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BREEDING AND GENETICS

Cotton Half-Seed Selection Strategy for Gossypol and Its Plus Isomer

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ABSTRACT

Successful breeding programs optimize time and resources to produce elite lines. Selecting individual plants in the F_2 generation is an efficient strategy if the trait is highly heritable and nondestructive methods exist to analyze the seed. Cotton (*Gossypium hirsutum* L.) seed has limited uses because of gossypol, a toxic compound found in the seed. Gossypol exists in two enantiomeric forms with the (+) less toxic than the (−) form. Reducing gossypol or increasing the (+) enantiomer in the seed would increase the amount that could be fed to livestock, chicken, or fish. Rapid, cost-effective methods were developed to measure (+) and (−) gossypol in the cotyledon (chalazal) half of a seed by high performance liquid chromatography (HPLC) on a reduced scale. Techniques were also developed to propagate the embryo (micropylar) half of the seed. The techniques were used to develop elite lines with varied gossypol levels produced one year earlier than possible with more conventional breeding strategies. The half-seed methods combined with a modified HPLC gossypol assay provided a simple, cost-effective method to breed for modified gossypol content and enantiomer composition. These methods can be combined with other testing or evaluation techniques to further optimize selection efficiency.

In any breeding program, time and valuable resources can be saved by selecting in early generations. However, in early generations the progeny will segregate for the trait of interest, and selection on a single plant basis is most effective. Previously when selecting for seed traits such as oil and protein content, fatty acid composition, or secondary metabolites, the chemical analysis techniques and instrumentation often required relatively large amounts of seed (i.e., potentially a significant proportion of the seed produced by an individual F_2 plant). This meant testing was done in later generations on bulk seed from multiple plants. Today, techniques and instruments have improved and testing of small samples is routine. The ability to test individual seeds in the F_2 generation allows identification of an individual genotype, and it also reduces the number of lines that need to be carried into subsequent generations. If the whole seed is destroyed during the chemical testing, no seedling can be produced. Therefore, this approach allows only part of the seed to be analyzed, saving the embryo and part of the energy reserves to advance the selected line to the next generation.

This half-seed breeding approach was first reported for rape seed (*Brassica napus* L.) to select for levels of undesirable fatty acids in the oil by gas chromatography (GLC) (Downey and Harvey, 1962). The seed coat from each individual seed was removed, the outer cotyledon was analyzed for erucic acid, and the remaining cotyledon and embryo tissues were germinated in wet paper towels and planted. The resulting plant produced seed for the next generation. Their strategy had two components: a way to assay the seed but still produce a viable plant and an assay protocol downsized to be compatible with the small amount of available seed tissue. This half-seed selection method has since been used by others to assess seed protein content in rape seed (Velasco and Möllers, 2002) and has been extended to related species, such as *Brassica carinata* A. Braun (Ethiopian mustard) (Getinet et al., 1994) and *Lesquerella fendleri* (A. Gray) S. Watson (Dierig et al., 2006). GLC analysis of a single seed or part of a seed also has been reported for other dicotyledonous species, such as sunflower, olive, and soybean, to improve their seed-oil fatty acid composition (Conte et al., 1989; Tahmasebi-Enferadi et al., 2004; Wang et al., 2007). To date, the technique has been used mostly to select for either protein or oil levels or the oil's fatty acid components. Other seed traits of interest include many secondary metabolites, for example,

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those that help protect the plant from pests and diseases are often measured by high performance liquid chromatography (HPLC). Reduced-scale HPLC methods would extend the potential for screening of individual seeds or leaves for these traits.

