual instars may damage different plant parts: squares/buds, small bolls or large bolls. For example, in the case of the fall armyworm, 3rd, 4th, and 5th instar larvae damaged bigger bolls less than they did mid-size or smaller bolls. It has also been shown that damage to squares by all instars resulted in a significant reduction in survival of fruit to harvest. Insect feeding on large bolls did not reduce the probability of survival of fruit to harvest; however, yield from damaged bolls may be much lower compared to yields from unaffected bolls. It is generally accepted that the fall armyworm is one of the lepidopteran species that is least susceptible to the Bt endotoxin proteins expressed in cotton.
- Endotoxin concentrations need to be quantified because the amounts of endotoxin found in the leaves and in the other parts of the cotton plant vary significantly. Thus, the ultimate efficacy of any particular single-gene or multiple-gene biotech cotton will depend on the protein expression levels in different plant parts (Adamczyk *et al*., 2008). When larvae feed on less effective Bt type cotton leaves, they need to consume greater quantities of leaf material to ingest the amount of endotoxin needed to be lethal for them. It has also been established that feeding on transgenic cottons significantly reduced pupal weight and emergence, and also delayed larval development. According to Kranthi *el al.* (2005) toxin levels decrease as the crop matures and is consistently very low or undetectable in squares. *H. armigera* and bollworm larval mortality was greater on leaves containing toxin than on other fruiting parts (Arshad *et al*., 2009). The amount of toxin may also vary among the parts of a single plant. In general, petals, leaves and squares have higher

Expression of Cry1Ac Endotoxin in Various Cotton Varieties at Two Locations

Variety	Concentration of Cry1Ac Endotoxin (ppm)	
	Mississippi State	Stoneville
DP 33B/458B/RR	2.03 a	2.95 a
Sure-Grow 125 BR	1.15 b	2.69 a
PM 1218 BG/RR	0.90 bc	1.87 bc
ST 4892 BR	0.64 bc	1.94 b
DP 451 B/R	0.76 bc	1.49 c
ST 4691 B	0.61 c	1.56 bc

concentrations of Bt toxins than anthers and ovules. Research has also shown that bolls in position 1 (close to the main stem) have higher concentrations of toxins than those in positions farther away from the main stem. Concentrations of Bt proteins steadily decline between nodes 9 and 17. The age of the plant is also important as some data show that toxin amounts decline in older plant parts, particularly at 110 days after planting. This situation will demand insecticide sprays against target bollworms and those that may be prevalent at the crop maturity stage. Chloroplast concentration is also claimed to have an effect on toxin expression and, consequently, on sensitivity to protection against target insects, particularly *Spodoptera frugiperda*.

- Different varieties may express different amounts of toxin. As a consequence, the efficacy of toxins may differ widely from one variety to another (Kranthi *et al*., 2005). Hofs and Vaissayre (2007) have done an extensive review of the factors responsible for toxin expression. The work done in the USA as early as 2001 clearly revealed that there were significant intervarietal and interlocational differences in toxin expression (http://msucares.com/newsletters/pests/cis/2002/cis1302.htm). The data also showed that, at one location, a variety having a higher endotoxin concentration than another may actually have a lower concentration at a different location.
- Differences in susceptibility can also occur as a function of the geographic location of the population. Even before the commercialization of biotech cotton, it was well established that high temperatures can cause physiological imbalances in the plant that can trigger the degradation of soluble proteins. Consequently, concentrations of Bt toxins in any given variety may diminish if hot temperatures prevail for a long time. Chen *et al*. (2003, 2005) showed how exposure to temperatures of over 37º C for a 24-hour period reduces concentrations of Cry1A proteins by more than 50%. Geographical effects are pronounced due to various abiotic conditions that include not only high temperatures but also drought, salinity, water logging, etc. A study carried out by the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences, China, showed that the toxin content in Bt cotton varieties changed significantly over time, depending on the part of the plant, the growth stage and the variety. Generally, the toxin protein was expressed at high levels during the early stages of growth, declined in mid-season, and rebounded late in the season.
- Plants contain many secondary compounds such as phenols, orthoquinones, terpenoids and tannins. Studies have shown that the concentrations of these compounds vary according to the age of the plant and exposure to external factors. Some of these compounds create synergies with Bt toxins (gossypol), while others (tannins) create negative interference. Kranthi *et al*. (2005) reported that, in times of stress, the relative increase in the concentration of gossypol makes up for the reduced concentration of Bt toxin. These findings show that changes in efficacy do not depend exclusively on the level of Bt toxin in the plant, but also on the plant's physiological condition. Similar lines of study on transgenic potatoes found that foliar glycoalkaloids in transgenic potato varieties affected the anti- insect benefits of the transgenes. This kind of research has not been done on cotton but it may probably be required. The conclusions of such studies might even help to enhance the plant's ability to produce greater amounts of toxins.
- Insect exposure to the same chemicals over a long period of time (i.e., frequent applications of the same insecticide year after year) enables the insect to develop the capacity to tolerate insecticide concentrations considerably greater than the recommended doses. Sucking, as well as chewing insects, are equally capable of developing such tolerances. Lepidopterans, for example, have developed resistance to insecticides in many countries and it is generally admitted that target insects can develop resistance to Bt toxins; in fact, resistance to the Bollgard Cry1Ac gene has been confirmed in many countries. Once a target insect develops resistance, the variations and fluctuations of endotoxin concentrations resulting from the many factors mentioned above will require greater numbers of insecticide applications, even on dual-gene biotech cotton.

New Insect-resistant Bt Based Technologies

The two new insect-resistant technologies that are expected to become available for commercial use in the next few years are Bollgard III® and TwinLink™. Both technologies are pending regulatory registration and the appropriate approvals, but they are already undergoing extensive testing in Australia and the USA. Last year, Monsanto applied to the US Environmental Protection Agency for permission to carry out field tests of genetically engineered triple-gene insect-resistant Bollgard

III cotton. Bollgard III combines the established Bollgard II gene (MON15985), which produces Cry1Ac and Cry2Ab2 toxins, with Syngenta's COT102 (Vip3Aa19), for the control of lepidopteran insects. The primary objective of the new technologies in the new products that are expected to come on line in the near future is to prevent the development of resistance for as long as possible and increase the spectrum of lepidopteran insects effectively controlled by the toxins. Bollgard III is expected to provide better protection against fall armyworms. Some early trials conducted on non-Bt cotton, Bollgard II and Bollgard III indicated that fall armyworm larvae were capable of penetrating 47%, 18%, and 3% of bolls on non-Bt cotton, Bollgard II, and Bollgard III, respectively.

