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BREEDING AND GENETICS

Effect of Chromosome Substitution from Alien Tetraploid Cotton Species in Upland Cotton on (+) and (-) Gossypol Enantiomer Levels in Cottonseed

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ABSTRACT

Cottonseed contains high quality protein meal for feed and oil for human consumption, but gossypol in cottonseed has potential toxicity and detrimental effects that limit cottonseed use as food for humans and monogastric animals. Therefore, identifying germplasm containing lower gossypol content is critical. The objective of this research was to investigate the influence of specific chromosomes or chromosome segments from *Gossypium barbadense*, *G. tomentosum,* and *G. mustelinum*, respectively, on (+) and (−) gossypol levels when substituted into *G. hirsutum*. A total of 11 genotypes were used in this study: nine chromosome substitution lines (CS lines) were investigated for cottonseed gossypol level in field experiments in 2013 and 2014; TM-1 (the recurrent parent of the CS line) and AM UA48 (cultivar) were used as controls. Results showed significant variation in gossypol level and its fractions among CS lines. This variation is a result of chromosome substitution, although it was also affected by environment (location) as location x genotype was significant. Significant positive relationships between total gossypol, (+) gossypol, and (−) gossypol were found. This research demonstrated significant differences among the nine CS lines, and some CS lines had significantly lower gossypol level in cottonseed. These results provide an alternative breeding approach for possibly selecting low levels of gossypol and improving cottonseed nutritional qualities using CS lines.

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Cotton, *Gossypium* spp., is considered America's number one value-added crop contributing more than $120 billion to the U.S. economy (Cotton Counts, 2019). The U.S. was ranked as the third leading cotton producing country in the world in 2016-2017, producing approximately 17.6 million bales of fiber in 2018 (Statista, 2019). Although cotton is used most visibly to produce natural textile fibers, cottonseed meal and oil are secondary byproducts used as human food, animal feed, and industrial raw materials. Cottonseed is considered a good source of plant proteins (23-28% dry seed weight) and oil (21-29% dry seed weight) (Bi et al., 1999). The U.S. was ranked third globally in cottonseed oil production (286,000 t), following India (1,487,000 t) and China (1,305,000 t) in 2013-2014 (USDA–FAS, 2015).

Throughout the plant, cotton is characterized by the presence of gossypol storage pigment glands that contain terpenoid aldehydes (Rathore et al., 2012). Gossypol is a phenolic compound produced by pigment glands distributed throughout plant, but concentrated in seeds (Alexander et al., 2008; Kenar, 2006; Rogers et al., 2002; Romano and Scheffler, 2008) and roots (Scheffler, 2016). Cottonseed contains several toxic chemicals as secondary metabolites, including gossypol, cinnamic acids, flavonoids, terpenoids, alkaloids, cyanohydrins, quinones, saponins, unsaturated lactones, benzoxazinones, allyl sulfides, thiocyanates, and polyacetylenes (Ingham and Harborne, 1976; Stoessl et al., 1976). Gossypol was first isolated in 1899 from *Gossypium*; has a 518.55 Dalton molecular weight; is a yellow pigment; and is insoluble in water and hexane, but soluble in acetone, chloroform, ether, and methyl ethyl ketone (Gadelha et al., 2014).

The chemical formula of gossypol is $C_{30}H_{30}O_8$, and the structural formula is 2,2´-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene) (Gadelha et al., 2014). Gossypol occurs naturally as a mixture of two enantiomers [(+) and (−) gossypol] that differ in their optical properties (Huang et al., 1987). Chemically, gossypol is composed of two

naphthalene rings with restricted rotation around the bond connecting the rings. Because of this restricted rotation, cottonseed is considered the second-best source of plant proteins after soybean, and the fifth-best oil-producing plant after soybean, palm tree, colza, and sunflower (Saravanan et al., 2010).

Although cottonseed meal contains high quality protein for animal feed and oil for human consumption, gossypol has a toxicity and detrimental effect on health when fed to humans and monogastric animals, including pigs, chickens, and fish (Blom et al., 2001; Henry et al., 2001; Idowu et al., 2012). Gossypol also plays an important beneficial role in defending the plant against various insect pests (Bell, 1974; Swain, 1977). Currently, most cottonseed feed is fed only to adult ruminants because their digestive system can tolerate gossypol (Romano and Scheffler, 2008). In addition, gossypol is an orally active, polyphenolic aldehyde product with potential antineoplastic activity present in unrefined cottonseed oil (refined cottonseed oil is stripped of gossypol). Gossypol is responsible for acute clinical poisoning, impairment of male and female reproduction, and interference with immune function (Gadelha et al., 2014).

Researchers reported that feeding chicken broilers with diets containing high levels of (−) gossypol was more detrimental to growth than (+) gossypol (Lordelo et al., 2005). Their results suggested that both gossypol enantiomers were toxic to broilers, but (−) gossypol was more harmful to efficient broiler production than (+) gossypol. Gossypol leads to a significant effect on inducing cell-cycle arrest at the G0/G1 phase, thereby inhibiting DNA replication and inducing apoptosis; inhibiting cell-signaling enzymes, resulting in inhibition of cell growth; and could act as a male contraceptive (https://pubchem.ncbi.nlm.nih.gov/compound/gossypol). Gossypol's toxicity effects on health are dependent upon the enantiomeric form of which the (+) form is the least toxic (Joseph et al., 1986; Lordelo et al., 2005; Yu, 1987). Previous studies indicated that the (+) and (–) forms of gossypol are equally effective against the plant pathogen *Rhizoctonia solani* J.G. Kühn (Puckhaber et al., 2002) and corn earworm (*Helicoverpa zea* [Boddie]) larvae (Stipanovic et al., 2006).

