INTROGRESSION OF THE ULTRA-LOW GOSSYPOL COTTONSEED TRAIT IN ELITE COTTON (*Gossypium hirsutum* L.) CULTIVARS

A Dissertation

by

ROSA NOEMI JAUREGUI

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Chair of Committee,	Steve Hague
Co- Chair of Committee,	Keerti Rathore
Committee Members,	C. Wayne Smith
	Seth C. Murray
Head of Department,	David D. Baltensperger

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ABSTRACT

Food security is one of the most important challenges that society will continue to face in a world with a rapidly expanding population coupled with a growing affluence. Cottonseed is an underutilized source of protein and oil which could play a key role in human and non-ruminant animal nutrition if the toxic polyphenol gossypol contained in lysigenous glands throughout the plant could be removed from the seed.

Gossypol is a terpenoid that protects cotton plants against insects and pests and if removed from the vegetative portion of the plant, the plant becomes highly susceptible to attacks. Scientists at Texas A&M University used RNAi technology to develop transgenic cotton plants, which have normal gossypol levels in vegetation but ultra-low levels in seed. The main objective of this study was to assess the traits function in multiple genetic backgrounds.

Transgenic lines were field tested in 2011 and 2012 near College Station, TX. Performance parameters such as yield, lint percent and crop maturity were measured as well HVI fiber properties. Also, recurrent parents were tested in a three-year field trial and several methods for screening for the ultra-low gossypol cottonseed (ULGCS) trait were evaluated.

Results from these studies suggest the ULGCS trait can be backcrossed into diverse upland cotton lines without interfering with the inherent performance

of the recurrent parent. Moreover, during the backcross process, selections can be made to improve fiber quality and abiotic stress tolerance. Availability of a food product such as cottonseed can have numerous positive effects on local economies where cotton is grown and in the lives of millions of people who cannot fulfill one of the most important and basic rights of humanity: access to food.

DEDICATION

I dedicate this dissertation to my wonderful family. To my beloved husband Riqui for his unconditional support and his absolute confidence in me. To my three young children: Rufino, Ricardo and Facundo because without knowing it they supported me and gave me strength. To my mother, father and sister for believing that I could do it and for always being there for all of us. What an unforgettable journey this has been! How lucky I was to share it with the best persons in the world, who stood beside me no matter what. For this and more I will always be grateful. I love you all.

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CHAPTER I

INTRODUCTION AND

LITERATURE REVIEW

The Food and Agriculture Organization (FAO) of the United Nations' Committee on Food Security (FAO, 2012) defines food security as the ability of a person to fulfill their daily nutritional requirements. Globally, one out of six people do not meet this requirement. Most of the world's undernourished people live in developing countries located in tropical and sub-tropical environments where cotton can be grown. Global cottonseed production in 2013/2014 was 45.1 MT (USDA, 2014); however, the presence of the toxic polyphenol gossypol severely restricts its use as a food source and even as feed for monogastric animals.

Gossypol is contained in lysigenous glands, a characteristic of the *Gossypium* genus. The toxicity affects monogastric animals including humans, although it can also affect young ruminants (Wang 2009). Among many biological activities, several studies have shown that gossypol can have severe effects on animal health including reproduction due to its contraceptive activity in males of different species including humans (Cai et al. 2010, Coutinho 2002).

In 1959 S.C. McMichael, a researcher from the USDA-ARS Cotton Field Station in Schafter, CA, reported that the cross of upland cotton with a Hopi

biotype from central Arizona (*Gossypium hirsutum* race punctatum) known as Hopi Moencopi produced cotton plants with glandless bolls, stems, hypocotyls and petioles controlled by one major gene from the Hopi Moencopi variety called gl_1 (McMichael, 1959). Further studies showed that glandless cotton plants with glandless seed could be obtained from segregating populations and that this trait was the result of combined effects of two genes, gl_2 and gl_3, in the homozygous recessive state (McMichael, 1960). These findings led to high expectations on the availability of gossypol-free cottonseed products. Numerous breeding programs were initiated in the U.S. and elsewhere to produce gossypol free cottonseed using these alleles (Sunilkumar et al. 2006).

Many studies conducted in the 1960's showed that when the cotton plant was deprived of its natural defenses (e.g. gossypol and related terpenoids) it was highly susceptible to pests. In a field study with two different glandless and glanded cotton genotypes, Jenkins et al. (1966) reported higher insect susceptibility of glandless cotton. Interestingly, the authors reported that insects that normally do not feed on glanded cotton, such as adult *Maecolaspis flavida* (Say) and *Gastrophysa cyanea* (Melsheimer) preferred the glandless lines and *Alabama argillacea* (Hübner) moths also preferred to oviposit on glandless lines over glanded lines. Lukefahr et al. (1966) reported that in laboratory tests, growth of both bollworm (*Heliothis zea* (Boddie)) and Tobacco budworm (*Heliothis virescens* (F.)) larvae was greater when fed with glandless square or cotyledon tissue regardless of plant variety. In the field, these authors reported

that both pillbugs (*Porcellia* sp.) and spotted cucumber beetles (*Diabrotica undecimpuncata howardi* Barber) infestations, which are not common cotton pests, were significantly higher in glandless plants regardless of variety, but fleahopper (*Psallus seriatus* (Reuter)) infestation and bollworm oviposition was not affect by the presence or absence of glands (Lukefahr et al. 1966).

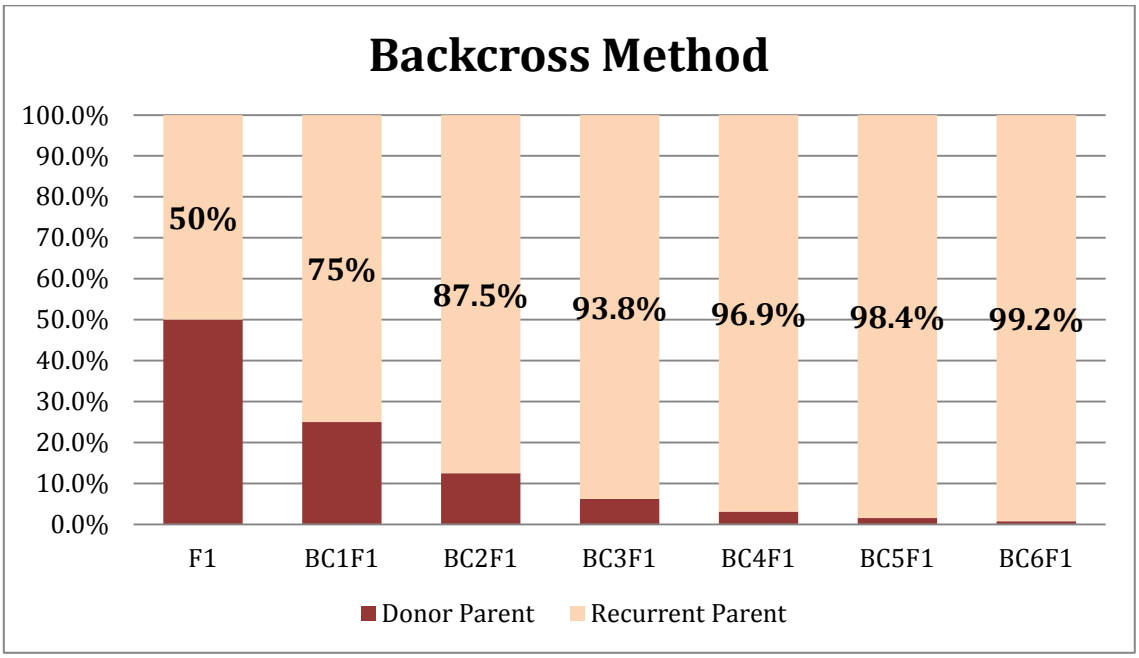
The main reason for the commercial failure of glandless cotton was the fact that the whole plant lacked its natural defenses, in the form of gossypol and related terpenoids, making it more susceptible to insect pests. Sunilkumar et al. (2006) utilized biotechnological tools to develop transgenic upland cotton (*Gossypium hirsutum*) cv. 'Coker 312' lines with ultra-low gossypol levels in the seed while maintaining normal gossypol/terpenoids levels in the rest of the plant. This research team from the Institute for Plant Genomics and Biotechnology at Texas A&M University (http://ipgb.tamu.edu/) achieved this by transformation with a gene silencing RNAi construct using the *Agrobacterium tumefaciens* system. This construct contained a sequence encoding a hairpin RNA of the (+)-δ-cadinene synthase, a key enzyme involved in the gossypol synthesis pathway (Zhou et al. 2013**,** Sunilkumar et al. 2006).

A key point of this approach is the use of the promoter sequence of the cotton α-globulin B gene. This is a highly seed specific promoter that allows expression of the RNAi construct only in the seed but does not affect normal expression of the (+)-δ-cadenine synthase enzyme in the rest of the plant (Sunilkumar et al. 2002). The transgenic 'Coker 312' lines were stable and

showed no detrimental effect due to the expression gene silencing construct. The efficacy and stability of the silencing prompted us to establish a backcross breeding project to introgress the valuable Ultra-Low Gossypol Cottonseed (ULGCS) trait into elite cotton lines. Through backcrossing, one or a few genes of interest can be transferred from a donor parent that contains the gene or genes of interest in an otherwise undesired genetic background to adapted or elite materials (recurrent parent) with other desirable characteristics. In every cycle of backcrossing the selected progeny containing the gene of interest is crossed to the recurrent parent so that at the end of the process the recurrent parent's genetic background is recovered with the addition of the trait of interest. In this case, ‘Coker 312’ was used as the donor parent having the ULGCS trait and six upland cotton lines were chosen as recurrent parents as described in the materials and methods section. Figure 1 shows how the backcross method can be used to recover the recurrent parent´s genetic background with the addition of one or a few traits derived from the donor parent. The ability to screen the progeny in order to find those individuals that carry the trait of interest is key to this breeding methodology. After the first crossing, F_1 seed is obtained with a genetic composition derived equally from each parent. When the F_1 progeny is crossed with the recurrent or elite parent, the BC_1F_1 progeny will be 75% similar to the recurrent parent and 25% similar to the donor parent on average. This means that in each cycle of backcrossing, the progeny is enriched in the recurrent parent´s genetic background by half the percentage of the donor

parent's genetic background in the previous generation. After six generations of backcrossing the recurrent parent´s genetic background is recovered to 99.2%. Each additional generation will make a very small difference in terms of increasing the recurrent parent´s genetic background in the progeny. In the BC_4F_1 more than 96% of the recurrent parent´s genetic background has been recovered.

Figure 1. Graph showing how the genetic background of the progeny is enriched in the recurrent parent´s genetic background.