The TwinLink™ Bt technology will offer an alternative to existing Bt technologies. The TwinLink™ insect resistance technology contains two Cry genes, expressing Cry1Ab and Cry2Ae proteins targeting lepidopteran pests on cotton. A number of trials were conducted in 2010 to further characterize lepidopteran control, confirm agronomic performance, compare varietal background performance, and determine protein expression profiles. The findings showed that plots of non-Bt cotton suffered 100% boll damage from bollworms. On the same date, plots of protected TwinLink™ technology sustained less than 10% damage to bolls, while bollworms caused about 15% boll damage in unprotected TwinLink™ plots. Average seasonal boll damage was less than 10% and 20% in protected and unprotected plots of TwinLink™ cotton, respectively. Yields from protected and unprotected plots of TwinLink™ cotton were similar, indicating that performance was only minimally increased with supplemental control of bollworms.

In another study, Cry1Ab and Cry2Ae protein concentrations were determined by protein extraction from terminal leaf tissue and quantitative, colorimetric ELISA procedure. Five locations and multiple genetic backgrounds were sampled for six consecutive weeks during the flowering and boll set period. The data indicate that, as with other Cry1 proteins, a slight decline of the TwinLink™ Cry1Ab protein takes place as the cotton plant matures. However, the Cry2Ae protein in TwinLink™ either maintained, or numerically increased its expression level through to maturity. These data indicate that under certain conditions, including extreme lepidopteran pressure (Greene *el al*., 2011), supplemental lepidopteran control may be needed to bolster the efficacy of the TwinLink™ technology

The discussion of the data above ndicates that it is not only the existing insect-resistant biotech cottons that may require additional spraying; the new Bt technologies (Bollgard III and TwinLink™) that are in the pipeline for approval (and expected, hopefully, to be in use within the next 2-3 years) will also need insecticide applications to get maximum yields. However, it is equally true that this situation is also linked to insect pressure and, as such, may vary from year to year. Which gene is effective against which lepidopteran will always be a factor in determining the need for insecticide applications, and at what economic net returns.

Conclusion

Insecticides are sprayed on non-biotech cotton based on certain thresholds for various pests. Thresholds may be based on a combined level of assessment of various pests. The use of a combined threshold might prevent the losses from one pest from reaching too high a level before insecticide applications are made. When a threshold is verified on non-biotech cotton it generally means that at least some damage has already been sustained prior to the initiation of insecticide application. Conversely, with insect-resistant biotech cotton, there is no threshold for any target pest and there is 100% pest control under all circumstances. If Bt proteins are not 100% effective, as has been shown to be the case with Bollgard II and WideStrike, which allow some bollworm damage in the field, spraying of insecticides may increase yields. Damage and benefits vary from year to year depending on the level of pest pressure. Data and annual surveys have shown that in the USA, Bollgard II and WideStrike varieties benefit from a single application of insecticide against the bollworm. Bollworm treatment increased lint yields by an average of 78 kg/ha and 125 kg/ha respectively with Bollgard II and WideStrike across all varieties and years evaluated. When bollworm insecticide costs, estimated insect protection and seed technology fees were factored into the equation, Bollgard II and WideStrike varieties provided economic returns as expressed in terms of bollworm control. Furthermore, everything seems to indicate that the more effective technologies expected to come on line within the next 2-3 years may also provide economic returns as expressed in fewer insecticide applications.

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Cotton Color: Measurement and Discoloration

The two most used independent parameters of cotton color are: degree of reflectance (Rd) and yellowness (+b). Reflectance indicates how bright or dull the cotton is, and yellowness indicates the degree of color pigmentation. The color code is determined by locating the point at which the Rd and +b values intersect on the Nickerson-Hunter cotton colorimeter diagram for Upland cotton.

The amount of sunlight, day and night temperatures during growth, agronomic inputs and their time of application are responsible for most of the variations in fiber quality parameters within a single variety. Fiber color is also affected by the same factors. Cotton grade is a composite assessment of color, trash and preparation. Each feature is judged separately, but a qualified classer integrates his assessment of the three diverse parameters into a composite grade. Color assessment is a primary category used to assign a grade to cotton. Trash used to be the second most important factor in determining quality, but advances in equipment have made it increasingly easy to eliminate plant materials from cotton, thus underscoring the significance of color as an important quality factor. While measuring fiber quality parameters has improved a great deal, adding to the reliability and repeatability of data, color measurement has not achieved similar successes. Part of the problem is that human color perception results from the interaction of three components: a light source, an object, and a detector (the eye and brain in the case of manual grading). So, when the color of a sample is measured, it is in fact a process whereby color, as perceived by humans, is measured and described by the naked eye or by a color-measuring instrument (HVI, Colorimeter, etc.). This article deals with the latest developments in fiber color measurement and progressive changes in color due to various reasons.

Measuring Color

Cotton color can be judged visually with the naked eye or measured mechanically with the help of different kinds of instruments. Naked-eye assessment of color, often referred to as classer's color grade, can be affected by the light under which a cotton sample is observed and by the general surroundings (table color, wall color, etc.). If the color of a sample has to be judged visually by a classer, the lab must make certain that the proper lighting is provided. Humans classify samples by visual comparison with a set of physical standards under standard illumination. The assessment is made in a room with dark grey walls, and samples are placed on a black table with an incident light intensity of 1,200 lx. The more experienced the classer is in judging cotton color, the more precise the assessment he/she can make. Classers require special training before qualifying for the job of assessing cotton color.

The US Department of Agriculture (USDA) started developing instruments to assess cotton color in the 1930's. The two criteria for color measurement (Rd and +b) were introduced at the time. According to Matusiak and Walawska (2010), Nickerson and Hunter developed an objective method of measuring color using a Colorimeter in the early 1940's. The Hunter scale used in a Nickerson Hunter Cotton Colorimeter indicates the percentage of reflection (Rd) in a vertical direction, which is a measure of the lightness of a sample, and in a horizontal direction the color code is determined by locating the point at which the Rd and +b values intersect on the diagram. During a color test, photodiodes absorb filtered light from the illuminated sample, and a microprocessor expresses the results in terms of the lightness and yellowness of the sample. Matusiak and Walawska reported (2010) that colorimetry technology was incorporated into HVI testing

equipment during the 1970's. HVI testing employs a dual Xenon light source to illuminate a sample window measuring 7.1 cm by 9.1 cm. The two lights located at an angle of 45° are flashed on a window containing a compressed cotton sample. The light reflected is measured by two detectors, and signals from these lights are used to calculate the sample's Rd and +b. The ITMF International Committee on Cotton Testing Methods recommends measurement of color by HVI.

According to the standardized procedures developed by USDA, there are 25 official color grades plus 5 categories of below-grade color. Color gives an indication of the fibers' ability to take dyes in the manufacturing process. The greater part of the cotton crop in the USA is classed as white 21, 31, or 41 color grades. Grades 32 or 42 would be light spotted grades. Pima cotton has no grades because it is usually ginned on roller gins, so preparation is different. Moreover, Pima also has a deeper yellow color than Upland cotton.