Cottonseed of *G. barbadense* L. contains up to 34 mg of gossypol/g (Percy et al., 1996). Previous studies suggested that (−) gossypol enantiomer is the most biologically active form and more toxic than the (+) gossypol (Bailey et al., 2000; Kakani et al., 2010). The (−) gossypol proportion ranges from 33.8 to 47.0% in seeds of Upland varieties (*G. hirsutum* L.) (Calhoun et al., 1995) and from 24.9 to 68.9% in seeds of *G. barbadense* (Percy et al., 1996). Research conducted with broilers showed that ground Pima (*G. barbadense*) cottonseed with a higher (−) to (+) ratio of gossypol was significantly more toxic, based on feed intake and body weight gain than ground Pima cottonseed with a higher (+) to (−) ratio of gossypol (Gamboa, 1997). Previous studies demonstrated that feeding broilers diets containing high levels of gossypol result in depressed weight gain (Lillie and Bird, 1950; Waldroup, 1981) and poor feed efficiency (Couch et al., 1955; Heywang and Bird, 1955). Previous reports showed that natural variation for total seed gossypol content in *G. hirsutum* and *G. barbadense* exists (Scheffler and Romano, 2008), ranging from 0.97 to 2.47% dry seed weight (Percy et al., 1996; Stipanovic et al., 2005). Entries in the 2017 National Cotton Variety Test ranged from 0.46 to 1.03% dry seed weight for (−) gossypol and 0.24 to 0.92% dry seed weight for (+) gossypol with total free gossypol from 0.95 to 1.78% dry seed weight (USDA-ARS, 2019). Others reported that considerable natural variation for total seed gossypol content exists among *Gossypium* species (0.0-3.6% dry seed weight) and *G. hirsutum* cultivars (0.6-2.5% dry seed weight) (Adams et al., 1960; Bell and Stipanovic, 1978; Stipanovic et al., 2005). The percentage of the (+) enantiomer varies, ranging from 50% in some varieties to greater than 90% in some exotic germplasm and *G. hirsutum* landraces.

The genes responsible for glanding in cotton, and therefore gossypol, include six independent loci: *gl1*, *gl2*, *gl3 gl4*, *gl5*, and *gl6*. Recent molecular studies from gene expression analysis also indicate multiple genes including several transcription factors are associated with these glands. These transcription factors have been designated the cotton gland formation (CGF) genes (Janga et al., 2019). Gao et al. (2020) reported that 22 transcription factors showed expression patterns associated with pigment glands and they characterized these genes. Previous study reported that no sequence differences were observed between glanded and glandless cotton for *CGF1* and *CGF2* gene pairs (Janga et al., 2019). They found that glandless cotton has a transposon insertion within the coding sequence of the *GoPGF* (synonym *CGF3*) gene of the A_t subgenome and extensive mutations in the promoter of the D_t subgenome homeolog in the tetraploid cotton. Overexpression of *GoPGF* resulted in a large increase in gossypol and related terpenoids

in cultured cells, whereas CRISPR/Cas9 knockout of *GoPGF* genes resulted in glandless phenotype (Janga et al., 2019). Classical genetic studies indicated that only two major genes (*Gl2* and *Gl3*) are believed to be involved in gland formation (Endrizzi et al., 1985; Gutierrez et al., 1972). *Gl2* and *Gl3* were localized to the A12 and D12 chromosomes of *G. hirsutum*, respectively (Lee, 1965; Percy et al., 2015; Samora et al., 1994). Different combinations of dominant (*Gl*) and recessive (*gl*) alleles produced lesser number of glands with varying distribution in different parts of the plant at different stages of development (Gutierrez et al., 1972; Lee, 1965; McCarty et al., 1996; McMichael, 1960; Scheffler and Romano, 2008, 2012). Considering the importance of pigment glands in cotton, genetic knowledge as well information about genetic variation of gossypol genes in the other tetraploid species is critical for the improvement of this trait in Upland cotton. There are limited studies on the gossypol gland with respect to gossypol (+) and (–) enantiomer levels especially other than on *G. hirsutum* species. One of the goals of the current research is to study the effects of the substituted chromosome from other alien species in TM-1 (*G. hirsutum*).

Several mapping studies identifying genes controlling the formation of glands producing gossypol have been conducted. Cheng et al. (2016) reported that the Gl^{e}_{2} gene can effectively inhibit the formation of the pigment glands, producing gossypol. They used three F_2 populations having two pairs of near isogenic lines differing in the gland trait for fine mapping Gl^{e}_{2}. They were able to identify DNA markers from the recently developed cotton genome sequence and were able to find that the Gl^{e}_{2} gene was located within a 15-kb genomic interval between two markers, CS2 and CS4, on chromosome 12. They added that Gl^{e}_{2} encodes a MYC transcription-factor family member with 475 amino acids, but expression analysis results indicated that the *MYC* gene expresses in glanded lines, but not in glandless lines. They concluded that the *MYC* gene is an essential positive regulator for pigment glands and low expression of this gene can inhibit pigment gland formation, facilitating the research on glandless trait and low-gossypol cotton breeding. Other researchers have been able to identify a total of 40 single nucleotide polymorphisms (SNP) including six indels in 16,328 bp of gland development-related protein (GDRP) coding and intron sequences (Cho et al., 2011). GDRP is a total of 85 unique mRNA sequences that were collected from NCBI GenBank (Cho et al., 2011). All SNP markers were genotyped against 188 recombinant inbred lines previously developed from the cross of TM-1 and 3-79 and mapped 36 SNP markers (24 loci) to a genetic map saturated with more than 2,000 molecular markers. The 24 loci mapped in this investigation were distributed throughout 15 chromosomes.