The genus *Gossypium* (Family Malvaceae, Order Malvalaes, Tribe Gossypieae) comprises more than 50 species of which four were domesticated

independently and are currently cultivated mainly for fiber and oil (Wendel and Cronn, 2003). Two of the cultivated species, *Gossypium herbaceum* and *Gossypium arboreum* are diploids (2n = 2x = 26) that originated in the Old World and are grown in marginal areas in Africa and Asia covering 0.77 million hectares and 1.75 million hectares respectively (Kulkarni et al., 2009). The other two cultivated species, *Gossypium hirsutum* and *Gossypium barbadense*, are allotetraploids (2n = 4x = 52) which originated in the New World and are cultivated worldwide (Wendel and Cronn, 2003). Together, these allotetraploid species constitute more than 90% of the world's cotton production (Seelanan et al., 1997; Smith and Cothren, 1999; Wendel and Albert, 1992; Wendel and Cronn, 2003). Ancestors of the allotetraploid species are not completely known to this day even though extensive research has been conducted to determine the evolutionary origin of these species. It is speculated that the genome donors of the allotetraploid species are probably extinct (Seelanan et al., 1997; Smith and Cothren, 1999; Wendel and Albert, 1992; Wendel and Cronn, 2003). The genomes of both *G. barbadense* and *G. hirsutum* are comprised of two sub-genomes: A and D. The A sub genome contains similarities with the A genome of the Old World diploids while the D sub genome contains similarities with the D genome of the New World diploids (Smith and Cothren, 1999, Wendel et al. 2010).

Cotton (Gossypium hirsutum L.)

Of the two predominant cultivated cotton species, *Gossypium hirsutum* is the most widely cultivated, dominating the global cotton commerce (Wendel and Cronn, 2003). It is commonly known as Upland cotton and accounts for over 90% of the world cotton production. Worldwide, 26,745,000 MT (www.fas.usda.gov) of cotton fiber was produced in 2011/12, with the United States (3,413,000 MT) being the third biggest producer after China (7,294,000 MT) and India (5,879,000 MT). In the United States, Upland cotton is grown in 17 states, from California to southeastern Virginia accounting for a US $100 billion/year industry, employing more than 300,000 Americans and constituting one of the largest contributors to the US national gross profit (Wendel and Cronn, 2003). Texas is the largest Upland cotton growing region in the United Sates, accounting for more than 40% of the national planted acreage in 2011 and ranking as the #1 state in Upland cotton production across the cotton belt (USDA, 2011).

Cottonseed

The primary use of cotton is its fiber of which cottonseed is an important by-product (Sunilkumar et al. 2006). For every kilogram of fiber, approximately 1.5 kilograms of seeds are produced, thus making cottonseed the third most

important oilseed crop after soybean and rapeseed worldwide (Cai et al. 2010, Romano and Scheffler, 2008; Wilkins et al., 2000). In 2010, more than 6 million MT cottonseed was produced in the United States (USDA, 2011). Cottonseed contains approximately 23% of good quality protein which, at an annual worldwide production of 44 million MT of cottonseed it is approximately 10 million MT of protein, which could help meet protein requirements of 420 to 600 million people annually in the world at a 65 g/day or 45 g/day rate, respectively (Rathore et al., 2011; Rathore et al. 2003), but potentially more important would be the impact on livestock feed.

Cottonseed also contains approximately 21% oil with a fatty acid composition of more than 50% of the essential fatty acid linoleic acid, 20% palmitic acid, 17% oleic acid and 2% stearic acid approximately, and a ratio of 2:1 of polyunsaturated to saturated fatty acids (Lukonge et al., 2007; Lusas and Jividen, 1987a; Rathore et al., 2003). In the United States, cottonseed oil production totals 1.2 billion pounds per year, ranking third after soybean and corn oil (Rathore et al., 2003). Lecithin content in cottonseed is the second highest among oilseeds after soybean, but contrary to soybean lecithin, the absence of more than two double bonds in any fatty acid is expected to decrease its tendency to oxidation in food and industrial processes (Lusas and Jividen, 1987a).

In addition, cottonseed contains important phytochemicals, such as polyphenols and flavonol glycosides including aglycones of quercetin and

kaempferol and carbohydrate moieties, which are natural antioxidants present in plants. Natural antioxidants have gained importance in the human diet over the last few years due to the greater understanding of their role in human health as anti-cancer and anti-aging agents, in the food industry particularly as lipid stabilizers improving quality and shelf life of products, and in animal health (Piccinelli et al., 2007; Ramadan and Moersel, 2006). A detailed study conducted by Oskoueian et al. (2011) showed that cottonseed meal has antioxidant activity with a 74% inhibition of formation of hydroperoxides (ferric thiocyanate test) and 69% inhibition of thiobutaric reactive substances (TBA test). The Xanthine oxidase inhibitory activity test also showed an inhibitory effect of cottonseed meal extract on this enzyme. The anti-inflammatory activity of cottonseed meal extract was assessed through the inhibition of nitric oxide in RAW 264.7 cells. In a similar way, Gao et al. (2010) studied the antioxidant activity of different peptide fractions derived from the hydrolysis of cottonseed protein with Neutrase. These authors found significant inhibition of linoleic acid peroxidation, significant 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity and significant hydroxyl radical-scavenging activity in four hydrolysate fractions tested. Fraction III of the hydrolysate, which showed the highest antioxidant activity in all assays, was rich in Phenylalanine, Histidine, Proline, Methionine, Isoleucine and Cysteine. Several amino acids such as Histidine and Proline have been shown to contribute to free radical scavenging and an increase in hydrophobicity of proteins and peptides has been shown to

increase antioxidant activity (Gao et al., 2010). In addition, cottonseed contains high levels of α-tocopherol (vitamin E), which in dairy cows has been shown to translate into higher levels of α-tocopherol in plasma (Risco et al., 2002).

However, in spite of its high nutritional value for humans, the main use of cottonseed is as ruminant feed, seed hull, seed meal rich in essential amino acids, and oil (Rathore et al., 2008; Wilkins et al., 2000). This is due to the presence of gossypol, a polyphenol that is toxic to humans and other non-ruminant animals (Lusas and Jividen, 1987a; Rathore et al., 2003; Romano and Scheffler, 2008). Cottonseed and cottonseed by-products that contain gossypol can be partially tolerated by cattle and fed to mature animals up to four kg per day usually without noticeable effects.

Gossypol is found throughout the cotton plant from the seed to the root in lysigenous pigment glands that are ovoid or spherical shaped structures with tough and resilient walls. In the seeds, these glands contain 35-50% gossypol constituting 2.4-4.8% of the weight of dehulled kernels (Lusas and Jividen, 1987a). Gossypol is a terpenoid aldehyde that serves an important role in cotton plants: it acts as a natural plant defense against diseases and pests (Cai et al., 2010).

Ruminants are capable of detoxifying gossypol in the rumen through different processes such as binding it to soluble proteins (Hawkins et al., 1985; Solaiman et al., 2009). However, if the detoxification capacity of the rumen is exceeded, ruminants can also suffer from gossypol toxicity including reduction in

fertility of males, red blood fragility and immune response depression (Solaiman et al., 2009).

In the dairy industry, whole cottonseed is regarded as an outstanding source of protein (23%), energy (fat, 20%) and crude fiber (24%) (Cotton Incorporated, 2012); Dayani et al., 2011b). Numerous studies have determined the impact of whole cottonseed both in nutrition of dairy cows and in milk yield and quality (Coppock et al., 1987; Dayani et al., 2011b; Mccollum and Galyean, 1985). The high energy content of whole cottonseed is important for lactating cows which have high energy demands; lint and hulls are a high fiber source which aid in providing a balanced diet (Cooke et al., 2007; Dayani et al., 2011b). Unlike many other feed sources that show negative correlations between high energy and fiber cottonseed provides both high fiber and high energy (Coppock et al., 1987). Dietary fiber aids in maintaining high acetic acid levels that support rumen health and milk fat levels (Coppock et al., 1987). Dayani et al. (2011b) reported a study by Kubik and Stoke (1990) which showed that whole cottonseed is protected by the hull in a way that it can by-pass rumen degradation allowing for the oil to be absorbed in the intestine impacting milk quality directly. For this reason, the linoleic acid-rich cottonseed can positively enrich milk. Moreover, because the linoleic acid found in cottonseed is conjugated, it has been shown to have anti-cancer properties as well as improvement of immune responses and bone mineralization, and provides protection against arteriosclerosis and diabetes (Dayani et al., 2011b).

It has also been suggested that feeding ruminants with high-oil sources such as whole cottonseed, which is rich in oil and unsaturated fatty acids that are toxic to protozoa, can decrease protozoa population in rumen and crude protein. This is desirable because ciliated protozoa present in the rumen can reduce the amount of amino acids that reach the animal's intestine, increase methane production (a major source of greenhouse gases), and consume feed energy that could otherwise be used by the animal (Dayani et al., 2011a).

Numerous aquaculture studies have been conducted on the feasibility of replacing fishmeal, the main source of protein in fish feeds, with protein sources of vegetable origin such as soybean meal and cottonseed meal (Gatlin et al., 2007; Pham et al., 2007). Fishmeal contains all essential amino acids making it a high quality source of protein, but it is expensive and can account for up to 50% of production costs of a fish farm (Pham et al., 2007). Pham et al. (2007) studied the effect of replacing fishmeal with a 1:1 soybean:cottonseed meal diet in increasing proportions from 10 to 40% and a control diet with fishmeal. The authors found no significant differences in fish growth and nutritional status of juvenile Japanese flounders (*Paralichthys olivaceus*) (weight gain, specific growth rate, protein efficiency ratio, feed conversion ratio and nitrogen retention among other characteristics) in substitutions of up to 40% of fishmeal with these protein sources supplemented with methionine and lysine which are essential amino acids present at low levels in soybean (*Glycine max*) or cotton. Nevertheless, as levels of cottonseed in the diet among the fish populations in

the study increased the authors found significant differences in gonadosomatic index (100 x (gonad size/body weight)), hematocrit count, hemoglobin content and higher total liver gossypol with higher dietary gossypol contents. These detrimental health symptoms are characteristic of gossypol toxicity, which is the main limiting factor for the use of cottonseed in aquaculture. Many feeding trials using cottonseed have been reported in other fish species and at different developmental stages. These studies suggest that cottonseed is a good replacement for fishmeal in terms of its nutritional value and feed acceptance, but gossypol in the diet has detrimental effects that can negatively affect growth and development, thus preventing the fish industry from utilizing cottonseed as a protein source that would reduce the cost of the feed and in turn, improve the revenues.