HVI color measurement also requires special lighting. Lighting through a north skylight installed over the shoulder is considered suitable for accurate assessment of color. It is also recommended that surroundings be off-white to light gray. Ceilings should not be more than 9.5 on the Munsell Neutral Color Scale. It should also be assured that the walls of classing rooms are no darker than 9.0 on the Munsell Neutral Color Scale.

USDA produces and supplies International and HVI Calibration Cottons to calibrate all the machinery involved in HVI measurement of quality characteristics. However, in the case of color, ceramic tiles, not cotton samples, are used to calibrate the machines. The USDA provides different tiles for different makes and models of HVI. It is widely accepted that there is no generally recognized traceability for these standards. Thus, effective July 1, 2000, the USDA Cotton Program accepted and implemented the HVI color grade as the official color grade standard. In support of these grades, the USDA Cotton Program maintains two master colorimeters in Memphis, Tennessee, to ensure the consistency of values on all HVI color calibration tiles and cotton color standards for Rd and +b. The master colorimeters are also calibrated with the help of tiles. In order to monitor long-term color calibration accuracy, master cotton color standards are kept in cold storage. These so-called "Freezer Cotton Standards" are measured by each master colorimeter on a quarterly basis (Knowlton, 2008). Permitted tolerances of color for Rd and +b are 1.0 and 0.5 units respectively. It would be desirable to have International Calibration Cotton for color that is reliable. Currently, USDA provides cotton color check boxes, but the problem is that color (mainly +b) changes as cotton becomes older. Therefore it is doubtful that a change from tile calibration to cotton calibration could be useful.

Manual and HVI color grading can produce different results. HVI data are affected by trash in cotton and the classer's grade is largely dependent on his/her ability. Experience has shown repeatability among classer grades is only about 70%, and even lower levels of repeatability have been reported between HVI and classers' grades. The two areas with the most significant opportunities and challenges for updating and improving rapid and precise measurement of cotton color are: 1) better understanding and application of the present color system (Rd and +b) to well-known color systems and 2) the development of verifiable or "traceable" cotton color standards from authentic sources. In that light, new procedures and color systems are being investigated to update and improve present color measurements, as well as the color standards protocols of USDA's Agriculture Marketing Service (Rogers and Thibodeaux, 2006).

Color Change and Measuring Color on-Site at Remote Locations

Cotton color is dependent on the specific variety and its genotypic interaction with the environment as well as on the inputs applied. Unfortunately, cotton color is not stable over time, whether in the field or packed in bales. The following factors have a significant impact on cotton color.

- Upland cotton is naturally bright white in color. Continuous exposure in the field to weathering and low action of microorganisms may cause cotton to lose its brightness and become darker, but this is true not only under field conditions; weathering can also change the color of cotton even after it has been ginned and packed. Once a boll opens, five to seven days should be allowed for the lint to die and dry in the field before it is picked.
- Lack of irrigation water or frost can force bolls to open prematurely. We know that immature bolls produce weak fibers with subnormal micronaire. Cotton harvested from immature bolls is also characterized by increased saturation of yellow color. Immature fibers are also prone to discoloration faster than mature fibers.
- Some bollworms affect only a portion of the boll. If a bollworm eats a few seeds in the boll, the hollow seed and its adjoining seeds may produce

Official Grade Designations for Upland Cotton

	White	Light spotted	Spotted	Tinged	Yellow Stained
Good Middling (GM)	11	12	13	-	-
Strict Middling (SM)	21	22	23	24	25
Middling (M)	31	32	33	34	35
Strict Low Middling (SLM)	41	42	43	44	-
Low Middling (LM)	51	52	53	54	-
Strict Good Ordinary (SGO)	61	62	63	-	-
Good Ordinary (GO)	71	-	-	-	-
Below Grade	81	82	83	64	85

spotted cotton with a yellowish tinge, enough to have a significant impact on the grade of cotton.

- Even if the boll matures normally and is harvested on time, machine picking may stain cotton with contaminants like green leaves, grease, oil, etc., to a degree sufficient to lower its grade.
- Every boll cannot be picked at its optimal picking time. Delays in picking and continuous dew on the open bolls can also bring about changes in the natural color of cotton.
- Moisture and temperature are the two most important factors affecting cotton color after picking and during storage.

Reports show that some cotton bales, especially those transported overseas, change color significantly; particularly noticeable is the variance in +b from the initial HVI measurement. Based on the same hypothesis, Rodgers *et al*. (2011) tested cotton color "on-site remote locations" using portable spectrophotometer and compared it with HVI data obtained from samples cut after ginning. Researchers performed several comparisons between spectrophotometer color parameters and HVI readings for +b and found the best overall results to be HVI +b and HunterLab MiniScan EZ spectrophotometer (MSEZ). They found an excellent linear match between HVI +b and MSEZ b* readings for both ceramic tiles and cotton biscuits, with slopes near unity and high correlations (> 0.97). It may be remembered that USDA provides two types of color standards: a set of five ceramic tiles and a set of 12 cotton fiber biscuits. Color matches between the tile and biscuit standards were slightly different, but significant, indicating that the spectrophotometer is capable of measuring colors to an acceptable degree of precision.

The data further revealed that the HVI +b values were generally ~1.3 units lower than the MSEZ b* values. When adjustment was made for this difference, excellent agreement was observed between HVI +b and MSEZ b*.

Color Change in Storage

The negative effects of weathering in the field or of storage on lint color are well recognized. Studies have shown that most of the coloring effect was due to yellowing. Yellowing was primarily linked to moisture content, days stored, average air temperature during storage and the temperature of the lint at the time it was stored. Density in storage, aeration and sunlight will also affect the yellowing process. Moisture content above 14 percent sharply increases yellowing. The second most important factor having an affect on lint color or yellowing is temperature.

Moisture

When cotton is brought in from fields, it usually has higher-than-optimal moisture content for proper ginning. Thus, the lint is usually dried to 6-8%. The dryer cotton is more easily cleaned, thus making for a better leaf grade. Drying, in principle, lowers fiber strength; therefore, excessive drying can cause more fiber breakage during ginning, which results in lower yarn quality. There is also a tendency to add water to seedcotton for different reasons, including bale weight. Higher moisture in seedcotton reduces fiber breakage and also requires less energy to press bales. Chun and Anthony (2004) added water at the gin lint slide at the rate of 0, 5.9, 9.1, 21.8 and 25.0 kg per bale as an over spray before pressing cotton into bales. They then stored the cotton for four months to see the effects of higher fiber moisture on microbial activity, cotton color and other fiber quality characteristics. The bales were packed at universal density of approximately 448.5 kg/m^3 and a bale size of 53.3 cm x 139.7 cm x 78.7 cm. After 116 days, the bales were opened and samples were taken and tested for various parameters. Some characteristics were affected negatively while others were affected positively. Cotton color decreased from middling to strict low middling in four months. Lint became darker and more yellow with the higher amount of moisture. The data also showed that the addition of moisture increased microbial activity, particularly mold, which can have further consequences for the color of the lint. The effects on color are shown below.