Two major gland genes, *Gl2* and *Gl3*, exist (McMichael, 1960). Lee crossed fully glanded (*Gl2Gl2Gl3Gl3*) cotton lines with glandless (*gl2gl2gl3gl3*) lines and found that some F_2 progeny had fewer gossypol glands and lower content of gossypol in cottonseed (Lee, 1962, 1973, 1974; Lee et al., 1968). Others showed that crossing genotypes with glands and no glands resulted in progeny with a range of gland numbers and distribution (Rhyne et al., 1959). Romano and Scheffler (2008) made crosses between glanded (GL) and glandless (gl) parents and evaluated the resulting progeny to determine the contribution and effectiveness of the genetic variation for low gossypol genotypes and gland distribution in the segregating progeny. They were able to identify F_7 generation progeny that had < 0.30% total gossypol in the seed, while having adequate glands at critical locations throughout the cotton plants. Working on two stocks of *G. hirsutum* differing in gossypol level, Lee (1962, 1965, 1977, 1978) and Lee et al. (1968) found that the line, 3-T (dimeric genotype, *Gl2Gl2G3Gl3)*, had an average of 2.78% gossypol in dried seed meal, whereas the same genotype in 'Acala 4-42' averaged 0.90%. Line 3-T (high-gossypol content), the monomerics *Gl2Gl2gl3gl3* and *gl2gl2Gl3Gl3*, had an average of 3.03 and 1.18% gossypol, respectively. They also found that the same genotypes in Acala 4-42 (low-gossypol content cultivar) averaged 0.69 and 0.24%. Introgression of the glandless seed trait from *G. sturtianum* Willis into Upland cotton was attempted without success (Altman et al., 1987; Romano and Scheffler, 2008; Vroh et al., 1999).

Molecular markers such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), and simple sequence repeats (SSRs) have been used in cotton genome mapping and genetic diversity analysis (Chen, 2007). The relatively low levels of polymorphism and limited association of these markers with candidate genes resulted in a lack of advancement in mapping studies of candidate genes. Van Deynze et al. (2009), however, reported that SNP has been shown to be more effective and the most abundant class of DNA polymorphism, providing valuable information on allelic variation

and mapping. Cho et al. (2011) reported that GDRP gene markers are more effective than predominantly used molecular markers such as SSRs because gene markers are directly indicative of genes of interest and no further linkage analysis is needed as in other types of markers, especially for mapping. Cho et al. (2011), who identified SNP markers, developed for 20 GDRP genes and used for mapping population of 188 recombinant inbred lines developed from an interspecific cross between *G. hirsutum* and *G. barbadense*, concluded that prior genetic maps have been saturated with SSR markers mostly developed from non-coding regions of the genome and generally were not linked with genes of known function or to traits of economic importance. Recently, Janga et al. (2019) were able to identify three CGF genes involved in gland formation by silencing any one or more of these genes, eliminating or reducing gossypol content in cottonseed. Janga et al. (2019), using RNA-seq analysis of embryos from near-isogenic glanded (*Gl2Gl2Gl3Gl3*) versus glandless (*gl2gl2gl3gl3*) cotton plants, were able to show high level of expression of 33 genes in embryos just before gland formation in glanded plants. They concluded that the *GoPGF* (*CGF3*) gene plays an essential role in the formation of glands in the cotton plant, and seed-specific silencing of CGF genes can eliminate glands and as a result, gossypol, making cottonseed safe as food for human consumption or feed for animals. Although using gene silencing technologies with a seed-specific promoter would eliminate gland formation and thus gossypol production in cottonseed, no commercial cultivars with no seed gossypol are available in the market.

In addition to its enantiomeric forms, two forms of gossypol, free and bound, have been observed (Alexander et al., 2008) and free gossypol content in whole cottonseeds varies depending on the cotton genotype (Alexander et al., 2008). In addition, treatment of cottonseed using heat and pressure can decrease the concentrations of free gossypol. For example, some genotypes produce cottonseed that contains concentrations of total gossypol greater than 14,000 mg/kg and 7,000 mg/kg of free gossypol (Alexander et al., 2008). However, after oil extraction from cottonseeds, up to 0.6% more gossypol is available following solvent extraction and approximately 0.06% is available when the extraction process involves mechanical pressure and heat treatment (Nicholson, 2012). It has been reported that seed processing methods, including heat treatment (Broderick and Craig, 1980) and extrusion process (Noftsger et al., 2000), can reduce free gossypol concentrations in cottonseed. Breeders have developed cotton lines that produce cotton plants devoid of gossypol glands (Fig. 1); however, these lines are not grown commercially because they are more vulnerable to attacks by insects. Stipanovic et al. (2005) reported that cottonseed with > 95% (–) gossypol could be a source of biopharmaceutical applications and (+) enantiomer shows little, if any, toxicity to nonruminant animals and cottonseed with > 95% (+) gossypol increases the use efficiency as feed for nonruminants. The general higher percentage of (+) gossypol compared with (–) gossypol across CS lines and locations in this study (55.5-62.0%), confirmed the genetic control of this trait. Stipanovic et al. (2005) reported that the (+) to (–) gossypol ratio in commercial Upland (*G. hirsutum*) cottonseed is approximately 3:2 and approximately 2:3 in commercial Pima cottonseed (*G. barbadense*).

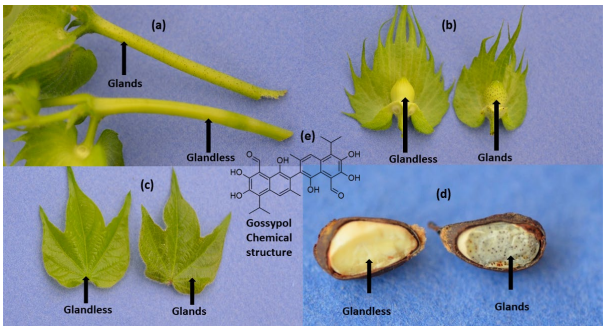

Figure 1. Glanded (containing gossypol) and glandless cotton: (a) petiole, (b) square (flower bud) development, (c) leaves, (d) seeds, and (e) gossypol chemical structure.