Some fish species can tolerate gossypol depending on their developmental stage. Rainbow trout (*Oncorhynchus mykiss*) fingerlings have been reported to tolerate up to 15% of cottonseed meal in their diet (Lee et al., 2002), while Blom et al. (2001) reported that adults of this same species can tolerate 50% fishmeal replacement with cottonseed meal. Another study by Dorsa et al. (1982) showed that juvenile channel catfish (*Ictalurus punctatus*) can intake up to 900 mg/kg of free gossypol. Although the effect of gossypol on fish is species- and developmental stage-dependent, all these studies suggest that the primary limiting factor for replacing fishmeal with high levels of

cottonseed meal is the presence of gossypol because of its detrimental effect on fish health and growth.

Gossypol toxicity

Several studies, both in ruminant and non-ruminant animals, have reported negative effects of feeding gossypol-containing feeds. Solaiman et al. (2009) studied the effect of feeding 0, 15.7, or 32.7% dry matter EasiFlo® cottonseed (Cotton Incorporated, Cary, NC) in addition to 49% Bermudagrass (*Cynodon doctylon*) hay to 12 Nubian goat buck juveniles (*Capra nubiana*) aged 6-8 months. The authors reported an increase at the 15.7% EasiFlo® cottonseed diet in intakes of dry matter, neutral detergent fiber and crude protein among other parameters measured. In accordance to other studies performed in lactating cows (Hawkins et al., 1985) and beef heifers, erythrocyte fragility increased with increased cottonseed content and time. In addition, enzymes such as alkaline phosphatase and creatine kinase also increased, which may indicate liver and bone injury, respectively due to gossypol toxicity (Solaiman et al., 2009).

In humans, gossypol toxicity has also been documented. The maximum content of free gossypol allowed by FDA in food products for human consumption is 450 ppm, while FAO-WHO allows 600 ppm. In broilers (*Gallus*

gallus domesticus), a non-ruminant animal the limit of free gossypol has been set at 100 ppm, and 40 ppm for laying hens (Lusas and Jividen, 1987a).

The availability of ultra-low gossypol cottonseed offers numerous advantages to the cottonseed processing industries as well as consumers. In the oil mill industry, a reduction in gossypol would yield lighter colored crude oil and meal and reduce the costs involved with solvent extraction process (Lusas and Jividen, 1987a). It was also reported that glandless cottonseed meal had higher levels of available lysine and improved protein nutrition efficiency which could be due to the fact that in glanded cotton, gossypol binds certain amino acids reducing their availability (Lusas and Jividen, 1987a; Lusas and Jividen, 1987b, Graham et al 1969). Glandless cottonseed flour has been compared to other protein sources such as soy, beef and milk and it has been shown that 100 g of cottonseed flour contains more protein than 100 g of ground beef and 100 g of skim milk. In addition, it has also been shown to be a growth promoter in several studies conducted in different animal species (Alford et al 1996 and references therein). Epidemiological studies conducted in Peru showed that malnourished infants fed with glandless-cottonseed flour had significant weight gain and nitrogen retention. In these studies it was concluded that at certain levels cottonseed protein could be the main source of protein for children and infants. (Alford et al. 1996 and references therein). Similar studies conducted in Africa and India also showed that cottonseed flour protein significantly improved children´s growth. Clinical studies carried out by the Texas Woman's University

showed that children fed with cottonseed flour had higher vitamin A levels (Alford et al 1996). Graham et al (1969) conducted a study in which cottonseed flour was compared with cow milk in terms of nitrogen balance when fed to malnourished infants and children in a hospital situation. The difference among the cottonseed flours in the study consisted of the method used for gossypol extraction, including cottonseed flour from glandless cotton which required no gossypol extraction. In this study the authors concluded that the protein source that resulted in the highest nitrogen balance in the population studied was cow milk followed closely by glandless cottonseed flour. Among the glanded cottonseed flours the azeotropic solvent extraction showed higher nitrogen balance than the heat processing and screw press extraction.

CHAPTER II

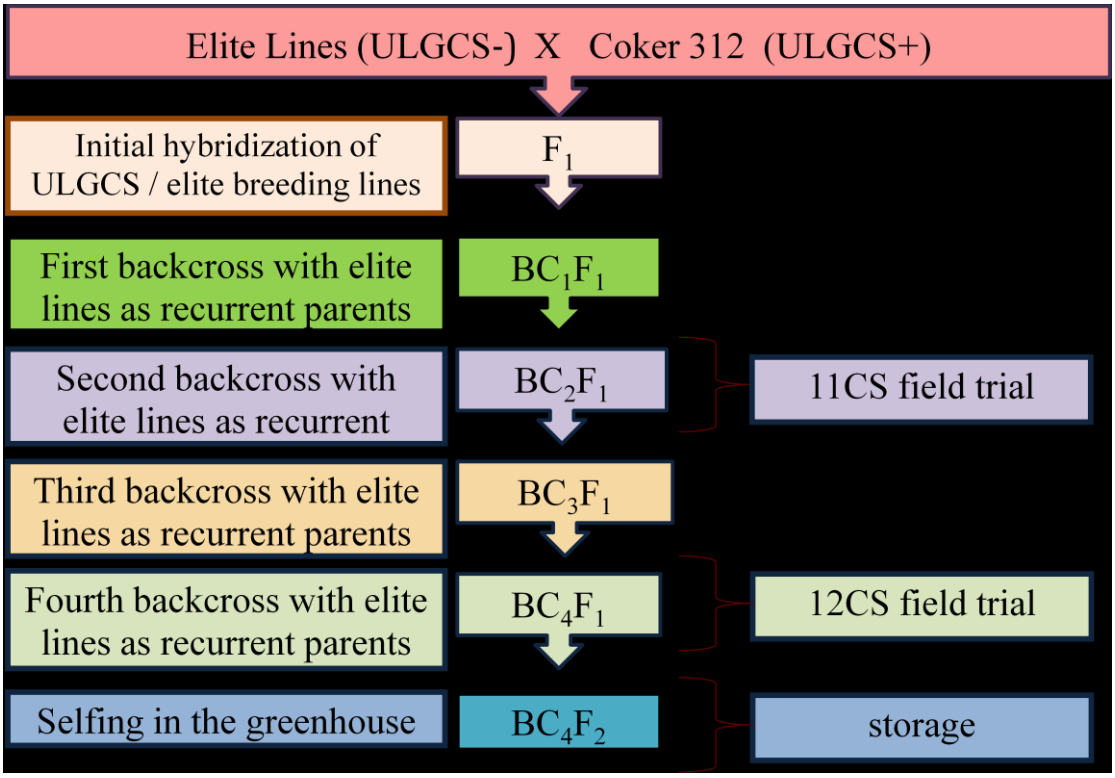
MATERIALS AND METHODS

Introgression of ULGCS in a greenhouse

For the introgression of the Ultra-Low Gossypol Cottonseed (ULGCS) trait, six elite lines were selected for use as recurrent parents. Four of these lines were developed at the Texas A&M University, Cotton Improvement Laboratory: 'TAMCOT 73', TAM Exp. 05-A-46, TAM Exp. 05-WL-27, TAM Exp. 08-WZ-91. Two lines were developed in East Africa: 'HGN 71' and 'HGN 78'.

The recurrent parent lines were first hybridized in a greenhouse in the winter of 2010 with 'Coker 312' (SeedCo Corporation, 1974)-RNAi line #49b, carrying the RNAi silencing construct responsible for the ULGCS trait, as the donor parent. A series of backcrosses were conducted until BC_4F_2 seed was obtained (Figure 2). Approximately 2 ½ generations were possible in one year. All hybridization activities were conducted inside a greenhouse at College Station, TX. During each cycle of backcrossing, four donor parent plants and ten recurrent parents were used for each genotypic combination.

Figure 2. Introgression of ULGCS using the backcross method.



Screening for the ULGCS trait

In every generation of backcrossing, seed carrying the ULGCS transgenic trait from the donor parent had to be selected for the next cycle of backcrossing. Twice as much seed as was needed to be advanced was planted in Jiffy-7®-peat pellet in the greenhouse. Because cotyledons comprise 60% of the cottonseed weight, they were used to test for gossypol as soon as they emerged (Bewley et al. 2006). Positive seedlings, showing the ULGCS trait, were transplanted either to 15 liter pots, if that generation was going to be grown in

the greenhouse, or taken to the field if it was one of the two generations that was tested in the field trial in College Station.

Identifying the best method for screening the ULGCS trait

Three different ULGCS selection methods were considered and tested for accuracy, speed, costs and type of tissue used: the *Phloroglucinol Assay for Gossypol, PCR* and *Fourier Transform-Near Infrared (FT-NIRS) Spectroscopy.*

Phloroglucinol assay for gossypol

The phloroglucinol assay for gossypol (Bell, 1967) is a colorimetric test that can be performed with seed or cotyledon tissue. Because it is a destructive assay, the seeds were germinated and the cotyledons were tested for the presence or absence of the transgene by examining gossypol levels. A disc 1 cm in diameter was excised from a cotyledon and placed into a 1.5 ml microcentrifuge tube. One metal grinding bead was added to each tube along with 1 ml 96% ethanol (EtOH) and left in the dark for 40 minutes. After incubation, the cotyledon pieces were ground mechanically and the tubes were incubated at 65°C in a water bath for 30 minutes. The samples were later centrifuged for 10 minutes at 13,000 rpm. In a clean tube, 90 µl 96% EtOH + 50 µl phloroglucinol solution (50mg/ml) + 10 µl supernatant + 100 µl Conc. Chloridric acid (HCl) were mixed. The tubes were incubated in the dark for 30

minutes. After this time, a pink colored solution indicated the presence of gossypol in the tissue sample, while a clear solution indicated low levels of gossypol. In this way, if the reaction had turned pink the seedling from which the sample had been obtained was discarded, but if the sample was clear, the seedling was selected in order to be used for a new backcross generation.

PCR

DNA isolation from cotyledons and PCR analysis to detect the 653-bp fragment including part of the δ-cadenine synthase transgene was performed following protocols from Sunilkumar et al. 2006 using a primer pair that would hybridize on one end to the cassette containing the transgene and on the other end to the δ-cadenine synthase gene. In this way only the transgenic enzyme gene would be amplified but not the native gene. PCR was conducted on BC_1F_1 seed in addition to a wild-type negative control and a transgenic donor parent as a positive control. In 2011, the phloroglucinol assay was used to screen cotton seedlings and PCR was used to confirm the presence of the transgene in these plants. In all cases, plants that scored positive for the transgene with the phloroglucinol assay were positive with the PCR method, thus confirming the robustness and reliability of the phloroglucinol assay. Therefore, in 2012, seedlings were tested only with the phloroglucinol assay. This strategy was also cost effective because the phloroglucinol assay is cheaper than the PCR method.