Effect of Moisture Addition on Cotton Color

Moisture Added	Rd	+b	Color Grade
No water added	75.7	8.5	31
5.9 kg/bale	74.7	8.9	31
9.1 kg/bale	73.6	9.3	41
21.8 kg/bale	70.6	10.1	42
25.0 kg/bale	69.3	10.6	43

Cotton is baled and packed well below its equilibrium moisture content in storage. So, if a bale is not properly pressed, lint can absorb moisture, consequently deteriorating color at a given stage. Similar findings on the detrimental effect of moisture on cotton stored for months have been reported in the literature, including Anthony (2002a).

Temperature and Bale Covering

Spinners recommend wrapping bales in cotton fabric for the purpose of avoiding contamination. However, cotton continues to be wrapped in polyethylene film, woven polypropylene, and woven burlap. The ranking of bale covering materials from least porous to most porous is: polyethylene film, woven polypropylene, cotton and woven burlap. Regarding the effect of surface area exposure on cotton color, it is reasonable to assume that the more porous a bale covering, the greater the fiber surface area that will be exposed to atmospheric conditions. The literature shows that fiber color +b values were significantly affected by bale covering type and atmospheric conditions over time. This is important because stored bales are used to form blended laydowns for mills to produce even colored yarns and fabrics. Some reports show that color +b of

0.15 had significant consequences while other studies show that only when the variance was over 0.38 +b units would there be significant consequences during finishing. High temperatures and high humidity work together to produce changes in fiber color +b. The relationship between fungus growth on fibers and their response to temperature explains why summer ageing can result in significant changes in yellowness +b.

Effect of Trash on Color Measurement

As mentioned above, cotton color is an interaction of reflectance and yellowness. Reflectance can be affected by trash in cotton. Dr. Malgorzata Matusiak of the Textile Research Institute of Poland presented a paper at a meeting of the International Committee on Cotton Testing Methods of the International Textile Manufacturers Federation (ITMF) in Bremen, Germany in March 2010 dealing with the 'Influence of trash on color measurement.' Dr. Matusiak tested 20 cotton samples on an HVI for Rd and +b with trash and without trash. She found that in 70% of the cases, color grade changed/ improved as a result of removing trash. At the same meeting, Mr. James Knowlton of the US Department of Agriculture also presented a paper on the same subject. He took four samples of cotton and measured color grade at four stages. The first test was on the sample on arrival. The second test was done after leaf trash was picked out to lower the leaf content by 1 to 2 leaf grades. The third test was done after picking leaf trash to lower the leaf contents by another 1 to 2 leaf grades. In the fourth test, grade standard cotton biscuits were used as a control parameter. Mr. Knowlton used a Master Xenon Color/ Trashmeter for testing color and leaf grade and concluded that:

- Cotton color classification is based on total sample color and not fiber color alone.
- Rd is affected by leaf content, Rd decreases with increasing leaf content.
- +b is not affected by leaf content.
- Quantifying the degree of color change to leaf change (based on instrument measurements) is possible in grade standard cotton biscuits, but not in raw cotton.

Mr. Hossein Ghorashi of Uster Technologies presented a similar paper at a meeting of the ITMF International Committee on Cotton Testing Methods held in Bremen, Germany on April 1-2, 2008. Mr. Ghorashi explained that they manipulated the USDA Color Grades Standards by adding trash particles systematically and then checking Rd and +b on HVI. The Uster Technologies data proved that there is a strong inverse correlation between Rd values and trash content levels as determined by the USDA Color Grades Standards. Conversely, +b values were not affected by the addition of trash. Mr. Ghorashi observed that the relationships developed as a result of his work might be used to correct HVI color measurements. There are, however, some questions about the relationship of trash content with Rd values. Trash is measured on the surface: if it is uniformly spread throughout the sample, that is fine, but it is quite possible that trash measured on the surface may not represent the actual amount of trash in the sample.

Reproducibility of Color Results

Reproducibility and repeatability of the color data is not satisfactory. It has already been shown that the amount of trash in cotton can affect its Rd value when tested on a HVI. One way to test color and avoid the effect of trash would be to test Rd by spectrophotometer. Matusiak and Walawska (2008) compared HVI data with the data from the Datacolor 650 spectrophotometer. The color coordinates determined on the spectrophotometer were L* -lightness, a* -green/red, b* -blue/ yellow, C* -chroma and h -hue angle. Measurements were made at different illuminants, D 65 (Daylight), A (Tungsten) and F 11(TL 84). Color indexes were calculated on the basis of the measured color parameters as per the CIE whiteness index, the Stephensen whiteness index and the D 1925 yellowness index. Matusiak and Walawska (2008) concluded that there is a strong correlation between the results of cotton color measurement by HVI and by spectrophotometer. However, cotton samples classified into the same color grade on HVI actually differ from each other in the range of L*, a* and b* on the spectrophotometer.

Rogers *et al*. (2006) studied the impact on color measurement of using various instruments and sampling procedures. The samples analyzed were color tiles, AMS (USDA Agriculture Marketing Service) tiles, and AMS cotton batts or "bricks." The samples were measured on both bench-top and portable instruments from various color instrument manufacturers. They observed that the primary variable that impacted the color agreement between units was the use of HVI glass in front of the sample. The impact of this glass on the consistency of the portable unit's readings was severe.

The HVI results also showed that color assessment by HVI can be different from the classer's grade. The level of confidence can differ as a result of many factors, but it is estimated that, on average, HVI data tally with the classer's grade only 70% of the time. This is true not only for spectrophotometer vs. HVI data; HVI data recorded from one machine also showed lower repeatability on other machines. Different HVI systems may also produce different readings. The ICAC Task Force on Commercial Standardization of Instrument Testing of Cotton has conducted round trials with laboratories around the world, and has come to the same conclusion. Sample preparation, HVI calibration, condition of color tiles, etc., are among the possible factors responsible for low repeatability.

Coloring Fabrics

Cotton is a hygroscopic fiber and swells in a high humidity environment, in water and in concentrated solutions of certain acids, salts and bases. Cotton is known to show excellent resistance to alkalis. During the process of chemical treatment in dyeing and finishing, degradation is usually caused by

oxidation, hydrolysis, or both. Most of the surface chemicals on cotton, including insecticides, defoliants and desiccants, if any, are washed away during the bleaching process. It is yarn, not single fibers, that undergoes the greatest number of chemical treatments. Most chemicals are applied to yarn or fabric for the sake of imparting color or avoiding shrinkage. Color fastness or Colorlock in a yarn or fabric is achieved through heating. Long exposure of fabric to visible and ultraviolet light, especially in the presence of high temperatures around 250-397° C and humidity can degrade the color of cotton. Yellowing or color change may result in a weakening of the fabric, or even its complete disintegration. There is less deterioration if cotton is mercerized, but mercerized cotton is somewhat more susceptible to oxidation and to hydrolysis. (http://cotton.missouri.edu/Classroom-Resistance.html).