A breeding strategy of developing low (–) gossypol cottonseed lines without compromising the nutritional or insect-resistance value might help overcome the problems of cottonseed as a secondary source of food products. Therefore, the ideal choice for use of cottonseed as secondary products for nutritional value would be to develop cotton lines with adequate level of high (+) gossypol and low (–) gossypol to prevent any detrimental effects on animal and human health while retaining the minimal level of (–) gossypol needed for insect resistance. However, limited genetic information is available on (+) gossypol and (–) gossypol in cotton genotypes. Breeders have been long aspired to introgress valuable genes from different species into Upland cotton. However, they have had limited success using conventional methods because of incompatibility at the whole genome level between the species. A unique set of euploid CS

lines in which a homologous pair of chromosomes or chromosome segment from alien species of *G. barbadense*, *G. tomentosum* Nutt. ex Seem., and *G. mustelinum* Miers ex Watt, respectively, have been substituted for the homologous *G. hirsutum* (TM-1) chromosome or chromosome segment (Saha et al., 2017). Each CS line is nearly isogenic to the recurrent parent, TM-1, for 25 chromosome pairs, and to each other for 24 chromosome pairs, except the substituted chromosomes or chromosome segment originated from the alien tetraploid species. Previous studies demonstrated that these CS lines provide a genetic tool to discover many beneficial alleles for important traits from the wild species for the genetic improvement of Upland cotton. In the current research, we used the CS lines to investigate the effect on the alien species substituted chromosome or chromosome segment on the level of gossypol in seed and the ratio of (+) and (–) forms of gossypol. The CS lines will have potential for targeted introgression of the low gossypol trait from the alien tetraploid species and will improve our genetic knowledge of gossypol in cottonseed. The objective of this research was to study the association of chromosome or chromosome segment substitutions from *G. barbadense*, *G. tomentosum*, and *G. mustelinum*, respectively, on (+) and (–) gossypol levels when they are substituted into *G. hirsutum*. The CS lines are genetically similar to TM-1, an Upland cotton (*G. hirsutum*) genetic standard, and to each other, except that each line differs by the replacement of a specific homologous pair of chromosomes or chromosome segments from the donor line of *G. barbadense*, *G. tomentosum*, and *G. mustelinum*, respectively.

MATERIALS AND METHODS

Nine euploid (2n) cotton CS lines represented by the substitution of chromosome 2, chromosome 4, and the short arm of chromosome 8 (8sh) from three tetraploid species of *G. barbadense* (CS-B), *G. tomentosum* (CS-T), and *G. mustelinum* (CS-M), respectively, into Upland cotton (TM-1, *G. hirsutum*) genetic background; TM-1, the recurrent parent of the CS line; and 'AM UA48', Reg. No. CV-129, (Bourland and Jones, 2012) were used in this research. In 2013, nine CS lines (CS-B02, CS-B04, CS-B08sh, CS-M02, CS-M04, CS-M08sh, CS-T02, CS-T04, CS-T08sh), TM-1, and AM UA48 were grown at Florence, SC (34.1° N, 79.4° W). The soil type was a Norfolk loamy sand (Fine-loamy, kaolinitic, thermic typic Kandiudults). In 2014, the same lines were grown at Mississippi State, MS (33.4° N, 88.8° W). A total of 11 entries, including controls were used in each location. The soil type at Mississippi State was a Leeper silty clay loam (Fine, smectitic, nonacid, thermic Vertic Epiaquept). Each entry was grown in single-row plots 12 m long with rows spaced 97 cm and plants spaced 10 cm apart (approximately 110 plants per row). A 25-open-pollinated-boll sample per plot was hand harvested at both locations from the first fruit positions near the middle nodes of plants to determine fiber properties. Samples were ginned on a 10-saw laboratory gin to separate seeds from the lint. Seeds from these samples were used to determine gossypol content (% dry seed weight). Four replicates of each entry were used.

Gossypol Analysis. Gossypol content in delinted mature cottonseed was determined by Ultra-Performance Liquid Chromatography (UPLC) (Waters Corporation, Milford, MA) with modifications according to Hron et al. (1999) based on American Oil Chemists' Society methodology. Briefly, a 100-mg dried, then ground, delinted seed sample was extracted with 2 ml of complexing reagent (2:10:88, R-(−)2-amino-1-propanol, acetic acid, N, N-dimethylformamide). The sample was derivatized by heating at 100° C on a heater block for 30 min then cooled to room temperature. The samples were vortexed for 30 sec and diluted with 8-ml mobile phase (85:15; acetonitrile: 10 mM KH_2PO_4 pH = 3). An aliquot was transferred to a microfuge tube and centrifuged for 2 min at 12,000 rpm. A sample of clean supernatant was transferred into a UPLC vial for gossypol quantification. Samples were analyzed by UPLC on a Waters Sample Manager FTN (Flow Through Needle)/Quarternary Solvent Manager, coupled to a Waters eλ Photodiode Array Detector (PDA) (Waters Corporation) set at 254 nm with scanning from 200 to 500 nm. A 2 µL injection was made on an Acquity UPLC HSS C18 column set at 40° C (1.8 µm, 2.1 mm × 100 mm i.d.) connected to a VanGuard pre-column (1.8 mm) (Waters Corporation) in which the flow rate was set to 0.8 mL/min with initial gradient conditions consisting of 30% mobile phase A [(10 mM KH_2PO_4 pH = 3) and 70% mobile phase B (acetonitrile)]. At 1.01 min, the gradient was programmed to 15:85 (A:B) and held constant for 1.49 min. The solvent was then returned to its original starting conditions of 30:70 (A:B) at 2.51 min and held for a total run time of 3.0 min. Standard curves were prepared using gossypol-acetic acid (95% racemic gossypol, Sigma-Aldrich (St. Louis, MO), and

standard solutions of 50:50 (+) and (–) enantiomers were used to create standard curves. Total gossypol was calculated as the sum of (+) and (–) enantiomers.