Cotton (*Gossypium hirsutum* L.) seed contains the terpenoid gossypol, which is toxic to monogastric animals (e.g., pigs and poultry), but is tolerated by cattle in moderate quantities (Eisele, 1986; Kim et al., 1996; Lordelo et al., 2005; Santos et al., 2003). As a consequence, even though cotton seed has approximately 21% oil and 23% protein with relatively high levels of the essential amino acid arginine and methionine (Lusas and Jividen, 1987), the value of the seed as a by-product of fiber production is currently limited. Gossypol is deposited in glands that are present throughout most of the vegetative and reproductive tissues of cotton plants (Adams et al., 1960). Total seed gossypol content varies among *Gossypium* species (0.0-3.6%) and among *G. hirsutum* cultivars (0.6-2.5%) (Adams et al., 1960; Bell and Stipanovic, 1977; Stipanovic et al., 2005). Gossypol exists in two enantiomeric forms, plus (+) gossypol and minus (−) gossypol. The percentage of the (+) enantiomer varies, ranging from 50% in some varieties to greater than 90% in some exotic germplasm and *G. hirsutum* landraces. Although environmental conditions can have a significant effect on gossypol levels, the ratio of enantiomers in seed is much less sensitive to environmental influences and appears to be largely under genetic control (Pettigrew and Dowd, 2011).

Biological activity differs between the two chiral gossypol forms. For example, the (−) enantiomer exhibits greater ability to inhibit the growth of cancer cell lines (Blackstaffe et al., 1997; Liu et al., 2002). By adding either pure (+) or (−) gossypol to non-cottonseed containing diets, Lordelo et al. (2005) found that (−) gossypol inhibited growth in broiler chickens to a greater extent than did (+) gossypol.

Gossypol and its (+) and (−) forms are good candidates for F_2 selection strategies. Previous research by Bell et al. (2000) and confirmed by our own genetic studies (data not shown), indicate that the proportion of (+) seed gossypol can be treated as a simply inherited trait. Bell et al. (2000) conducted a series of genetic tests with backcross progeny from crosses between landrace 'moco' cotton with > 90% seed gossypol in the (+) enantiomer form and adapted lines with < 60% (+) gossypol. They concluded that the (+) gossypol trait was controlled by two genes and easily transferred. Our studies, conducted prior to initiating the half-seed study, indicated that there was one gene with a large effect on the proportion of (+) gossypol. Our results also indicated there was likely a second gene with a positive modifying effect. The Bell et al. (2000) study used HPLC analysis of petals to test for (+) and (−) gossypol. However, results from the study showed that the correlations between the petal and seed values were low. In addition, the plants had to be grown long enough to produce flowers before selection was possible, which normally requires at least 10 wk. When working with photoperiod sensitive cotton, which includes the moco lines, it can take 24 to 48 wk to initiate flowering.

To evaluate and select for gossypol content in cotton, we developed methods not only to assay for the individual enantiomers on the cotyledon fraction of an individual F_2 seed, but also to produce plants from the embryo part of the seed. An HPLC-based screening method developed to analyze 100-mg cotton seed samples (Scheffler and Romano, 2008) was scaled down to allow half a seed to be analyzed reliably with fewer chemical reagents, thereby reducing analysis cost. Techniques were also developed to produce plants (and subsequently seed) from the other half of the seed. With these methods, only selected plants needed to be advanced to the next generation saving time and resources.

The highly heritable genetic diversity available in cotton can be exploited to improve cotton cultivars by reducing total gossypol content and shifting the (+) and (−) enantiomer profile. A reduction in seed gossypol level or a change in the proportion of enantiomers could allow for increased utilization of this high quality protein source in cattle and dairy feed, and enable its use in feed for other domestic livestock or chickens and fish.

MATERIALS AND METHODS

Plant Material. Twelve cotton varieties/lines representing the range of gossypol content in cotton were used in tests to evaluate the half-seed method (Table 1). The varieties and lines ranged from common varieties to landraces and hard-seeded photoperiod sensitive lines, such as JS 1 (PI 163604) or JS 3 (PI 196458), which make up a majority of the high (+) gossypol lines. To simplify subsequent explanations, the plant material will be referred to collectively as "lines".