Cotton fabrics form part of a variety of end use products in which functional performance and visual appearance are of paramount importance. Thus, textiles known as industrial, technical or by any other term, must maintain their physical and mechanical properties throughout the service life of the material. Colorants or dyes are mostly applied in an aqueous solution. Many times, not one but many colors are applied and it is very important that all colors are applied uniformly. Textiles are dyed only after surface impurities, for example fiber wax, spin finishes, and particulate dust have been removed by appropriate treatments. Such treatments, i.e. de-sizing and scouring, impart stable whiteness to a fabric. Bleaching is also done to add stable whiteness to fabrics. Mercerization improves luster, tensile strength, dimensional stability and moisture regain. But dead fiber or fibers with little or no secondary wall benefit comparatively less from mercerization. Color deterioration indicates reduced processing ability of the fiber in addition to lower market price. Color deterioration renders fibers relatively more unable to take dyes than equivalent non-color-deteriorated fibers. Even when color-deteriorated cotton is able to take dyes as well as non-color-deteriorated cotton, the ability of discolored fibers to hold dyes is diminished. Furthermore, when discolored cotton is dyed it may not take the same finish as non-deteriorated cotton.

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Role of Biotechnology in Sustainable Development of Cotton

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Abstract

The prospects of biotechnology to provide cost-efficient sustainable cotton production under a safe environment for the 21st century are enormous. The role of plant biotechnology in the improvement of cotton is a rapidly evolving area and very broad. The specific objective of this paper is to provide a report on three specific areas including transgenic technology, marker assisted selection and cotton genome sequencing with reference to the role of biotechnology in cotton improvement. The transgenic technologies in cotton are specifically targeted to overcome two major problems in cotton production: 1) the high cost of weed management and 2) the severe yield reduction caused by fruit-feeding lepidopteran insects, especially bollworm. The success story of transgenic technologies using *Bt* and herbicide tolerant genes in the improvement of cotton remains an outstanding scientific accomplishment. To improve the efficiency and delay the development of insect resistance to the Cry1Ac gene in cotton lines, a pyramiding method of at least two or more genes. Cry2Ab, Cry1F and Cry1Ac genes have been applied in the second generation of *Bt* genes after 2009. The transgenic insect and herbicide resistant cotton

lines have demonstrated the great potential of biotechnology in sustainable cotton production. Recent studies showed that the new emerging tool of RNAi technology will also have great impacts in improving seed quality as a food source and also in providing resistance genes against pests and diseases including nematodes. Marker assisted selection (MAS) will provide breeders an efficient selection tool to augment traditional phenotypic selection with a gel-based DNA marker in the laboratory. The Simple Sequence Repeat (SSR) and Single nucleotide polymorphic (SNP) markers will be used as potential markers in MAS to expedite cotton-breeding programs. Recently, a team of scientists from both public and private sectors initiated a partnership to put forward a report on the problems and prospects of cotton genome sequencing. Scientists at the University of Georgia, USA and at the Texas Tech University, USA have led multidisciplinary international teams, which are nearing completion of the sequence of the ancestral D and A genome diploid species respectively of upland cotton. Knowledge gained from decoding the cotton genome will improve understanding of genes at the molecular levels and help to unlock the mystery of genetics for improving yield and fiber quality. Considering both environmental and economical impacts of the new technologies and cotton as a major cash crop in both developed and developing countries it is high time to consider establishing an independent international cotton research center. The center should recommend the complex, system-oriented solutions based on a knowledge-intensive plan using new emerging biotechnology tools for global food and fiber security.

Agricultural biotechnology will play a major role in providing cost-efficient sustainable cotton production in a safe environment for the 21st century. The world population is projected to be over nine billion people by 2050 (Wakelyn and Chaudhry, 2010) and the rapid increase in population demands doubling the current production levels for global food and fiber security within the next four decades. Farmers will have to fulfill this demand under conditions of rapidly declining agricultural resources including land and water. Biotechnology will be at the forefront of agricultural invention and innovation to solve many of these challenges. The power of new technologies to accelerate agricultural development is nowhere more visible than cotton because of the success story of biotech cotton.

Cotton, *Gossypium* spp., is the most important natural fiber source for the textile industry and is the major cash crop in 70 countries including USA, China and India (Smith and Coyle, 1997). It is a source of many renewable products including textiles for clothing, home insulation materials to save energy, protein- and energy-rich animal feeds, Human food in the form of cooking oil and efficient use of energy through the use of the mulch and biomass (Cotton Incorporated, 2010). Scientists are also exploring the future potential of cottonseed as an important food source for people due to its high nutritional value (Sunilkumar *et al*., 2006).

Cotton is one of the important crops that hold incredible promise to enjoy the benefits of plant biotechnology. It is one of the few crops to enjoy the benefits of genetic engineering since the introduction of Bt cotton in 1996. Currently, biotech cotton is grown on over 60% of world cotton production areas (Wakelyn and Chaudhry, 2010). The discussion on the role of plant biotechnology in the improvement of cotton is a rapidly evolving area and very broad encompassing basic and strategic research and its application. This paper will focus on three specific areas on the role of biotechnology in cotton improvement considering its great impact: 1) use of transgenic technology in economically and environmentally sustainable cotton production, 2) marker assisted selection to expedite cotton breeding programs, and 3) the future of cotton genome sequencing to unlock the secrets of genetics for the improvement of cotton.

Transgenic Technologies

Transgenic technology uses exogenous DNA or RNA sequences by recombinant DNA technology to create transgenic organisms that express novel and agriculturally useful traits. The transgenic technologies in cotton are specifically targeted to overcome two major limitations in cotton production: 1) high cost of weed management in cultivation due to slow cotton seedlings' growth compared to weeds (herbicide tolerance) and 2) the reduction in fiber yield due to severe infestation from fruit-feeding lepidopteran insects, especially the bollworm (Hake, 2010).

A recent report estimated that the value of plant protection chemicals used at the global level is about $32 billion per year and 16% of all global insecticides are used to protect cotton (Kranthi and Kranthi, 2010). Cotton's share in pesticide consumption has declined by about 43% from 1986 to 2009 since the introduction of genetically engineered *Bt* cotton (Wakelyn and Chaudhry, 2010). It has also been reported recently in a special issue of *Nature* that the use of *Bt* cotton helps to improve yield by over 60% of that of conventional varieties and avoids at least 2.4 million cases of pesticide poisoning in Indian farmers each year, saving US$14 million in annual health costs (Whitfield, 2003). The success story of *Bt* genes in the improvement of cotton remains one of the most shining accomplishments in agriculture in the history of mankind. ICAC published several reports on the use of transgenic technologies in cotton from the time of introduction to the present from experts (ICAC 2000, ICAC 2004, Hake 2010, Kranti and Kranti, 2010). Many of the thoughts in this paper with reference to transgenic cotton are collected from these papers and a recently published book *Cotton: Technology for the 21st Century* by ICAC (Wakelyn and Chaudhry, 2010). Readers are encouraged to review these for detail references.