Experimental Design and Statistical Analysis. Cotton lines were grown in a randomized, complete block design with four replications within each location. Statistical analyses to study the effect of location, line, and their interactions, utilized PROC MIXED (SAS, 2002-2010). Replicates within location were considered as random effects. Multiple-comparison procedures (mean separation test) were conducted by generalized linear model at significance level of 5% in SAS for testing a hypothesis on the basis of a difference between sample means (SAS, 2002–2010). Because location-by-genotype interactions were significant for gossypol, results were presented separately. A significant difference in genetic effects between a specific CS line and TM-1 was considered a chromosome effect attributable to the specific substituted chromosome or chromosome arm from the donor parent of *G. barbadense*, *G. tomentosum*, and *G. mustelinum*, respectively, because the individual CS line is considered in almost isogenic genetic background of TM-1.

RESULTS AND DISCUSSION

Statistical analysis showed that genotype/line (G), location (L), and their interactions (G x L) were highly significant ($p = 0.0001$) for (+) gossypol, (–) gossypol, and total gossypol (Table 1). Significant interactions between CS line and location are revealed by the change in ranking of gossypol fractions in different locations, indicating a gene-by-environment interaction. Because CS line and location interaction was significant for all variables, results were presented by location (Tables 2 and 3). The distribution of gossypol content in CS lines (Fig. 2a, b, c; Fig. 3a, b, c) showed a normal probability distribution and was bimodal, indicating the distribution of gossypol in CS lines is complex. There were positive significant ($p < 0.0001$) correlations between (+) and (–) gossypol; between (+) and total gossypol; and between (–) gossypol and total gossypol (Table 4).

Experiment in South Carolina (Table 2). The concentration of (+) gossypol ranged from 0.81% (AM UA48) to 1.22% (CS-M04) of total dry seed weight (tdswt), a difference of 50.6% (Table 2). The TM-1 parent had a concentration of 1.08% tdsdwt and was intermediate between the highest and the lowest (+) gossypol CS lines. Minus gossypol content ranged from 0.62% (AM UA48) to 0.88% (CS-B04), a change of 41.9%. TM-1 had a concentration of 0.71% and was between the highest and the lowest (–) gossypol CS lines. Total gossypol concentration ranged from 1.43% (CS-B02) to 2.06% (AM UA48), a difference of 44%. TM-1 had 1.78% gossypol, ranking between the highest and the lowest CS lines for total gossypol. The percentage of (+) gossypol was higher than (–) gossypol, ranging from 55.5% (CS-B04) to 61.3% (CS-M08sh). Comparing with CS lines and TM-1, AM UA48 ranked the lowest (+) and (–) gossypol, but highest in total gossypol.

Experiment in Mississippi (Table 3). The concentration of (+) gossypol ranged from 0.89% (CS-M04) to 1.47% of total seed weight (CS-M02 and CS-M08sh), a difference of 62.2% (Table 3). TM-1 contained 1.26% and was between the highest and the lowest (+) gossypol. Minus gossypol ranged from 0.64% (CS-B04) to 1.01% (CS-B02), a difference of 57.8%. TM-1 had 0.84%, ranking it between the highest and the lowest (–)gossypol. Total gossypol ranged from 1.54% (CS-M04) to 2.46% (CS-M02), a difference of 59.7%. The level of total gossypol in TM-1 was 2.11%, ranking it between the highest and the lowest total gossypol. The contribution of (+) gossypol is higher than (–) gossypol percentage, ranging from 55.5% (CS-B02) to 62% (CS-M08sh). Comparing with CS lines and TM-1, AM UA48 ranked the third lowest in (+), (–), and total gossypol.

Table 1. Analysis of variance (F and *p* values) of the effects of location, genotype (11 entries: CS lines, parent TM-1, and commercial cultivar AM UA48), and their interactions for seed gossypol content (%dry seed weight) in different locations (Florence, SC, 2013 and Mississippi State, MS, 2014)

Effect		(+) Gossypol		(-) Gossypol		Total gossypol	
	DF[z]	F Value	*P* > F	F Value	*P* > F	F Value	*P* > F
Location	1	27.37	< .0001	22.52	< .0001	26.63	< .0001
Genotype	10	20.60	< .0001	14.01	< .0001	17.49	< .0001
Location × Genotype	10	2.23	0.017	2.71	0.0037	2.52	0.007
Residuals		0.012		0.006		0.034	

[z] DF=degree of freedom.

Table 2. Effects[z] of chromosome substitution on (+) gossypol, (–) gossypol, and total gossypol contents (%dry seed weight) in cottonseed, and (+) gossypol and (–) gossypol contributions to total gossypol (%). The experiment was conducted in 2013 in Florence, SC

Cotton genotype	(+) Gossypol (%)	(–) Gossypol (%)	Total (%)	(+) G contribution to total (%)	(–) G contribution to total (%)
CS-B02	0.96e	0.66f	1.43g	59.0c	41.0d
CS-B04	1.09c	0.88a	1.63e	55.5f	44.5a
CS-B08sh	0.92ef	0.63g	1.97b	59.3c	40.8d
CS-M02	1.02d	0.73c	1.55f	58.0d	42.0c
CS-M04	1.22a	0.84b	1.76d	59.3c	40.8d
CS-M08sh	1.16b	0.73c	1.89c	61.3a	38.8f
CS-T02	0.89f	0.67ef	1.56ef	57.5d	42.5c
CS-T04	1.05cd	0.73cd	1.78d	59.0c	41.0d
CS-T08sh	1.08c	0.70de	1.78d	60.8ab	39.3ef
TM-1	1.08c	0.71cd	1.78d	60.5b	39.5e
AM UA48	0.81g	0.62g	2.06a	56.8e	43.2b

[z] Means within a column followed by the same letter are not significantly different at the 5% level.