Fourier transform-near infrared (FT-NIRS) spectroscopy

Near Infrared Reflectance Spectroscopy (NIRS) is an analytical technique that has become useful in agriculture research as a tool for quantitative and qualitative analysis. It is non-destructive, fast, and cost efficient if a calibration can be developed, however it is less accurate than wet-chemistry analysis. Because of these advantages, it seemed likely that using the FT-NIRS method to screen for low gossypol seed would greatly speed up and simplify the selection process. For this reason, a calibration curve using 100 whole and ground seed samples was developed. For each seed sample 64 scans were collected using a rotating cup over the integrating sphere in the Thermo Scientific Antaris II FT-NIRS Analyzer requiring approximately 1 min per sample. After this, the amount of gossypol in the samples was determined with the phloroglucinol assay as described earlier, except that the final step involved measuring the absorbance of each sample using a spectrophotometer at 550 nm in order to determine μg of gossypol equivalents/mg tissue. The two calibration curves were developed using TQ Analyst software provided by Thermo Scientific. In order to asses the predictive value of the FT-NIRS method thirty five additional seed samples were scanned and their gossypol levels were obtained using the phloroglucinol levels.

Testing parental lines in field trials in 2010-2012

In order to test the performance of the six elite lines that were selected for use as recurrent parents (TAMCOT 73, TAM Exp. 05-A-46, TAM Exp. 05-WL-27, TAM Exp. 08-WZ-91, HGN 71, and HGN 78) a three-year field trial was performed in College Station in 2010-2012. The entries were planted in a randomized complete block design (RCBD) with four blocks. The two-row plots were 3 m long and 1 m wide. Normal practices were followed for furrow irrigation and pest control. The lines were tested for general performance, including yield, lint percentage (calculated as lint weight/ seedcotton weight) and fiber quality traits. Harvesting was done with a one-row picker on one row and 30 boll-sampling for fiber quality was done on the second row. Fiber quality was measured with a High-Volume Instrumentation (HVI) at the Texas Tech University Fiber and Biopolymer Research Institute in Lubbock, TX, in 2011 and at Cotton Inc. in 2012.

Testing ULGCS introgressed lines in field trials in 2011 and 2012

Field trials were conducted at the Texas A&M University Field Laboratory near College Station, TX, in 2011 and 2012. Twelve entries were planted in a randomized complete block design (RCBD) with four blocks with a total of 48 plots. These entries included the six recurrent parents and corresponding BC_2F_1

lines. Each block consisted of 12 plots that were 3.05 m in length and 1 m in width. Plants were germinated in the greenhouse, tested for low-gossypol, and the selected seedlings were transplanted in the field at a density of 10 plants per plot. Plots were separated by non-transgenic plants with red foliage that were also planted at the same density. Three border rows were planted on the west side of the field with 'Coker 312' and 'TAMCOT 73' and two border rows were planted on the east side with the same varieties. Hand weeding was done on a weekly basis and herbicide treatments and irrigation were performed as needed.

Lines were tested for general performance, including yield, fiber quality and crop maturity. Yield was calculated as total lint harvested. Fiber quality traits were measured with a High-Volume Instrumentation (HVI) at the Texas Tech University Fiber and Biopolymer Research Institute in Lubbock, TX, in 2011 and at Cotton Inc in 2012. Crop maturity was calculated as the number of green bolls/total number bolls in each plant approximately three weeks prior to harvest. All plots were hand harvested to determine yield per plot and a 50 g sample from each plot was submitted for fiber quality tests.

The trial was repeated at the same location in 2012 with a similar protocol except the BC_4F_1 lines were tested instead of BC_2F_1 lines.

This field trial was grown separate from any other cotton to avoid natural crossing as a way to contain the transgenic trait. After the trials were completed all plants including the borders had to be harvested, the seedcotton on the

ground and all green material above ground was collected and autoclaved as a containment method for the transgenic trait.

The backcross process was carried out in the greenhouse so the generation used in each field trial was the most advanced backcross generation available when it was time for planting in the field. In addition, the selection of individual plants that carried the ULGCS trait required a large amount of seed in order to grow four blocks of the same line making the availability of seed from the previous season a limiting factor to grow the same generation twice. For this reason in the year 2011 the recurrent parents and the BC_2F_1 generation were tested in the same field trial while in the year 2012 it was the BC_4F_1 generation that was tested with the recurrent parents.

Data analysis

All data from field trials and fiber quality traits were analyzed in an analysis of variance (ANOVA) using JMP® software, version 10.0.0 64-bit edition Copyright © 2012 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. Mean comparisons were performed using least square means (LS Mean) differences Tukey HSD Test and significant differences between means were shown with different letters. When only two means were compared the

least square means (LS Mean) differences Student´s t test was used and LSD was calculated; differences between means were shown with different letters.

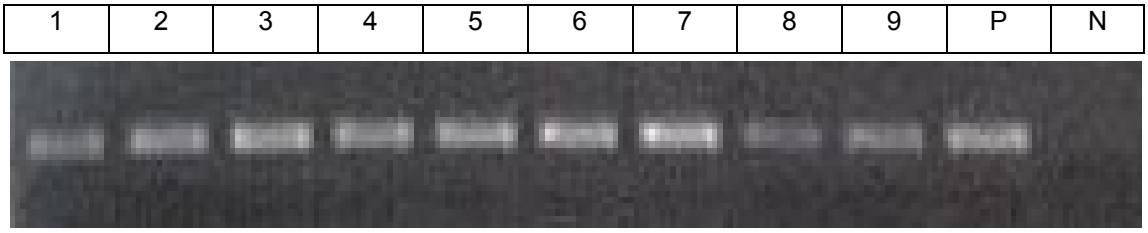
CHAPTER III

RESULTS

Screening for the ULGCS trait

From the three methods that were considered the *Phloroglucinol Assay for Gossypol, PCR* and *Fourier Transform-Near Infrared (FT-NIRS) Spectroscopy,* the method that was chosen to select for new plants carrying the ULGCS trait was the Phloroglucinol Assay. This assay proved to be simple and effective in detecting samples with ultra-low levels of gossypol. In order to test the efficacy of the methods, a number of samples were tested with the Phloroglucinol assay and also with PCR (Figure 3). Since results from PCR and the phloroglucinol assay were identical, it was concluded that a colorimetric assessment of the presence/absence of the transgenic trait using the Phloroglucinol assay was 100% accurate. In addition to this, approximately 60 samples could be run using this assay in one day, it is less costly than PCR, and it provided quantitative characterization of the gossypol. However, because of the destructive nature of the assay, the seed had to be planted and a sample from each cotyledon had to be collected from the greenhouse so the use of these resources and the time consumed needs to be taken into account.

Figure 3. PCR results for delta-cadenine synthase gene in BC1F1 progeny.



Wells 1-9: TAM 73 BC_1F_1 lines; (P) positive control; (N) negative control

For this reason the use of FT-NIRS was also explored. FT-NIRS equipment is expensive but can have many applications in a research environment for evaluating composition. In addition, it is a non-destructive and fast method which does not involve the use of expensive, hazardous chemicals. After testing one hundred whole and ground cottonseed samples in the Thermo Scientific* Antaris II FT-NIRS Analyzer the samples were tested for gossypol levels using the Phloroglucinol assay. Two calibration curves were developed selecting the spectral regions and statistical values in TQ Analyst which would result in the highest correlation coefficients (Figures 4 and 5).

Figure 4. Calibration curve for BC1F1 whole seed using FT-NIRS spectroscopy and phloroglucinol assay.

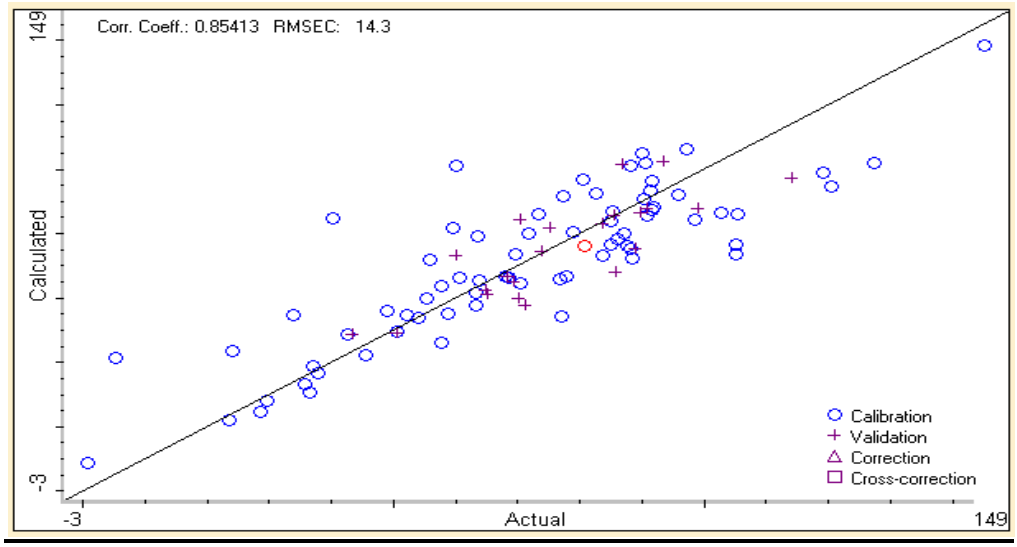
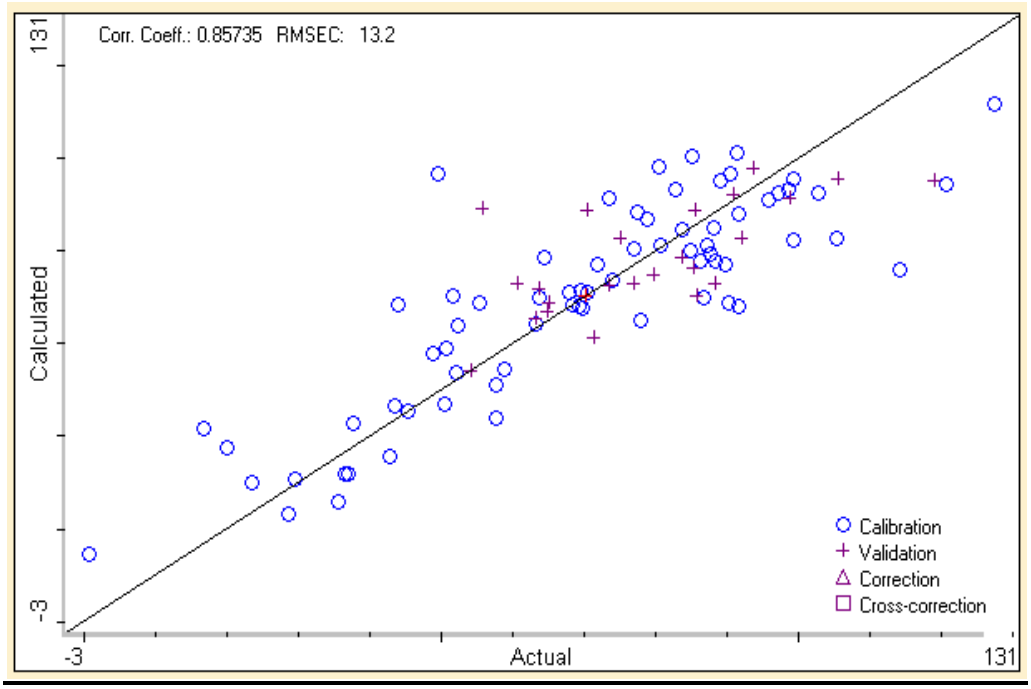
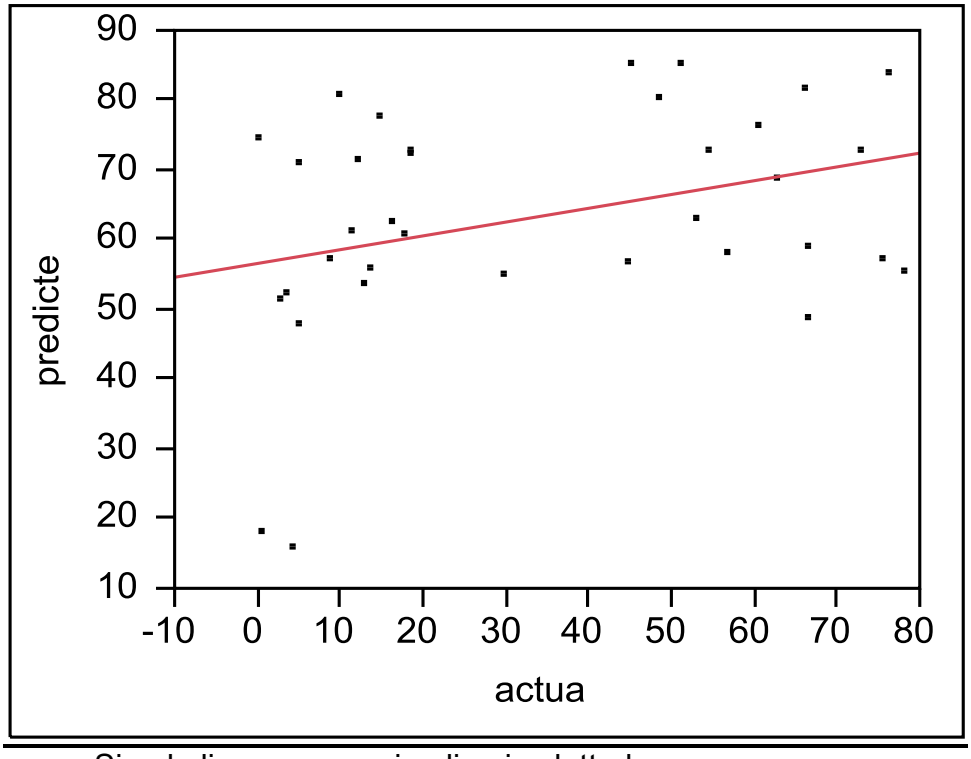