As of 2010, twelve countries including Argentina, Australia, Brazil, Burking Faso, China, Colombia, India, Indonesia, Mexico, Pakistan, South Africa and United States have officially approved biotech cotton planting since its inception in 1995 (Hake, 2010). Over the past two decades, demand for increased yields forced farmers to use more insecticides in

pest management. This caused more insect species to develop resistance to insecticides and as a consequence high levels of pest resistance, especially bollworm resistance, caused a crisis in cotton pest management (Kranti and Kranti, 2010). The United States of America was among the first to commercially release *Bt* cotton incorporating the *Cry1Ac* gene (derived from soil bacterium *Bacillus thuringiensis*) in 1996. The *Bt* toxin expressed in biotech cotton protects the fruit from lepidopteran insects but the toxin is safe to all other non-target organisms including beneficial insects, birds, fish, animals and humans (Hake, 2010). It has been estimated that most of the cotton area in Australia, China, India, Mexico, South Africa and USA is under transgenic cotton now covering over 15 million hectares worldwide (Kranti and Kranti, 2010). However, the single gene *Bt* technology (Bollgard 1™) registered with the U.S. Environmental Protection Agency has been voluntarily withdrawn due to concerns about the development of resistance to the toxin by selected insects (Dodds and Bond, 2010). To improve the efficiency and delay the development of insect resistance to the *Cry1Ac* gene in cotton, a strategy of pyramiding at least two or more genes like *Cry2Ab*, *Cry1F* and *Cry1Ac* has been applied to the second generation of *Bt* genes after 2009.

The high cost of weed management was always a major concern to cotton growers. The discovery of glyphosate-resistant cotton technology helped in the development of transgenic cotton lines with enhanced tolerance to glyphosate, an herbicide used to control weeds in cotton fields. The first herbicide tolerant gene to the herbicide bromoxynil in cotton varieties, sold under the trade name BXN, was developed using the *bxn* gene from the natural soil bacteria *Klebsiella pneumonia* subspecies *ozaenae* (Dodds and Bond, 2010). The plant containing this gene produces an enzyme, which detoxifies bromoxynil to its primary metabolite. The transgenic cotton lines resistant to glyphosate were developed by incorporating the glyphosate-insensitive EPSPS enzyme gene from *Agrobacterium* spp. strain CP4 (Green, 2009). Glyphostae-resistant and second generation glyphosate-resistant cotton (Roundup Ready Flex™) covered about 35% of total cotton acreage in USA and 80% of total cotton areas in Australia in 2006 (Holtzapffel *et al.,* 2008; USDA-AMS 2008; Wreth *et al*., 2008). Since 2009, almost all commercial transgenic cotton varieties in U.S.A. contain second generation of glyphosate-resistant technology in addition to the stacked insect resistant *Bt* genes (Dodds and Bond, 2010). The transgenic insect and herbicide resistant cotton varieties have raised our expectations for a continued flow of scientific accomplishments in sustainable cotton production for the 21st century.

RNAi Technology in Cotton Improvement

Scientists in cotton research will soon witness the legacy of biotechnology in another new emerging area of RNAi technology. Recently, the development of a new technology in which a double-stranded RNA (dsRNA) is introduced into an organism to induce sequence-specific RNA interference (RNAi) of a target transcript has become a powerful tool to discover gene function (Serenella *et al.*, 2007). RNAi is a new emerging technique based on homology-dependent post transcriptional gene silencing, induced by double stranded RNA (dsRNA). Recently, several papers have been published describing the merits of this method in plant sciences (Nui *et al.*, 2010; Sindhu *et al.*, 2009; Kranti and Kranti, 2010). A source of dsRNA must be introduced through transformation techniques into a plant's DNA to create transgenic plants with RNAi-mediated traits, which can pass those traits on to the next generation. Selective inactivation of the genes using encoded DNA through RNAi technology will have great potential in future cotton improvement due to its high specificity, stability and efficacy especially in the area of plant resistance against pests, pathogens and nematodes and improvement of seed quality.

For every one kilogram of fiber, the cotton plant produces about 1.65 kg of seeds which is an important source of high quality protein (23%) and oil (21%), and cotton is the third largest field crop in terms of edible oil seeds in the world (Sunilkumar *et al*., 2006). The cotton seed grown worldwide can provide enough protein to feed 500 million people per year (Star Tribune, 2009). However, the seeds also contain gossypol glands that provide resistance against insects, but gossypol lowers blood potassium to dangerous levels in humans and can harm the heart and liver in people and animals (Star Tribune, 2009). Cotton seed is used primarily as animal feed because the bovines' stomachs gradually digest the poisonous gossypol rendering it harmless to the animal. Recently Dr. Keerti S. Rathore's group at the Texas A&M University used the RNAi technology and made a breakthrough discovery to develop cotton plants eliminating gossypol production in the seed, leaving gossypol production to continue in stems, leaves, and flowers to protect the plant against insects (Sunilkumar *et al*., 2006). This discovery provides a new tool for the use of cotton seeds as an important source of food products.

The RNAi technology also has great promise in controlling pests and pathogens in cotton. Researchers have discovered potential genes that are lethal for reproduction or fitness when silenced to free living nematodes. Scientists showed that silencing four genes from parasitic nematode through RNAi technology led to a reduction in the number of mature nematode females in transgenic *Arabidopsis thaliana* (Sindhu *et al*., 2009). RNAi-mediated plant resistance has greater potential over conventional *Bt* resistant transgenic cotton plants (Niu *et al*., 2010). For example, many pests and pathogens share distinct lineages and homologues of important genes, so silencing the appropriate target genes may provide resistance against a broad group of multiple pests or pathogen organisms. Also the resistance is more stable because it is based on RNA hybridization on a few nucleotides rather than protein-protein interaction and the potential of mutation to impede RNA hybridization is less (Escobar *et al*., 2001). So there will be less potential of pests overcoming the resistance.

Theoretically, all pests and pathogens carry genes with detrimental knockdown phenotypes and identifying these target genes will provide a scope to use RNAi technology to make transgenic plants resistant against these pests and pathogens promoting eco-friendly crop protection methods (Niu *et al*., 2010). One of the difficulties in RNAi technology is to identify genes that can be effective through a suitable delivery system. For example, dsRNA rapidly breakdown in the digestive system of mammals and fail to uptake RNA into cells.