Table 1. Plant material used in the study with source and type information

Variety/ Line	Reference	Comment
FM 832	PVP 9800259	variety
SG 747	PVP 9800118	variety
Coker 312	PI 529278	variety
H1220	PI 578226	variety
MD51ne	PI 566941	elite line
Acala 1517-99	PI 612326	variety
Mac7	McCarty et al., 1996	high (+) gossypol line
DES 119	PI 606809	variety
Pun-3	PI 561960	landrace
JS 1	PI 163604	landrace high (+) gossypol
JS 3	PI 196458	landrace high (+) gossypol
A1006	ACSI sister line of FM 832	variety

PVP–Plant Variety Protection Number

PI–Plant Introduction Number

Seed Preparation and Propagation. Prior to dehulling, the seeds were pretreated with a 50% bleach solution for 10 min followed by four 30 sec rinses with sterile water. Individual seeds were then then placed in 2-mL microcentrifuge tubes and each seed was covered with 1 mL of sterile H_2O (Fig. 1). The tubes were incubated for 16 to 24 h at 28°C. To dehull the seed, each was removed from the tube and pinched on the chalazal end, which allowed the kernel to slide free of the weakened water-imbibed hull. The dehulled seed was cut transversely in half with the embryo (micropylar) half transferred into one microcentrifuge tube (to be planted) and the cotyledon (chalazal) half put in a second tube (for gossypol analysis).

To geminate the embryos, plastic pots were filled with a well-drained potting mix (Metro Mix 360 or similar, Sun-Gro, Agawam, MA) and premoistened for 24 h. Each dehulled embryo half seed was planted in a pot at a 1-cm depth. If necessary, the embryo half can be stored at 4°C for up to 5 d before planting, but for best results the half seed should be planted within 2 d after cutting and dehulling. Plants with the desired gossypol profile are kept and allowed to set seed. Forty to 60 seed can be produced in a 10 x 10-cm pot if it is fertilized monthly with a 10-10-10 fertilizer, although it becomes difficult to keep them adequately watered. More seed are produced with less maintenance if the pots are deeper (15 x 15 x 41 cm) (e.g., tree seedling plastic pots, Stuewe, Tangent, OR). Greenhouse growing conditions were 16 h daylight at 32°C and 8 h dark at 21°C. Seed were produced approximately 140 d after planting. To maximize generation advancement, F_2 seed were harvested from the field in late September, divided into "half seeds" and grown in the greenhouse during the winter. These greenhouse grown plants produce seed in time to plant in the field the following spring.