Figure 5. Calibration curve for BC1F1 ground seed using FT-NIRS spectroscopy and phloroglucinol assay.



The accuracy of the predictive model was assessed with a simple linear regression (Figure 6). The slope of the linear curve and the R square close to zero (Table 1) show that the predictive model was not accurate enough to use the FT-NIRS gossypol concentration prediction as a seed selection method in spite of obtaining a good correlation in the calibration curves both for whole kernels (Figures 4) and ground seed (Figure 5). It was also attempted to classify seed in a qualitatively way such as “presence/absence” of gossypol but the results were not improved in spite of many treatments.

Figure 6. Scatterplot of FT-NIRS spectroscopy predicted values and phloroglucinol assay actual values of u equivalents of gossypol.



Simple linear regression line is plotted.

Table 1. Coefficient and probability value for linear regression equation for FT-NIRS spectroscopy predicted values and phloroglucinol assay actual values of µ equivalents of gossypol.

Order of equation	N	Intercept	Intercept Probability	Model Probability	R Square
1	35	56.58	<0.0001	0.051	0.11099

Cotton (*Gossypium hirsutum* L.) parental lines field trials

The analysis of variance showed significant seedcotton yield variation for all parental genotypes tested in 2010, 2011 and 2012 College Station field trials (Table 2 and Table 3). Because the interaction between genotypes and years was significant, the genotypes were analyzed by year as well. The yield trend that was seen in the three-year analysis was present also in the analysis per year.

Table 2. Seedcotton yield mean squares of six parental lines of cotton (*Gossypium hirsutum* L.) near College Station, TX, in 2010, 2011 and 2012.

Source	Df	Mean squares
Genotype	5	2,465,746***
Block[year]	9	800,738**
Year	2	7,105,986***
Genotype x Year	10	940,615***
Error	45	218,321

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

Table 3. Seedcotton yield means of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

Genotype	Seedcotton†
	kg ha^{-1}
TAMCOT 73	3,049 a
05-WL-27	3,017 a
05-A-46	2,914 ab
08-WZ-91	2,021 bc
HGN 78	1,848 c
HGN 71	1,137 c
Mean	2,712
CV %	17.2

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

In 2010 and 2012 ‘TAMCOT 73’ was found among the highest yielding genotypes while ‘HGN 71’ and ‘HGN 78’ were among the lowest yielding genotypes. In the 2011 growing season, no differences were found among genotypes and the test average was considerably less than the other two years (Table 4 and Table 5).

Table 4. Seedcotton yield mean squares by years of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

Year	Source	df	Mean Squares
2010	Block	3	176,731
	Genotype	5	2,465,596***
	Error	15	166,466
2011	Block	3	1,545,503***
	Genotype	5	175,135
	Error	15	95,524
2012	Block	3	680,167
	Genotype	5	3,821,754***
	Error	15	392,963

***Significant at the 0.001 probability level.

Table 5. Seedcotton yield means by years of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

Genotype	Seedcotton†		
	Year		
	2010	2011	2012
	kg ha^{-1}		
05-A-46	3,164 ab	1,322 a	4,538 ab
05-WL-27	3,267 a	1,355 a	4,401 ab
08-WZ-91	2,270 bc	1,541 a	5,029 ab
HGN 71	1,386 c	1,025 a	2,561 c
HGN 78	2,098 c	1,163 a	3,656 bc
TAMCOT 73	3,298 a	1,560 a	5,179 a
Mean	2,580	1,328	4,228
CV %	15.8	23.3	14.8

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

Lint percentage, or gin turnout, is the ratio of lint weight to seedcotton weight. In general, but not always, higher lint percentage indicates higher lint yields and is an important yield component. In the three-year field trial analysis, there was a significant interaction between genotypes and years (Table 6 and Table 7). For this reason, the performance of the genotypes in terms of lint percentage was further analyzed by years.

Table 6. Lint percentage mean squares of six parental lines of cotton (*Gossypium hirsutum* L.) near College Station, TX, in 2010, 2011 and 2012.

Source	df	Mean squares
Genotype	5	21.08***
Year	2	6.28
Block (Year)	7	6.52*
Genotype x Year	10	10.38**
Error	35	2.74

* Significant at the 0.01 probability level.

** Significant at the 0.001 probability level.

*** Significant at the 0.0001 probability level.

Table 7. Lint percentage means of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

Genotype	Lint percentage†
	%
08-WZ-91	39.6 a
05-A-46	39.3 a
TAMCOT 73	38.7 a
05-WL-27	36.4 ab
HGN 71	36.4 ab
HGN 78	33.7 b
Mean	38.1
CV %	4.3

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

TAMCOT 73, 08-WZ-91 and 05-A-46 had significantly higher lint percentage than the other three parental lines both in 2010 and in 2011. In 2012 there were no significant differences among parents (Table 8 and Table 9).

Table 8. Lint percentage means by years of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

Year	Source	df	Mean squares
2010	Genotype	5	21.08***
	Block	3	1.38
	Error	15	0.57
2011	Genotype	5	23.82***

Table 8. Continued

Year	Source	df	Mean squares
2012	Block	3	4.31
	Error	15	1.8
	Genotype	5	49.48
	Block	1	28.6
	Error	5	12.09

*** Significant at the 0.001 probability level.

Table 9. Lint percentage means by years of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

Genotype	Lint percentage†		
	Year		
	2010	2011	2012
	——————————%——————————		
05-A-46	39.6 a	41.0 a	41.2 a
05-WL-27	36.6 b	39.4 ab	35.4a
08-WZ-91	39.8 a	40.7 a	41.0 a
HGN 71	36.6 b	37.5 bc	30.3 a
HGN 78	33.9 c	34.8 c	34.3 a
TAMCOT 73	38.9 a	407 a	43.0 a
Lint percentage Mean	37.6	39.0	37.5
CV %	2.0	3.4	9.3

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

The six parental lines were tested for five fiber quality traits: micronaire, upper half mean length (UHML), uniformity, strength and elongation. The analysis of variance across years showed significant differences between years for UHML, uniformity and elongation but the interaction between genotype and year was non-significant for all traits (Table 10).

Table 10. Mean squares of micronaire, upper half mean length, uniformity, strength and elongation of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

		Mean squares				
Source	df	Mic	UHML	UI	Strength	Elongation
Genotype	5	0.109*	1.011	4.8**	401.754*	0.691***
Year	2	0.100	12.540***	8.320**	98.436	4.636***
Block (Year)	7	0.037	2.134***	2.824	256.555	0.221
Genotype x Year	10	0.037	0.638	1.479	174.017	0.111
Error	35	0.041	0.422	1.306	122.966	0.097

* Significant at the 0.01 probability level.

** Significant at the 0.001 probability level.

*** Significant at the 0.0001 probability level.

TAMCOT 73 had the highest value for all traits being significantly different from HGN 71, the genotype with the lowest value for micronaire and elongation. HGN 71 showed an intermediate performance for strength not being significantly different from TAMCOT 73. The genotype with the lowest strength value was 05-A-46. HGN 78 was together with HGN 71 the genotype with the lowest

performance in micronaire and elongation. There were no significant differences among genotypes for upper half mean length and uniformity (Table 11).