Table 3. Effects[z] of chromosome substitution on (+) gossypol, (–) gossypol, and total gossypol contents (%dry seed weight) in cottonseed, and (+) gossypol and (–) gossypol contributions to total gossypol (%). The experiment was conducted in 2014, Mississippi State, MS

Cotton genotype	(+) Gossypol (%)	(–) Gossypol (%)	Total (%)	(+) G contribution to total (%)	(–) G contribution to total (%)
CS-B02	1.26d	1.01a	2.27c	55.5h	44.5a
CS-B04	0.93g	0.64g	1.57g	59.5d	40.5e
CS-B08sh	1.18e	0.81e	1.99e	59.3de	40.8de
CS-M02	1.47a	0.99a	2.46a	59.5d	40.5e
CS-M04	0.89h	0.66g	1.54g	57.4f	42.6c
CS-M08sh	1.47a	0.90c	2.37b	62.0a	38.0h
CS-T02	1.31c	1.00a	2.31bc	56.5g	43.5b
CS-T04	1.39b	0.96b	2.34b	59.0e	41.0d
CS-T08sh	1.26d	0.79e	2.06de	61.3b	38.8g
TM-1	1.26d	0.84d	2.11d	60.0c	40.0f
AM UA48	1.11f	0.76f	1.87f	59.3de	40.8de

[z] Means within a column followed by the same letter are not significantly different at the 5% .

Table 4. Pearson Correlation Coefficients (*p* and *R* values) between (+) and (–) gossypol; between (+) and total gossypol (%); and between (–) gossypol and total gossypol for cotton grown in different locations

Experiment 1, Florence, SC, 2013	(+) Gossypol	(–) Gossypol
(–) Gossypol	R = 0.85231	
	p < .0001	
Total Gossypol	R =0 .97859	0.94113
	p < .0001	< .0001
Experiment 2, Mississippi State, MS, 2014	**(–) Gossypol**	**(–) Gossypol**
(–) Gossypol	R = 0.862	
	p < .0001	
Total Gossypol	R = 0.9765	0.95074
	p < .0001	< .0001

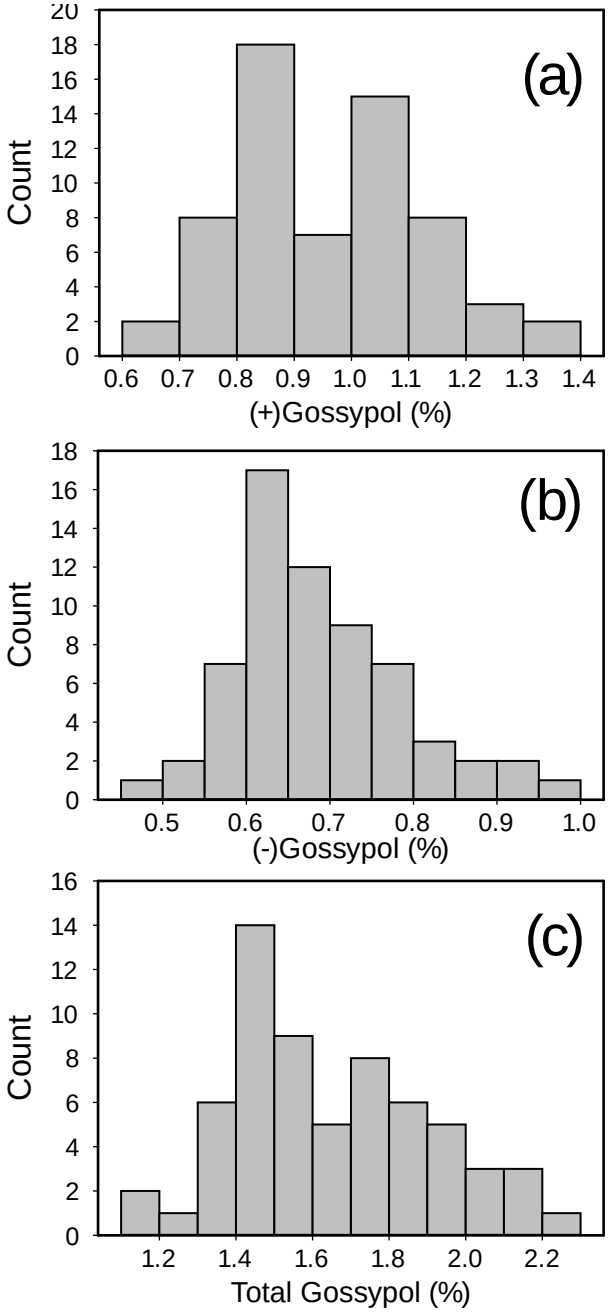

Figure 2. Effect of chromosome substitution on (a) (+) gossypol, (b) (–) gossypol, and (c) total gossypol contents. The experiment was conducted in 2013 in Florence, SC.