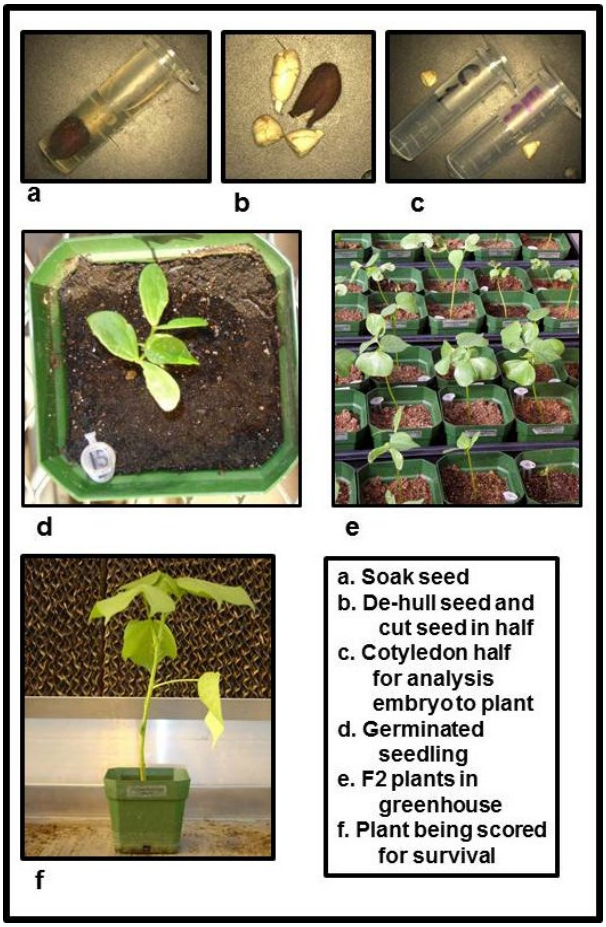

Figure 1. Summary of protocol for the half-seed analysis and plant propagation.

Cut Seed Survival Test. Ten lines were evaluated to determine if seed sectioning would decrease embryo survival. Four treatment conditions were evaluated. Treatment 1 was a standard control, that is, a dry seed planted directly in the soil without with any soaking or dehulling. Treatment 2 seed were soaked but was not dehulled. Treatment 3 seed were soaked and dehulled but not cut in half. Treatment 4 seed were soaked and dehulled and then cut and the embryo halves were planted. Eight seeds were evaluated for each of the 10 lines and grown in the greenhouse as described above. The plants were scored for survival at the 6 to 7 leaf stage (Fig. 2). All the plants that survived to the 6 to 7 leaf stage produced from 40 to 100 seed for the next generation evaluation.

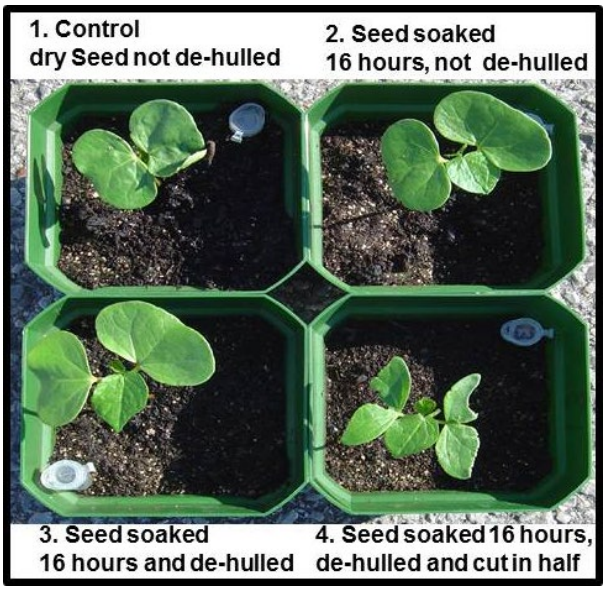

Figure 2. Four treatments used to evaluate survival rates for the half-seed method.

Efficiency would be increased if the embryo half of the seed could be stored long enough to allow completion of the HPLC analysis, thereby eliminating the need to plant all the embryos. A series of replicated tests were conducted to see how long the wet embryos could be safely stored. For this, the embryo halves were stored in microcentrifuge tubes at 4 or 28°C for a number of days before planting. For the 4°C test, samples were stored in the dark for 0, 1, 2, 3, 4, 5, 6, 8, or 10 d. For the 28°C test (room temperature), samples were kept in the dark for 0, 3, 5, or 7 d. Germination percentages were recorded for each combination of storage time and temperature.

Reduced-Scale Gossypol Analysis of Half Seeds. A typical gossypol analysis uses a 100-mg subsample of ground tissue. This sample is typically mixed with 2 mL of a complexing reagent, which is then heated to form a Schiff's base adduct of the gossypol with a chiral amine (Scheffler and Romano, 2008). After reaction and cooling to room temperature, 8 mL of mobile phase is added and this solution is used for HPLC analysis. For the lines used in this work, dry weight of the cotyledon half seeds weighed between 20 and 50 mg. Hence, a slightly scaled-down procedure was needed to yield a comparable analysis. In addition, it was desirable to streamline the process to use it on many seeds; hence, there was an advantage to conducting the assay in 2-mL microcentrifuge tubes. This required some further modifications to the procedure to maintain the molar ratio of gossypol to complexing amine and the volume ratio of complexing reagent and mobile phase.