Table 11. Genotype means for micronaire, upper half mean length, uniformity, strength and elongation of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

Genotype	Micronaire†	UHML†	Uniformity†	Strength†	Elongation†
	--	mm	%	mN/tex	%
05-A-46	4.4 ab	29.48 a	81.2 a	280.0 b	6.5 ab
05-WL-27	4.4 ab	29.29 a	83.3 a	301.1 ab	6.7 a
08-WZ-91	4.4 ab	29.23 a	81.1 a	291.0 ab	6.6 a
HGN 71	4.2 b	28.27 a	81.3 a	298.4 ab	5.75 c
HGN 78	4.2 ab	29.61 a	83.1 a	301.a ab	5.9 bc
TAMCOT 73	4.6 a	29.61 a	83.3 a	308.9 a	6.6 a
Mean	4.5	28.81	82	301.4	6.25
CV %	4.4	2.3	1.4	3.7	5.0

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

Upper half mean length, uniformity and elongation showed differences between years. Both upper half mean length and uniformity had the highest value in 2012. On the contrary, elongation had the highest value on the driest of the three years studied (Table 12).

Table 12. Year means for upper half mean length (UHML), uniformity and elongation of six parental lines of cotton (*Gossypium hirsutum* L.) evaluated near College Station, TX, in 2010, 2011 and 2012.

Year	UHML†	Uniformity†	Elongation†
	mm	%	%
2010	29.53 b	81.1 b	6.5 b
2011	26.99 c	80.1b	7.5 a
2012	31.49 a	83.9 a	6.5 c
Mean	28.8	82.0	6.3
CV %	3.0	1.7	8.5

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

ULGCS field trials

In the ULGCS field trials the objective was to compare how the backcrosses performed related to the corresponding recurrent parents and also to evaluate how the transgenic lines performed in the field when compared to non transgenic lines. For this reason, the lines were analyzed in a model that included both the genotype and the type (transgenic or non transgenic) in addition to year and block (Equation 1).

Equation 1. ANOVA model for the ULGCS field trials near College Station, TX in the years 2011 and 2012.

Y = Genotype + Type + Genotype x Type + Year + Block(Year) + Error

Table 13. Lint yield mean squares of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies near College Station, TX, in 2011 and 2012.

Source		Mean Squares		
	df	Lint yield	Lint percentage	Crop maturity
Genotype	5	60,915	0.007281***	529.8***
Type	1	499,606*	0.000651	160.2
Genotype x Type	5	73,369	0.000511	136.4
Block[Year]	6	149,585	0.000476	92.6
Year	1	5,978	0.009801***	26,533.5***
Error	77	89,896	0.000382	59.6

* Significant at the 0.05 level.

*** Significant at the 0.001 level.

The analysis of variance for yield showed no differences among genotypes or between years but it showed differences between recurrent parents (non transgenic) and the corresponding backcross lines (transgenic). In terms of lint percentage, there were differences among genotypes. TAMCOT 73, 08-WZ-91 and 05-A-46 had the highest values, but there were no differences between the recurrent parents and the backcross lines. The analysis of variance for crop maturity showed differences between lines but only HGN 71 had a significantly lower crop maturity (Table 13 and Table 14).

Table 14. Genotype means for lint yield, lint percent and crop maturity of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Genotype	Lint yield†	Lint percentage†	Crop maturity†
	kg ha^{-1}	%	%
05-A-46	1,560.0 a	39.2 a	70.9 a
05-WL-27	1,517.0 a	36.1 b	76.0 a
08-WZ-91	1,467.2 a	38.6 a	75.1 a
HGN 71	1,392.6 a	34.9 b	60.6 b
HGN 78	1,426.2 a	34.3 b	71.5 a
TAMCOT 73	1,440.8 a	38.8 a	75.2 a
Mean	1,467.3	37.0	71.6
CV %	20.4	5.3	10.8

Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

Transgenic backcross lines had a significantly higher lint yield on average which suggests a favorable effect of the transgenic trait in terms of plant productivity with no differences on lint percentage and crop maturity suggesting that transgenic lines respond to crop cycle similarly to the non transgenic recurrent parents (Table 15).

Table 15. Type means for lint yield, lint percent and crop maturity of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Type	Lint yield†	Lint percentage†	Crop maturity†
	kg ha^{-1}	%	%
Transgenic progeny	1,539.4 a	37.0 a	72.9 a
Recurrent parents	1,395.1 b	37.3 a	70.3 a
Mean	1,467.3	37.2	71.6
LSD	121.9	0.8	3.1
CV %	20.4	5.8	10.8

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Student´s t Test.

Table 16. Year means for lint yield, lint percent and crop maturity of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Year	Lint yield†	Lint percentage†	Crop maturity†
	kg ha^{-1}	%	%
2011	1,475.2 a	36.0 b	54.9 b
2012	1,459.4 a	38.0 a	88.2 a
Mean	1,467.3	37.0	71.6
LSD	121.9	0.8	3.1
CV %	20.4	5.8	10.8

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Student´s t Test.

The differences between years were reflected on lint percentage which was significantly lower in 2011 than in 2012 and in crop maturity. Maturity was higher in 2012 which could be due to when the boll counts were made within the growing seasons. Crop maturity is calculated as the percentage of open bolls vs. the total number of bolls (open + green) when 50% of the crop has open bolls. Differences are usually relative among genotypes and can be used to match the best maturity rate with the agronomic production system (Table 16).

The six parental lines and the corresponding ULGCS lines were analyzed for five fiber quality traits in the same way as the parental lines test. Micronaire, upper half mean length and strength showed interaction between genotype and type (Table 17). For this reason differences between genotypes were further analyzed in an ANOVA by type. Uniformity and elongation showed differences between genotypes and while uniformity also showed differences between types elongation showed differences between years (Table 17).

Table 17. Mean squares of micronaire, upper half mean length (UHML), uniformity, strength and elongation of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Source		Mean Squares				
	Df	Mic	UHML	Uniformity	Strength	Elongation
Genotype	5	1.09***	1.18	12.11***	4,510.04***	2.74***
Type	1	0.08	14.52***	10.47***	3,915.86***	0.05
Genotype x Type	5	0.15*	2.49**	1.06	814.38*	0.12
Block[year]	6	0.08	0.29	0.41	347.72	0.09
Year	1	0.55**	0.92	0.79	5,240.15***	74.20***
Error	77	0.06	0.59	0.62	291.69	0.13

* Significant at the 0.05 level.
** Significant at the 0.01 level.
*** Significant at the 0.001 level.

Both the transgenic progeny and the recurrent parents of genotypes 05-A-46 and 08-WZ-91 had a lower uniformity percentage while the two African lines had the lowest elongation percentage (Table 18).

Table 18. Genotype means for uniformity and elongation of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Genotype	Uniformity†	Elongation†
	%	%
05-A-46	82.6 b	5.6 a
05-WL-27	83.9 a	5.7 a
08-WZ-91	81.8 b	5.6 a
HGN 71	83.4 a	4.7 b
HGN 78	84.0 a	5.0 b
TAMCOT 73	83.8 a	5.6 a
Mean	83.3	5.4
CV %	0.9	6.8

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

The transgenic progeny had a higher uniformity percentage than the recurrent parents (Table 19) while elongation percentage was lower in the year 2012 than in 2011 showing that this trait was affected by differences between years (Table 20).

Table 19. Type means for uniformity and elongation of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Type	Uniformity†	Elongation†
	%	%
Transgenic progeny	83.6 a	5.4 a
Recurrent parents	82.9 b	5.4 a
Mean	83.3	5.4
LSD	0.3	0.2
CV %	0.9	6.8

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Student´s t Test.

Table 20. Year means for uniformity and elongation of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Year	Uniformity†	Elongation†
	%	%
2011	83.4 a	6.3 a
2012	83.4 a	4.5 b
Mean	83.3	5.4
LSD	0.3	0.2
CV %	0.9	6.8

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Student´s t Test

Those fiber traits which had shown an interaction between genotype and type (main effects) were studied separated by type. In this way the effect of

genotype can be observed without it being masked by type. The mean squares table shows there were differences between genotypes in micronaire and strength both in the transgenic progeny and the recurrent parents while upper half mean length shows differences in the recurrent parents only (Table 21).

Table 21. Mean squares by type of micronaire, upper half mean length (UHML) and strength of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Source	df	Mean Squares					
		Transgenic progeny			Recurrent parents		
		Mic	UHML	Strength	Mic	UHML	Strength
Genotype	5	0.99***	0.97	2,984.33***	0.45***	1.83*	1,475.61***
Block[year]	6	0.06	0.21	90.12	0.09	0.41	544.96*
Year	1	0.32**	0.77	26.58***	0.23	0.01	1,386.78*
Genotype x Year	5	0.02	1.83	1,259.28**	0.09	0.42	366.68
Error	30	0.05	0.85	320.88	0.06	0.37	221.06

*** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

*** Significant at the 0.0001 probability level.

The genotype with the highest and the lowest micronaire value were TAMCOT 73 and HGN 71 respectively in both types (Table 22). In the transgenic progeny there were no differences between genotypes for upper half mean length but there were differences between the recurrent parents where TAMCOT 73 and 05-A-46 had the highest UHML and 08-WZ-91 had the lowest

UHML (Table 22). Strength also shows differences between types (Table 22). The transgenic progeny shows higher values than the recurrent parents with the transgenic progeny of HGN 71 having the highest strength (Table 22).

Table 22. Genotype means by type of micronaire, upper half mean length (UHML) and strength of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Genotype	Transgenic progeny			Recurrent parents		
	Mic	UHML	Strength	Mic	UHML	Strength
	--	mm	mN/tex	--	Mm	mN/tex
05-A-46	4.6 ab	29.7 a	294.7 b	4.4 a	29.8 a	298.0 b
05-WL-27	4.3 b	30.1 a	339.7 a	4.5 a	29.1 ab	326.1 a
08-WZ-91	4.4 b	30.4 a	324.6 a	4.4 a	28.6 b	300.8 b
HGN 71	3.8 c	31.0 a	361.9 a	3.9 b	29.6 ab	327.3 a
HGN 78	4.4 b	30.3 a	325.3 a	4.2 ab	29.6 ab	319.4 ab
TAMCOT 73	4.7 a	29.7 a	329.4 a	4.5 a	29.9 a	327.3 a
Mean	4.4	30.2	329.3	4.3	29.4	316.5
CV %	5.1	3.1	5.4	5.7	2.1	4.7

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Tukey HSD Test.

The ANOVA by type showed differences between years for micronaire and strength which were lower in 2012 than in 2011 while the recurrent parents showed differences for strength which was also lower in 2012 (Table 23).

Table 23. Year means by type of micronaire, upper half mean length (UHML) and strength of six parental lines of cotton (*Gossypium hirsutum* L.) and its six introgressed backcross progenies evaluated near College Station, TX, in 2011 and 2012.