Although the transgenic technologies provide enormous benefits to produce higher agricultural yields with fewer resources and less environmental impact, sweeping adoption of these techniques without appropriate regulation concerns many about its potential hazardous environmental and health impacts. This is especially important considering that many countries have not developed appropriate safety and regulatory policies. As a consequence, many countries face new problems with pest management. For example, new sucking pests have emerged as major pests in India due to low usage of insecticides causing significant economic losses to cotton production (Kranti and Kranti, 2010). It is important to practice integrated pest and weed management with transgenic cotton varieties using proper regulatory control for cost-effective sustainable cotton production. For example, the widespread use of glyphosate-resistant technology has lead to a shift to some weed species that are tolerant to glyphosate in the USA (Holtzapffel *et al*., 2008). Weeds can develop herbicide resistance due to selection pressure by excessive use of herbicides applied to the population. *Palmer amaranth* is one of the most important herbicide-resistant weeds found in the US cotton fields (Bennett, 2007). There is a possibility that cross contamination of pollen can also transmit the herbicide resistant trait to the wild relatives located in the same areas of cultivated cotton.

Marker Assisted Selection to Expedite Cotton Breeding Programs

The principle of plant breeding is based on the selection of desirable traits and assembling more desirable combinations of traits in a specific plant. Marker assisted selection (MAS), a tool in plant biotechnology, provides breeders with an efficient selection systems to replace traditional phenotypic-pedigree-based selection with a gel-based DNA marker in the laboratory. MAS is an indirect selection process where a trait of interest is selected, not based on the trait itself, but on a DNA marker linked to the trait of interest. Most of the economically important traits in cotton are controlled by complex quantitative trait loci (QTL) consisting of many genes affecting the phenotypes. DNA markers are "landmarks' on the genome that can be selected for their close proximity to a QTL of interest. The selection of DNA markers linked to the QTL of interest increases the efficiency of breeding, decreasing costly, lengthy and subjective phenotypic selection and accordingly reducing significantly backcross generations.

MAS will have great potential in the following areas of cotton breeding program: 1) marker assisted pyramiding, 2) marker assisted backcrossing, 3) study of heterosis, 4) assessment of genetic diversity and parental selection, 5) cultivar identity and assessment of seed purity, and 6) marker assisted evaluation of breeding materials (Bertrand and Mackill, 2008**).** MAS will be very effective in cotton when breeders will use it in early generations because plants with undesirable gene combinations can be discarded and a lesser number of high-priority lines can be used in subsequent generations. Also if the linkage between the marker and the selected QTL is not very tight, the greatest efficiency of MAS is in early generations due to the increasing probability of recombination between the marker and QTL. The major disadvantage of applying MAS at early generations is the cost of genotyping a larger number of plants in the population (Bertrand and Mackill, 2008).

Identification of informative DNA markers is the first critical step to develop a marker assisted breeding program and to expedite the variety development program. The major limiting factor in the use of DNA markers is the limited number of informative markers useful for MAS in cotton. The success of MAS is based on the information on the association of the markers with the traits of interest based on molecular map. Most of the molecular maps in cotton are based on recombination map of a population developed from the crosses of specific parents of interest in a program. However, it has been reported that QTLs identified in a particular mapping population may not be effective in different genetic backgrounds (Liao *et al*., 2001). Some collaborative studies to develop molecular methods of association mapping are reported by Abdurakhmonov *et al*. (2008 and 2009). This novel method of association mapping strategy provided a statistically more powerful tool in molecular map of cotton because it is based on the survey of large populations compared to the most commonly used recombination mapping method based on few selected individual crosses. This is the first report on the use of association mapping strategy in cotton to the discoveries of: 1) genetic diversity in several hundreds cotton lines from Uzbekistan based on large number of molecular markers for fiber and agronomic traits, 2) the genome-wide linkage disequilibrium (LD) value in cotton genome, and 3) association of several DNA markers with important fiber and agronomic traits and their chromosomal locations. This research helped the geneticists to "mine" useful genes among large number of populations for germplasm enhancement. Currently, we are using MAS based on our association mapping study selecting the DNA marker from the donor parents associated with the improved trait of interest for improving fiber quality traits in some Uzbek cultivars. The study demonstrated that successful application of genetic association analysis using large number of populations will accelerate the discovery rate of gene/QTL and informative useful markers in cotton.

Selection of suitable markers is one of the key factors for the success of a MAS program and it must be based on a simple and efficient detection system, highly polymorphic

and distributed across the genome. SSR and SNP markers are considered as the marker of choices for MAS in many crop species. Recently scientists have created the Cotton Microsatellite Database (CMD) [http://www.cottonssr.org] with the support from Cotton Incorporated (Blenda *et al*., 2006). This is a curated and integrated web-based relational database providing centralized access to publicly available cotton microsatellites, an important resource for basic and applied research in cotton breeding. At present CMD contains publication, sequence, primer, mapping and homology data for nine major cotton microsatellite projects, collectively representing more than 3,000 microsatellites. In addition, Monsanto also donated about 4,000 cotton SSR markers and associated information to Texas AgriLife Research, an agency of the Texas A&M System, in 2009 (Xiao *et al*., 2009).

SNP marker discovery opens up a new paradigm in MAS especially considering many publicly available gene sequences now in GenBank. Single nucleotide polymorphic markers (SNPs) are normally associated with many candidate genes. Discovery of the SNP marker is very difficult in a polyploid species like cotton. It is like solving two jigsaw puzzles at the same time, because cotton has two duplicated sets of chromosomes. Many of the seed industries are now using SNP markers as a marker of choice in MAS in corn and other crops. However, there is almost no such information available in public cotton databases. Recently we discovered a strategy to identify SNP markers in tetraploid cotton species (An *et al*., 2007, 2008; Buriev *et al*., 2010, 2011). SNP markers are normally biallelic. However, detection of haplotype is essential to detect multiple alleles based on unique sets of SNP markers in a candidate gene. It is a difficult task to identify a haplotype that could distinguish allelic differences at a single locus in a polyploid species like cotton because of the presence of duplicated loci. Researchers used cluster analysis of the sequences from *G. hirsutum* and the diploid A and D genome ancestral species from a candidate gene of interest using Neighbor Joining clustering method and have grouped the tetraploid sequences into two sub genomes based on their association with the diploid ancestral species. The sequences of tetraploid genotypes from an individual clade of the phylogenetic tree were aligned and compared to detect the putative SNPs. The unique combination of SNPs in a sequence within a clade of the phylogram was considered as haplotype. Each clade in the dendogram is considered as a putative locus. The hypothesis was based on the assumption that sequences at each locus will be more similar compared to the sequences between the loci. Sometime this strategy of identifying haplotype based on clustering analysis without prior genetic knowledge may separate significantly different alleles of a locus into two different clades, thus misrepresenting allelic differences as locus differences. Such a condition may fail to identify some of the true SNPs. Our strategy provided a very conservative estimate of putative SNPs in *MIC-3* gene family (Buriev *et al*., 2010). The advantage of this method is the reduced number of false SNP in the analysis by avoiding the problem of comparison among the orthologue and paralogue sequences. This discovery of SNP markers was further confirmed using deletion lines to identify their chromosomal locations (Ann *et al*., 2007, 2008; Buriev *et al*., 2010, 2011).