The fresh cut cotyledon half of the seed was frozen (−20°C) and lyophilized for 4 d at −20°C to reach a constant dry weight. Each half seed was weighed by transferring into an empty, tared microcentrifuge tube. If desirable, the dried seed could be stored in the dark at −20°C prior to analysis. To analyze the sample, one 2.5-mm diameter glass grinding bead was added to the centrifuge tube followed by 0.38 mL of a complexing reagent that consisted of 8% *R*-(–)-2-amino-1-propanol, 10% glacial acetic acid, and 82% *N*,*N*-dimethylformamide. The tube was then capped and wet ground in a microcentrifuge bead beater mill (BioSpec Products, Bartlesville, OK), which operated for 1 min at 75% power. This was sufficient to pulverize the seed tissue to pass through a 20-mesh sieve. Each tube was then briefly centrifuged with a benchtop microcentrifuge at 2000 g for 30 sec to ensure that the entire solid content was immersed by the complexing reagent but not packed down to a pellet that might impede diffusion of the amine into the sample, and the tube was heated in a dry block bath at 95 to 100°C for 30 min to form a Schiff's base complex between gossypol and the amine. After the reaction, the centrifuge tubes were allowed to cool to room temperature and 1.52 mL of the mobile phase (described below) was added. The sample was briefly vortexed and then centrifuged at 12,000 g for 10 min to pellet the seed debris on the bottom of the tube. A portion of the clear liquid (~ 1 mL) was then transferred to an HPLC autosampler vial for analysis.

Samples were analyzed on an HPLC system (Waters, Milford, MA) consisting of a model 717 autosampler, model 600 pump, and a model 2998 photodiode array detector operated at 254 nm. An

Intersil ODS-2 column (5 µm, 4.6 mm x 100 mm i.d.) (GL Sciences USA, Rolling Hills Estates, CA) connected to a MetaGuard precolumn (4.6 mm) (Agilent Technologies, Santa Clara, CA) was used to affect the separation. Injection volumes were 20 µL. The flow rate was set to 1mL/min for 5 min under isocratic conditions with a mobile phase consisting of 78% acetonitrile and 22% of a 10 mM (pH 3) potassium phosphate buffer. If the sample concentration was above the range of the standard curve with a 20-µL injection, the volume was halved and the calculations were adjusted accordingly. Gossypol-acetic acid (89.62% racemic gossypol) was used to prepare standard response curves (Dowd and Pelitire, 2001). A series of standard solution concentrations were used to construct standard curves for each of the enantiomers. Total gossypol was calculated as the sum of the individual enantiomers.

Utility of the HPLC Microanalysis Protocol. To test the approach, 11 lines were selected that covered a range of gossypol concentrations and (+) isomer percentages (Table 1) and analyzed as described above. For each line, 10 seed were dehulled and halved. To determine if there were differences in estimated seed gossypol levels based on analysis of only the cotyledon half seed, both seed halves were analyzed. Whole seed gossypol levels were then calculated from the gossypol values of each half seed and the dry weights for each seed half.