Year	Transgenic progeny			Recurrent parents		
	Mic	UHML	Strength	Mic	UHML	Strength
	--	mm	mN/tex	--	mm	mN/tex
2011	4.5 a	30.25 a	334.42 a	4.3 a	29.44 a	321.85 a
2012	4.3 b	30.00 a	319.85 b	4.2 a	29.42 a	311.10 b
Mean	4.4	30.21	329.25	4.3	29.43	316.47
LSD	0.1	0.38	7.43	0.1	0.25	6.20
CV %	5.1	3.1	5.4	5.7	2.1	4.7

†Means not connected by the same letters are significantly different at the 0.05 probability level as calculated by least square means (LS Mean) differences Student´s t Test.

CHAPTER IV

CONCLUSIONS

There are several aspects of the work done to introgress the Ultra-Low Gossypol Cottonseed transgenic trait from Coker 312 into six elite cotton cultivars with different origins that are worth being discussed.

Screening methods for ULGCS trait in backcross cottonseed

The selection process of transgenic seed from a population of transgenic and non transgenic seed was a challenge in itself. From a breeding perspective the selection of seed to plant the next generation of individuals needs to be accurate, efficient and the least expensive and time consuming as possible. In every generation of backcrossing, two types of seed were generated due to segregation of the transgenic trait; seed that would not carry the ULGCS trait and seed that would be hemizygous for the trait, meaning that it carried a copy of the transgene received from the transgenic donor parent gamete. Because testing a small portion of the seed for the ULGCS trait and germinating it to continue with the backcrossing proved to be difficult in terms of seed germination, decision was made to perform the screening using the seedling

cotyledon tissue. This is a plausible alternative since cottonseed is predominantly cotyledonary tissue (McMichael, 1960).

Three different methods to identify ultra-low gossypol seeds, *Phloroglucinol Assay for Gossypol, PCR,* and *Fourier Transform-Near Infrared (FT-NIRS) Spectroscopy*, were tested to determine which one was the most beneficial in terms of:

a. Accuracy: whether it would be an effective method to select transgenic vs non-transgenic plants
b. Speed: turn around time from seed harvest to planting of the new generation needed to be fast in order to advance the most generations in one year
c. Tissue used: determining presence of the transgene in the seed before planting is desirable because fewer resources would be used in planting transgenic individuals only
d. Costs: the method with the lowest cost was desired

The phloroglucinol assay for gossypol was identified as the best method to select ULGCS seed to be advanced for the next cycle of backcrossing. This method was selected because it was quantitatively accurate, based on the results from the experiments conducted to compare phloroglucinol assay with the PCR method (Chapter III-Results). The time per sample was lower than with the PCR method, and the cost was lower than the PCR. Moreover, PCR had no advantage regarding the tissue-type tested because it also involved the use of

cotyledons. FT-NIRS appeared to be the most promising because it is a non-destructive, fast and low cost method that has been applied successfully by different research groups (Lohumi et al. 2013, Kumar and Andy 2013). Samples usually do not require prior preparation and the test is less costly than the traditional wet-chemical methods. Near-infrared spectroscopy uses combination and overtone bands, which are harmonics of absorption frequencies in the mid-infrared region, to differentiate compounds and it uses the size of peaks in the spectrum to indicate abundance of the materials present in the sample. The advantages of FT-NIRS over NIRS are related to limitations of the dispersive NIRS instrumentation and the production of an interferogram in the FT-NIRS, which is the combination of all the infrared frequencies, allowing for reduced analysis time per sample. Through the Fourier transformation the interferogram is transformed into a spectrum of frequencies vs. intensity. In addition to this, FT-NIRS instruments show higher resolution spectra than dispersive NIRS that can be measured quickly and easily. FT-NIRS instruments also use an internal reference laser known as Conned Advantage that allows for higher wavelength accuracy and precision greater than 2 cm^{-1} (Sousa-Correia et al., 2007, Clermont and Luchetta, 2002). However, in spite of the good correlation obtained in the calibration curve the gossypol values predicted by the FT-NIRS were not significantly correlated to the true gossypol values of the seed analyzed (Chapter III-Results). In addition, it was attempted to use FT-NIRS to classify seed qualitatively in high-gossypol vs. low-gossypol but despite many treatments

results were not improved. This poor correlation made the FT-NIRS method the least reliable method to screen the backcross progeny for ULGCS seed. Birth and Ramey (1982), concluded in their study of gossypol in cottonseed using Near-Infrared reflectance that the fact that glands containing gossypol are unevenly distributed in the seed could be a limiting factor for the accurate quantification of gossypol using spectroscopy techniques.

Field yield trials and fiber quality tests

Two types of yield trials were conducted in this study: a three-year yield trial of the parental lines selected as recurrent parents for the introgression of the ULGCS trait and two one-year yield trials in which two different backcross generations were compared with the elite recurrent parents. As it can be expected the three years in which the studies took place were different in terms of total rainfall and average temperature during the growing season from May to October (U.S. climate data). The year 2010 and the year 2012 had similar average temperatures throughout the growing season but were different in terms of distribution of total rainfall. Although the accumulated rainfall was similar in both years, the year 2010 had high precipitations at the beginning of the growing season and at the end, during the months of June and September but received only 1 inch of rain in July and almost no rain in August (0.33 inches). These two months are critical in cotton because during this time flowering and seed set

occur which are two of the main factors affecting cotton yield and cotton fiber quality. In addition, July and August are the hottest months of the growing season, adding heat stress to a crop which is already suffering from water stress due to the reduced water availability. On the other hand, rainfall during the year 2012 was distributed more evenly along the growing season with rainfall in July and August reaching 4.56 inches and 1.71 inches respectively. The year 2011 was highly different both from its preceding and following years. In this year total rainfall from May to October was almost half the amount of the years 2010 and 2012 and the average temperatures registered in June, July, August and October were higher than in the same months in 2010 and 2012. The Comptroller of Public Affairs considers the 2011 Texas drought as the most severe drought since 1895 (Texas Comptroller of Public Affairs)). In general, in the three years tested the four elite cultivars from the Texas A&M Cotton breeding program had higher yield and Lint percent than the two cultivars with African origin, showing that adapted germplasm performs better even in harsh years. In terms of fiber quality, the cultivars with African origin were usually, although not always, on the lower end for all the traits. This may also be due to adaptation of these cultivars to the College Station, TX environment. The fact that yield is usually the main focus of cotton breeding programs while improving fiber quality traits is not a strong driving factor may also contribute to low performance on some of the traits analyzed in the African lines and in some of the Texan cultivars. When the parental lines were grown with the backcross

progeny the same trend was observed in terms of adaptation of the tested cultivars to the environment and improved performance of all genotypes in 2012. Importantly only one of the genotypes tested showed significant differences for two fiber quality traits between a parental line and its corresponding backcross progeny. This suggests that the introgression of the ULGCS did not affect the genetic background of the lines that were used as recurrent parents. More importantly, the results from this research work confirm the stability of the ULGCS trait in advanced backcross generations that Rathore et al. 2012 had studied in advanced transgenic generations.

Impact of the ultra-low gossypol cottonseed trait

Overall, this study shows that the ULGCS seed trait can be introgressed into elite lines from different origins in a stable manner and without interfering with the genetic background of the recurrent parent. Moreover, during the backcross process, selections can be made to improve fiber quality and/or biotic and abiotic stress tolerance in addition to the yield. Cotton is grown in areas of the world where people suffer from significant protein deficiencies. Thus, making the availability of cotton with seed that can be used for monogastric animals´ consumption, including human beings should increase the value of this crop dramatically. Even in the United States turning the cotton crop into a dual crop – fiber and seed- can have great impact on local economies which are

influenced by the cotton industry. Cottonseed with ultra-low gossypol levels could also impact other industries because it can be fed to the monogastric animals that convert feed into protein more efficiently than the ruminants. This is also an advantage for subsistence farmers who could sell the fiber and keep the seed to feed chicken, pigs and other farm animals.

In addition, provided that appropriate alterations in the silencing cassette are made and the marker gene can be eliminated, this type of technology has a great potential in the generation of intragenic plants which may be more easily accepted by the public and even organic farmers because all the genetic material introduced will be of cotton plant origin within the species' sexual compatibility group (Rommens et al. 2007 and Rommens et al. 2004). This could accelerate the acceptance by many developing countries that currently ban the use of GMOs, making this valuable product available to the people who need it the most.

REFERENCES

Alford B.B., G.U. Liepa, and A.D. Vanbeber. 1996. Cottonseed protein: what does the future hold? Plant Foods for Hum. Nutr. 49:1-11.

Bell, A.A. 1967. Formation of gossypol in infected or chemically irritated tissues of Gossypium species. Phytopathology 57: 759–764.

Bewley J.D., M. Black, and P. Halmer, editors. 2006. The encyclopedia of seeds: Science, Technology and Uses. CABI Publishing Series, Oxfordshire, UK. ISBN: 9780851997230.

Birth G.S., and H. H. Ramey Jr. 1982. Near-Infrared reflectance for Analysis of cottonseed for gossypol. Cereal. Chem. 59:516-519.

Blom J.H., K.J. Lee, J. Rinchard, K. Dabrowski, and Ottobre J. 2001. Reproductive efficiency and maternal-offspring transfer of gossypol in rainbow trout (*Oncorhynchus mykiss*) fed diets containing cottonseed meal. J. Anim. Sci. 79:1533-1539.

Cai Y.F., Y.F. Xie, and J.G Liu. 2010. Glandless seed and glanded plant research in cotton. A review. Agron. Sustainable Dev. 30:181-190.

Clermont T., and L. Luchetta, 2002. Thermo Nicolet Industrial Solutions Note. ISN: 0001.

Cooke K.M., J.K.Bernard, and J.W. West. 2007. Performance of lactating dairy cows fed whole cottonseed coated with gelatinized starch plus urea or yeast culture. J. Dairy Sci. 90:360-364.

Coppock C.E., J.K.Lanham, and J.I. Horner. 1987. A review of the nutritive-value and utilization of whole cottonseed, cottonseed meal and associated by-products by dairy cattle. Anim. Feed Sci. Technol. 18:89-129.

Cotton Incorporated. 2012. Whole cottonseed a super feed for dairy cows. http://www.cottoninc.com (accessed 14 Oct. 2014).

Coutinho EM. 2002. Gossypol: a contraceptive for men. Contraception 65:259-263.

Dayani O., P. Dadvar, and M. Afsharmanesh. 2011a. Effect of dietary whole cottonseed and crude protein level on blood parameters and performance of fattening lambs. Small Ruminant Res. 97:48-54.

Dayani O., G.R. Ghorbani and, A.K. Esmailizadeh. 2011b. Supplementation with whole cottonseed changes milk composition and milk fatty acid profile in dairy cows. Anim. Prod. Sci. 51:95-101.

Dorsa W.J., H.R. Robinette. E.H.Robinson, and, W.E. Poe. 1982. Effects of dietary cottonseed meal and gossypol on growth of young channel catfish. Trans. Am. Fish. Soc. 111:651-655.