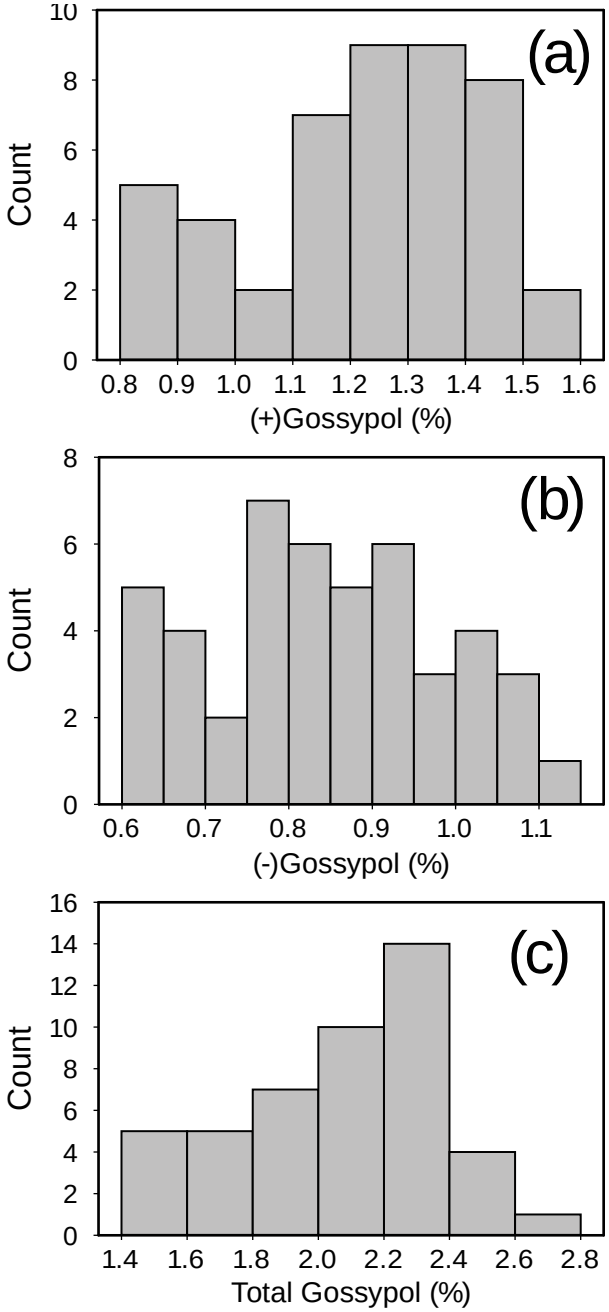

Figure 3. Effect of chromosome substitution on (a) (+) gossypol, (b) (–) gossypol, and (c) total gossypol contents. The experiment was conducted in 2014 in Mississippi State, MS.

The content of (+), (–), and total gossypol was generally significantly higher in MS (2.08 %) than in SC (1.74%) (statistically significant at 5%). This difference could be due to environmental factors, including heat and rainfall in each location. For example, during the critical months of growth (May through September) the temperature was higher in Mississippi State, MS than in Florence, SC and precipitation was higher in Mississippi State, except in July, which are possible sources of variability (Pons et al., 1953; Stansbury et al., 1956; US Climate Data, 2019). The contribution of (+) gossypol to total gossypol was higher than (–) gossypol, ranging from approximately 55 to 65% across locations.

Potential Chromosomal Association with Gossypol Content. A comparative analysis of the CS lines with TM-1 suggested that the chromosome substitution had significant effects at both location

on total, (+), and (–) gossypol levels. This indicated that the respective substituted chromosomes contain allele(s) in the CS line that affected gossypol content in cottonseed. CS-M04 had the lowest (+) gossypol percentage (0.89%) in SC and the highest (+) gossypol percentage (1.22%) in MS compared to other CS lines and TM-1. Results also revealed that CS-B02 had a similar contrast effect across the two locations for (–) gossypol percentage in cottonseed with the lowest percentage in SC and highest percentage in MS, suggesting that genotype and locations each played a major role on level of gossypol. Results showed that CS-M02 had the highest total gossypol percentage in cottonseed in MS. However, CS-B08sh had the highest total gossypol percentage in cottonseed compared with other CS lines and TM-1 in SC, indicating the potential effect of different genetic action for this trait by the allele(s) carried by the same substituted chromosome segment from two different alien species in TM-1 genetic background at two locations. It is important to note that in CS-M08sh the contribution of (+) gossypol percentage to the total gossypol was the highest and (–) gossypol percentage was the lowest compared to TM-1 and other CS lines at MS, implying the opposite genetic effects for the alleles of the two enantiomers carried by the substituted alien species chromosome segment of CS-M08sh in TM-1 genetic background. Our comparative results from the overall average of three specific substituted chromosome or chromosome segment revealed that both (+) gossypol and (–) gossypol percentage were highest in CS-M lines among the lines at both SC [average (+) gossypol 1.3 and (–) gossypol 0.77] and MS [average (+) gossypol 1.28 and (–) gossypol 0.85] locations. Results from the overall average of CS lines and TM-1 showed that CS-B lines had the overall lowest (+) gossypol percentage (0.99) and CS-T line had the overall lowest (–) gossypol percentage of the total gossypol (0.7) at SC.

A previous report revealed that Upland cotton improvement is seriously impeded by its narrow genetic base (Bowman and Gutiérrez, 2003). One of the choices for diversity would be to use interspecific introgression from the primary gene pool of the tetraploid cotton species, including *G. barbadense*, *G. tomentosum*, and *G. mustelinum*. However, several biological and technical challenges are associated with interspecific introgression due to the incompatibility at the whole genome level between the species. Utilizing these wild and unadapted gene pools to introduce useful genetic variation for important traits like (+) gossypol and (–) gossypol components in Upland cotton requires new and innovative approaches to discover and introgress the novel beneficial alleles from the wild and unadapted species.

The detailed technique on the development of the CS lines based on an integrated method of cytogenetic, molecular, and breeding methods was described in previous studies (Saha et al., 2012, 2015; Stelly et al., 2005). The CS lines have been used in previous studies to discover many novel traits and targeted introgression of useful traits from wild tetraploid species and unadapted cotton germplasm (Saha et al., 2006, 2010,2012, 2017). The near-isogenicity of BC_5S_n CS line with the recurrent parent TM-1was useful as a tool to uncover genetic mechanism associated with different components of gossypol by comparing the CS line with TM-1 or AM UA48 as ranked the lowest in (+) and (–) gossypol, but highest in total gossypol in SC, and ranked the third lowest in (+), (–) gossypol, and total gossypol in MS.

Plant growth and development are affected by the genetic composition of the plant, the environment of the location, and the interaction between them. Our result showing the significant effects of CS line and location and its interaction for (+) gossypol, (–) gossypol, and total gossypol percentage, has also been reported that, on average, *G. barbadense* has higher gossypol concentrations than *G. hirsutum* (Gadelha et al., 2014). Although gossypol, including the proportion of (+) and (–) gossypol production, is genetically controlled and varies between and within species (Percy et al., 1996), environmental conditions can affect the accumulation of gossypol (Percy et al., 1996; Scheffler, 2016; Stansbury et al., 1956). For example, Pons et al. (1953) studied the effects of variety and environment in eight cottonseed varieties. They found significant variation of gossypol content in 8 varieties grown at 13 locations over 3 years. They reported that negative correlation between gossypol content and temperature and positive correlation between gossypol content and rainfall. They concluded that varieties differed in gossypol due to the response to temperature and rainfall (Pons et al., 1953; Stansbury et al., 1956). Other reports agree with our results in that cottonseed traits, including gossypol, were genetically controlled, and genotype by environment interaction effects were significant (Liu et al., 2012; Ye et al., 2003). Robinson et al. (2001) investigated the effect of variety on gossypol content and found modest variation in gossypol levels among four major varieties of cottonseed grown in the southwest U.S.