Testing the Reliability of Selecting for Total Gossypol and the (+) Gossypol Enantiomer on Half a Seed in the F_2 Generation. One of our goals in breeding cotton seed is to develop elite lines with > 90% (+) gossypol and total percent gossypol at least as low as the parent. The breeding strategy previously used (Fig. 3) required seed to be harvested individually from each F_2 plant and the progeny first evaluated for gossypol and selected in the F_3 or F_4 generation. The half-seed method allows selection in the F_2 generation; therefore, only the selected lines needed to be advanced to the F_3 and later generations. In breeding programs, the top 20% is often used as the cutoff for selecting lines to advance. With the half-seed method, if the initial F_2 population had 50 plants, then the best 10 would be selected and 40 fewer lines would need to be advanced past the F_2 generation. Three F_2 populations were developed to compare efficacy of the half-seed method with conventional techniques. The high (+) gossypol parent was either Mac7-0113 or Mac7-1238. Both lines were single plant selections of the original Mac7 (McCarty et al., 1996). The adapted parent, with good agronomic characteristics, was either H1220, MD51ne, or A1006 (sister line of FM832). To allow comparison of the two strategies, F_3 seed from all greenhouse-grown F_2 plants were harvested and planted the following spring in a 4.5-m row. The F_3 rows were evaluated for agronomic traits. For conventional gossypol analysis, a 100-mg subsample from a 16-seed bulk sample per row was analyzed for total gossypol and (+) and (−) gossypol enantiomers. Selections were made separately based on the F_2 (half-seed) data or the F_3 progeny row data and the selections compared. To evaluate the longer term success of the method, the populations were advanced to the F_8 generation. F_4 seed was harvested from each row and a 16-seed bulk sample assayed. The F_4 seed as well as the later generations were evaluated in replicated trials to estimate yield and fiber quality and to evaluate seed gossypol profiles.

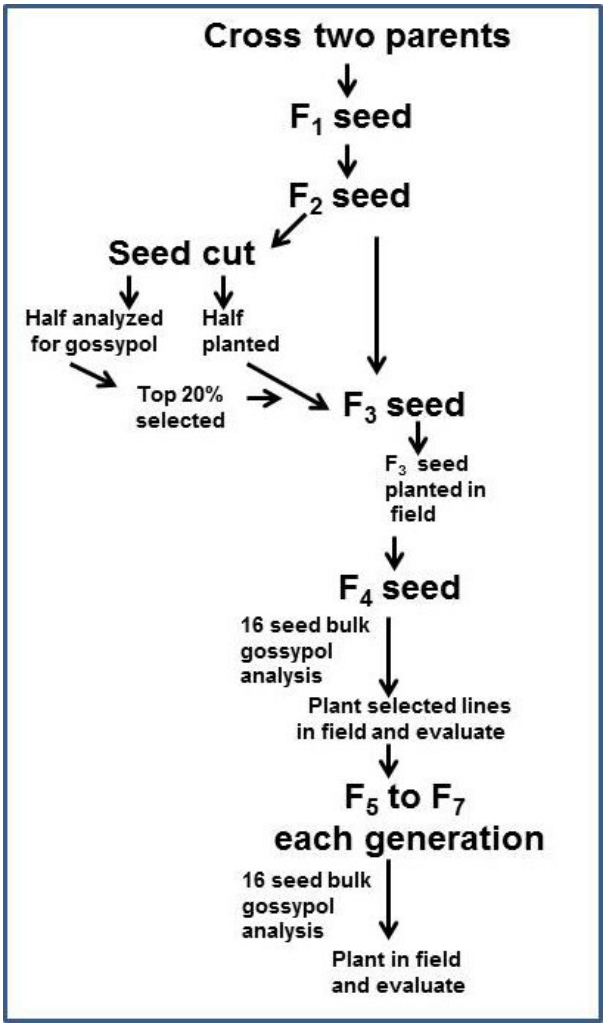

Figure 3. Comparison of conventional and half-seed method breeding strategies.

RESULTS AND DISCUSSION

Plant Propagation with the Half-Seed Method. The ability to grow plants successfully with the half seed method (Fig. 1) was evaluated against three control treatments for 10 cotton lines (Table 2). Among the treatments, the survival rate for the half-seed method (treatment 4) was as good as any of the control treatments (Table 2). The most notable difference was the days to emergence where treatment 4 was consistently 1 to 2 d earlier than treatment 1 and a day earlier than treatments 2 and 3. This was likely because the half seed had already imbibed water and did not have to break through the hull before emerging. There were differences between the entries, but a subsequent germination test indicated that the differences were likely due to differences in seed viability. The FM 832 had 85% germination compared with greater than 95% germination for the other entries. Coker 312 and Pun-3 had problems with fungal infection.