SNP markers will provide a tool to associate candidate genes in MAS in cotton molecular breeding programs. Such discoveries providing the knowledge of candidate genes associated with complex traits will also have an indirect effect to explore the possibilities of genetic manipulation of the specific candidate genes to improve important traits. SNP markers discovery will have great impact in MAS of cotton breeding program.

Cotton Genome Sequence

Decoding the cotton genome is essential for efficient use of genomic technologies in the improvement of upland cotton. Recently, a multidisciplinary international team including scientists from both public and private sectors initiated a partnership as a community to put forward a report on the problems and prospects of cotton genome sequencing (Chen *et al*., 2007). Readers are encouraged to study the report of Chen *et al*. (2007) for detailed reference on cotton genome sequencing. This paper will summarize some of the key information from this report.

Cotton is enriched with many available genomic resources including bacterial artificial chromosomes (BACs), ESTs, linkage maps, and integrated genetic and physical maps for sequence analysis and assembly (Chen *et al*., 2007). Sequencing cotton genomes will unveil the relationship of functional genome and agronomic performance, significance of polyploidy and genome size variation within the *Gossypium* genus.

The haploid cotton genome sizes are estimated to be approximately 880 Mb for *G. raimondii*, approximately 1.75 Gb for *G. arboreum*, and approximately 2.5 Gb for *G. hirsutum* (Chen *et al.*, 2007). It is essential to develop a comprehensive strategy for cotton genome sequencing based on economics, technology, and priorities. Due to continuing progress in high throughput sequencing technology and cost reductions, multiple and parallel approaches can be used to reveal complete genome information of *Gossypium* genomes. It is critical to have a comprehensive strategy for complete sequencing of one or more representatives of each A, B, C, D, E, F, G, K, and a tetraploid-derived AD (n = 26) *Gossypium* genome group to understand the complexity at the molecular level in the evolution of the cotton genome.

Recently, scientists at the University of Georgia under the leadership of Dr. Andrew Patterson and at Texas Tech University under the leadership of Dr. Thea Wilkins have led multidisciplinary international teams that are nearing completion of the sequence of the ancestral D and A genome diploid species respectively of upland cotton. Recently a report indicated that a joint venture of Monsanto Company (NYSE:MON) and San Diego-based Ilumina Inc. (NASDAQ: ILMN) will help to unveil the cotton genome sequence using Illumina's next generation sequencing technology (Monsanto web page, 2010). The report also stated that the

two companies have completed sequencing a wild Peruvian cotton species, *Gossypium raimondii*, and will donate their findings to the public. The U.S. Department of Energy's Joint Genome Institutes (http://www.jgi.doe.gov/) has taken a major step to support as a pilot study for shotgun sequencing of *G. raimondii* for a 0.5× coverage (Chen *et al*., 2007). USDA/ARS scientists are planning to sequence tetraploid cotton genome using a BAC-based sequencing approach in collaboration with scientists of the Anyang Cotton Research Institute in China (personal communication).

The AD genome sequence may offer superior opportunities to elucidate the types and frequencies of changes that distinguish a polyploid cotton from a diploid cotton. Sequences from A and D genome diploid species will be very helpful in tetraploid AD genome sequence assembly and will provide valuable information in gene content and expression patterns and polyploid genome evolution. Sequencing representatives from each diploid clade will be important to understand molecular patterns and biological events associated in evolution including the genomic and morphological diversity within the genus to adapt to a wide range of ecosystems in warmer and arid regions of the world (Chen *et al*., 2007). Knowledge gained from decoding the cotton genome will improve our understanding of gene function and ultimately benefit growers with improved yield and fiber quality. Information from cotton genome sequencing will be very helpful to develop tools for making cotton plants resistance against biotic and abiotic stresses.

Due to the globalization of agriculture it is expected that commodity prices are likely to decline, and efficient production will be the key factor in the competitive world market. Accordingly, researchers may have to target manipulating the genetic system using biotechnology tools to adopt cotton plants in environmental diversity, making the most out of different natural resources including limited water supply - rather than using costly inputs to change the environment.

Cotton is also a major cash crop in many developing countries, which are the source of rapid population growth and environmental degradation and some farmers cannot afford to adopt the high-input packages of biotechnology. However, we live in an interconnected global village and failure to capture the benefits of biotechnology will have ripple effects all over the world in the future. But the challenges are not only biological - they are also institutional, financial, political and social. Considering both environmental and economical impacts of the new technologies, cotton as a major cash crop in both developed and developing countries, the role of private industries in new technologies and the complex agricultural problems in different countries perhaps it is high time to consider establishing an international cotton research center. It is essential to face these challenges for the 21st century through a strong research partnership between public and private institutions. This international center will serve as a facilitator who can negotiate appropriate arrangements between the public and private sectors as catalysts in such partnerships. This center will make sure the benefits of tomorrow's breakthrough discoveries are shared properly in both developed and developing countries. This center should study the benefits of relative investments in favorable versus marginal environments in cotton because many cotton farmers in the world live in marginal areas and cannot afford the high input packages of biotechnology. Based on this study the center can guide to develop ecologically friendly principles such as crop rotation, intercropping, and crop management systems to local conditions to maximize the benefits of biotechnology. This center will set up a regulatory system based upon sound science and agro-ecological factors of the specific country, develop cost-effective methods for quarantine or regulatory purposes and assist farmers in the detection of transgenic purity, an efficient mechanism of transgenic seed delivery. It is essential to develop a strategy to counteract the negative attitude towards transgenic cotton. The center should recommend the complex, system-oriented solutions based on knowledge-intensive plans rather than just the simpler seed-centered technologies. The success story of biotechnology in cotton raised our expectations for a continued flow of scientific miracles to promote sustainable cotton production under a safe environment for the 21st century.

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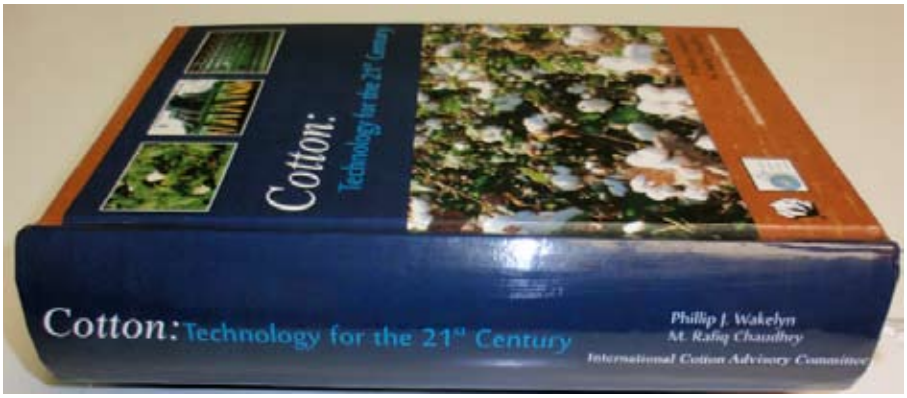

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