Our results showed that (+),(–), and total gossypol varied significantly at two locations and also among the CS lines, agreeing with previous research (Pons et al., 1953; Liu et al., 2012; Robinson et al., 2001; Stansbury et al., 1956; Ye et al., 2003).

The genetic basis of gossypol glands has been well studied (Endrizzi et al., 1985; Kohel and Lee, 1984; Lee, 1965; Ma et al., 2016). It is considered that gossypol gland formation is controlled by at least six independent loci, *gl1, gl2, gl3, gl4, gl5, and gl6*. McMichael (1959, 1960) developed a recessive mutant that eliminates all glands on aerial plant parts and seeds providing the possibility of cultivating glandless cotton using conventional breeding methods. The glandless cotton phenotype is determined by two pairs of duplicate recessive genes (*gl2gl3*) that are located on chromosome A12 and D12, respectively (Endrizzi et al., 1985; Lee, 1965). Previous report revealed that a single dominant glandless mutation following the irradiation of Giza 45 (*G. barbadense*) seeds produced gandless plants and seed. The genetic analysis of this line showed that this phenotype, known as Gl_2^e mutant, caused by a dominant allele at the Gl_2 locus that is epistatic to Gl_3 (Kohel and Lee, 1984; Tang et al., 1996). A new cotton line, homozygous for this new gene, was released as Bahtim 110 in Egypt (Afifi et al., 1966) and Hai-1 in China (Tang et al., 1996). These two mutant lines were used to develop many glandless cultivars with little or no gossypol in seeds in both *G. hirsutum* and *G. barbadense* (Tang et al., 1996; Wang et al., 2000). Breeders can select and develop cotton lines with different levels and ratios of positive (+) or negative (−) enantiomeric forms of gossypol. The (−) gossypol enantiomer is eliminated more slowly from seeds (Wu et al., 1986) and more biologically active form (Gadelha, 2014). It is more toxic than the (+) gossypol (Bailey et al., 2000; Kakani et al., 2010; Lordelo et al., 2005). Previous research studied the ratio of (+) to (−) gossypol in commercial cottonseed cultivars. Ratio of (+) to (−) gossypol was reported to vary between 95:5 and 31:69 in *G. hirsutum* and *G. barbadense* (Puckhaber et al., 2002; Yildirim-Aksoy et al., 2004); ratio of (77:23) in *G. arboreum* L., (44:56) in *G. barbadense*, and (68:32) in *G. herbaceum* L. (Jaroszewski et al., 1992). Cass et al. (1991, 2004) reported the (+) to (-) gossypol ratios in seed to be (57:43 and 65:35) in *Gossypium mustelinum*, (63:37) in *G. herbaceum*, and (48:52) in *G. herbaceum* x *G. hirsutum* cross. Few studies were attempted with the wild cotton species of *Gossypium* to determine the variations ratio of (+) to (−) gossypol ratio (Stipanovic et al., 2005, 2008). Among the goals of breeding programs (Scheffler and Romano, 2008) are to develop elite lines containing low total gossypol content in the seed and an increased (+) gossypol enantiomer, which provides pest protection and low toxicity levels in animal feed (Scheffler and Romano, 2008; Stipanovic et al., 2005).

The results suggested that it would be possible to utilize some of this germplasm for appropriate gossypol fraction to increase the nutritional quality of cottonseed meal for human and livestock feed consumption by targeted introgression of beneficial gene pool associated with the substituted chromosome or chromosome segment from the alien species using the CS lines. The CS lines also provided an analytical tool to understand the genetic mechanism associated with total gossypol, (+) gossypol and (−) gossypol fractions. This genetic information will help in future strategies of breeding program to improve cottonseed nutritional values. Our results from the overall average of three specific substituted chromosome or chromosome segment (Chromosome 2, 4, and 8 short arm) of three different species (*G. barbadense*, *G. tomentosum*, and *G. mustelinum*) and TM-1 (*G. hirsutum*) showed that both (+) gossypol percentage and (−) gossypol percentage of the total gossypol was highest in CS-M lines.

Correlation showed that there was a positive significant ($p < 0.0001$) correlation between (+) and (−) gossypol; between (+) and total gossypol; and between (−) gossypol and total gossypol, indicating that the increase in total gossypol leads to increases in (+) and (−) gossypol content across years and locations, confirming the genetic control of this pattern. In our study significant differences in gossypol contents and its fractions ranging from 0.81 to 1.47% for (+) gossypol, and from 1.43 to 2.46% for total gossypol across locations.

CONCLUSIONS

In summary, the results provided information on the association of gossypol (+) and (–) enantiomer levels associated with the respective substituted chromosome or chromosome segment of *G. barbadense*, *G. mustelimum*, and *G. tomentosum* in the CS line. This research provided valuable information that can be used for a cotton breeding program to improve the nutritional values of cottonseed as some of the CS lines contain a wide range in gossypol levels. A

set of novel CS lines were used in this research and (1) discovered the potential association of three specific chromosomes or chromosome segments from the species of *G. barbadense*, *G. tomentosum*, and *G. mustelinum* with (+) gossypol and (−) gossypol content, respectively; and (2) developed novel CS lines germplasm from interspecific crosses with a range of gossypol components for targeted introgression of genes to potentially provide a way to utilize cottonseed without the harmful effects of gossypol when fed to humans or monogastric animals.This information will not only help in understanding the genetic mechanism associated with this economically important traits but also provide a tool to use some of the CS lines for targeted introgression of useful genetic variation associated with this trait with reduced genetic drag effect from other wild tetraploid species in the breeding program.

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