Gao D.D., Y.S. Cao, and H.X. Li. 2010. Antioxidant activity of peptide fractions derived from cottonseed protein hydrolysate. J. Sci. Food Agric. 90:1855-1860.

FAO. 2012. Agricultural Development Economics Division (ESA), Committee on World Food Security. http://www.fao.org/cfs/cfs-home/en/ (accessed 1 Sep. 2014).

Gatlin D.M., F.T. Barrows, P. Brown, K. Dabrowski, T.G. Gaylord, R.W. Hardy, et al. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquacult. Res. 38:551-579.

Graham G. G,, E. Morales, G. Acevedo, J. M. Baertl, and A. Cordano. 1969. Dietary Protein Quality in Infants and Children. II. Metabolic S tudies with Cottonseed Flour. Am. J. Clinical Nutrition. 22:577-587.

Hawkins G.E., K.A. Cummins, M. Silverio, and J.J. Jilek. 1985. Physiological-effects of whole cottonseed in the diet of lactating dairy-cows. J. Dairy Sci. 68:2608-2614.

Jenkins J.N., F.G. Maxwell, and Lafever. 1966. Comparative preference of insects for glanded and glandless cottons. J. Econ. Entomol. 59:352-356.

Kubik, D. J., and Stock, Rick, G90-978 Byproduct feedstuffs for beef and dairy cattle. 1990. Historical Materials from University of Nebraska-Lincoln Extension. Paper 311. http://digitalcommons.unl.edu/extensionhist/311 (accessed 8 Jan. 2015).

Kulkarni V.N., B.M. Khadi, M.S. Maralappanavar, L.A. Deshapande, and S.S. Narayanan. 2009. The worldwide gene pools of *Gossypium arboreum* L. and *G. herbaceum* L., and their improvement. Springer, New York. p. 1-29.

Kumar S., and A. Andy. 2013. Fourier transform-near infrared reflectance spectroscopy calibration development for screening of oil content of intact safflower seeds. Int. Food Res. J. 20:759-762.

Lee K.J., K. Dabrowski, J.H. Blom, S.C. Bai, and P.C. Stromberg. 2002. A mixture of cottonseed meal, soybean meal and animal byproduct mixture as a fishmeal substitute: growth and tissue gossypol enantiomer in juvenile rainbow trout (*Oncorhynchus mykiss*). J. Anim. Physiol. Anim. Nutr. 86:201-213.

Lohumi S., C. Mo, J-S Kang, S-J Hong, and B-K Cho. 2013. Nondestructive evaluation for the viability of watermelon (Citrullus lanatus) seeds using Fourier transform near infrared spectroscopy. J. Biosystems Eng. 38:312-317

Lukefahr M.J., L.W. Noble, and J.E. Houghtal. 1966. Growth and infestation of bollworms and other insects on glanded and glandless strains of cotton. J. Econ. Entomol. 59:817-820.

Lukonge E., M.T. Labuschagne, and A. Hugo. 2007. The evaluation of oil and fatty acid composition in seed of cotton accessions from various countries. J. Sci. Food Agric. 87:340-347.

Lusas E.W., and G.M. Jividen. 1987a. Glandless cottonseed - a review of the 1st 25 years of processing and utilization research. J. Am. Oil Chem. Soc. 64:839-854.

Lusas E.W., and G.M. Jividen. 1987b. Characteristics and uses of glandless cottonseed food protein ingredients.2. J. Am. Oil Chem. Soc. 64:973-986.

Mccollum F.T., and M.L. Galyean. 1985. Influence of cottonseed meal supplementation on voluntary intake, rumen fermentation and rate of passage of prairie hay in beef steers. J. Anim. Sci. 60:570-577.

McMichael S. 1959. Hopi cotton, a source of cottonseed free of gossypol pigments. Agron. J. 51:630-630.

McMichael S.C. 1960. Combined effects of glandless genes gl2 and gl3 on pigment glands in the cotton plant. Agron. J. 52:385-386.

Oskoueian E., N. Abdullah, R. Hendra, and E. Karimi. 2011. Bioactive compounds, antioxidant, xanthine oxidase inhibitory, tyrosinase inhibitory and anti-inflammatory activities of selected agro-industrial by-products. Int. J. Mol. Sci. 12:8610-8625.

Pham M.A., K.J. Lee, S.J. Lim, and K.H. Park. 2007. Evaluation of cottonseed and soybean meal as partial replacement for fishmeal in diets for juvenile Japanese flounder *Paralichthys olivaceus*. Fish. Sci. 73:760-769.

Piccinelli A.L., A. Veneziano, S. Passi, F.D. Simone, and L. Rastrelli. 2007. Flavonol glycosides from whole cottonseed by-product. Food Chem. 100:344-349.

Ramadan M.F., and J.-T.Moersel. 2006. Screening of the antiradical action of vegetable oils. J. Food Compos. Anal. 19:838-842.

Rathore K.S., G. Sunilkumar, L. Puckhaber, R.D. Stipanovic, Monjur H.M., E. Hernandez, and C.W. Smith. 2003. Improvements in the nutritional quality of the cottonseed. In IK Vasil, editor, Proceedings of the 10th IAPTC&B Congress June 23–28, 2002 Orlando, Florida, U.S.A Plant Biotechnology 2002 and Beyond. Springer, Dordrecht. p. 417-420.

Rathore, K. S., L. M. Campbell, L. Tollack, G. Sunilkumar, and E. Lopata-Finch. 2011. Genetic engineering of a cotton plant: progress and the remaining bottlenecks, a quarter of a century later. In Vitro Cell. Dev. Biol.—Animal. 47 (Suppl 1):S17–S30.

Rathore, K. S., S. Sundaram, G. Sunilkumar, L. M. Campbell, Ll Puckhaber, S. Marcel, S. R. Palle, R. D. Stipanovic, and T. C. Wedegaertner. 2012. Ultra-low gossypol cottonseed: generational stability of the seed-specific, RNAi-mediated phenotype and resumption of terpenoid profile following seed germination. Plant Biotechnol. J. 10:174–183.

Rathore P., M. Singh, D. Pathak, J.S. Gill, and A.K. Atwal. 2008. Genetic analysis for seed oil content and component fatty acids in American cotton (*Gossypium hirsutum* L.). Indian J. Genet. Plant Breed. 68:340-343.

Risco C.A., A.L. Adams, S. Seebohm, M.J. Thatcher, C.R. Staples, H.H. Van Horn et al. 2002. Effects of gossypol from cottonseed on hematological responses and plasma α-tocopherol concentration of dairy cows. J. Dairy Sci. 85:3395-3402.

Romano G.B., and J.A. Scheffler. 2008. Lowering seed gossypol content in glanded cotton (*Gossypium hirsutum* L.) lines. Plant Breed. 127:619-624.

Rommens C.M., J.M. Humara, J. Ye, H. Yan, C. Richael, L. Zhang, R. Perry and K. Swords. 2004. Crop improvement through modification of the plant's own genome. Plant Physiol. 135:421–431.

Rommens C.M., M.A. Haring, K. Sowrds, H.V: Davies and W.R: Belnap. 2007. The intragenic approach as a new extension to traditional plant breeding. Trends Plant Sci. 12:397–403.

SeedCo Corporation. 1974. Cotton (*Gossypium hirsutum* L.) variety U.S. Plant Variety Protection PVP 7200100. Date issued: 26 July. http://www.ars-grin.gov/ (accessed 23 Apr. 2015)

Seelanan T., A. Schnabel, and J.F. Wendel. 1997. Congruence and consensus in the cotton tribe (Malvaceae). Syst. Bot. 22:259-290.

Smith C.W., and J.T. Cothren, editors. 1999. Cotton : origin, history, technology, and production. John Wiley and Sons, Inc. ISBN: 978-0-471-18045-6.

Solaiman S.G., N.K. Gurung, Q. McCrary, H. Goyal, and W.H. McElhenney. 2009. Feeding performance and blood parameters of male goat kids fed EasiFlo (R) cottonseed. Small Ruminant Res. 81:137-145.

Sousa-Correia C., A. Alves A., J.C. Rodrigues, S. Ferreira-Dias, J.M. Abreu, N. Maxted, et al. 2007. Oil content estimation of individual kernels of *Quercus ilex* subsp *rotundifolia* (Lam) O. Schwarz acorns by Fourier

transform near infrared spectroscopy and partial least squares regression. J. Near Infrared Spectrosc. 15:247-260.

Sunilkumar G., J.P. Connell, C.W. Smith, A.S. Reddy, and K.S. Rathore. 2002. Cotton *α*-globulin promoter: isolation and functional characterization in transgenic cotton, *Arabidopsis*, and tobacco. Trans. Res. 11:347–359.

Sunilkumar G., L.M. Campbell, L. Puckhaber, R.D. Stipanovic, and K.S. Rathore. 2006. Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. PNAS. 103:18054-18059.

Texas Comptroller of Public Affairs. http://www.window.state.tx.us/specialrpt/drought/ (accessed 12 Oct. 2014).

U.S. Climate Data. www.usclimatedata.com (accessed 12 Oct. 2014).

USDA. 2011. Annual cotton Review, USDA-NASS Oklahoma Field Office (accessed 8 Nov. 2014).

USDA. 2014. Foreign Agricultural Service. Oilseeds: World Markets and Trade. http://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf (accessed 8 Nov. 2014).

Wang, X., C.P. Howell, F. Chen, J. Yin, and Y. Jiang. 2009. Chapter 6 Gossypol-A polyphenolic compound from cotton plant. In: Steve L. Taylor, editor, Adv. Food Nutr. Res. 58: 215-263. ISBN 9780123744418, http://dx.doi.org/10.1016/S1043-4526(09)58006-0.

Wendel J.F., C.L. Brubaker, and T. Seelanan. 2010. The Origin and evolution of *Gossypium*. In: J. McD.Stewart et al. editors, Physiology of cotton. Springer, Netherlands. p. 1-18.

Wendel J.F., and V.A. Albert. 1992. Phylogenetics of the cotton genus (*Gossypium*) – Character-state weighted parsimony analysis of chloroplast-DNA restriction site data and its systematic and biogeographic implications. Syst. Bot. 17:115-143.

Wendel J.F., and R.C. Cronn. 2003. Polyploidy and the evolutionary history of cotton. Adv. Agron. 78:139-186.

Wilkins T.A., K. Rajasekaran, and D.M. Anderson. 2000. Cotton biotechnology. Crit. Rev. Plant Sci. 19:511-550.

Zhou M., C. Zhang, Y. Wu, and Y. Tang. 2013. Metabolic engineering of gossypol in cotton. Appl. Microbiol. Biotechnol. 97:6159-6165.