Table 2. Comparison of cut and dehulled seed to three control treatments. Percent survival by line and treatment for the 10 lines. Eight seed were tested for each line by treatment combination

Line	(1) No pre-treatment	(2) Soaked	(3) Soaked & dehulled	(4) Soaked, cut, dehulled	% Survival by line
FM 832	6	5	5	7	72
SG 747	8	8	8	8	100
Coker 312	8	5	5	5	72
H1220	6	6	8	8	88
MD51ne	8	6	7	8	91
Acala 1517	7	8	8	8	97
Mac7	8	8	8	8	100
DES 119	8	8	8	8	100
Pun-3	7	6	5	7	78
JS 3	8	8	7	6	91
% Survival by treatment	92	85	86	91	

A second test was conducted with new seed for FM 832 (94% germination) and a revised protocol that included a bleach pretreatment before soaking the seed for dehulling. This test highlighted the importance of having fresh, vigorous seed. Normally this is not a problem, as the seed used for this method would be less than one year old and greenhouse grown. Seed, especially field grown, can be contaminated with fungi or bacteria that cause a decrease in the survival of the germinating seeds. Pretreating the seed with a bleach solution followed by multiple rinses with sterile water mitigates most contamination problems. In the second test (data not shown), the survival rate of the treatment 4 embryo half seeds remained as good or better than the other three treatments, and the survival rate for individual lines was $\geq$ 89% for treatment 4.

Results from the series of replicated tests where the embryo half was stored in the microcentrifuge tube at 4 or 28°C for varying numbers of days before planting, showed that at 28°C germination was < 40% at day 3 and 0% on day 7. At 4°C, germination was 85% at day 4, but decreased to < 40% by day 6 and was 0% after day 10. As it takes at least a couple of days to freeze-dry the cotyledon seed halves and another day or two for HPLC analysis, it proved easier to plant all the embryos and then thin the unwanted seedlings within 10 d of planting. The pots and soil from the unselected plants could then be re-used.

Chemical Analysis for the Half-Seed Method. The method was evaluated with eight seed from each of 11 lines prepared following the half-seed protocol and analyzed for gossypol at the reduced scale described in the methods section. For the 88 samples, the correlation between the total gossypol for the embryo half of the seed and the total gossypol in the cotyledon half of the seed was high (Pearson R-value = +0.985, $p < 0.0001$), but a slightly increased level of gossypol in the cotyledon half of the seed was apparent, which increased with increasing gossypol content (data not shown). This difference appears to be related to the lack of glands in the immediate area surrounding the embryo. This region is often observed to be devoid of glands in seeds cut longitudinally to illustrate seed glanding patterns (Leahy, 1948). Total gossypol values obtained from the cotyledon half compared with gossypol levels for the whole seed (calculated from the analyses on both seed halves) were also highly correlated (Pearson R-value = +0.995, $p < 0.0001$) (Fig. 4). Although the cotyledon half -seed values were still slightly greater than the whole-seed values, the difference was considerably smaller. Despite this slight difference in absolute levels, the high correlation indicates that the gossypol

level in the cotyledon half is useable as a selection guide for estimating gossypol levels in whole seed. Comparing the ratio of gossypol enantiomers (expressed as the percentage of (+) gossypol) between the cotyledon seed half and embryo seed half (data not shown) or between the cotyledon seed half and the whole seed (Fig. 5), also showed good agreement, the later comparison having a Pearson R-value of +0.989, $p < 0.0001$. The half-seed HPLC method gave gossypol values similar to a previous study where 100-mg samples were compared directly with 50-mg samples (Scheffler and Romano, 2008).

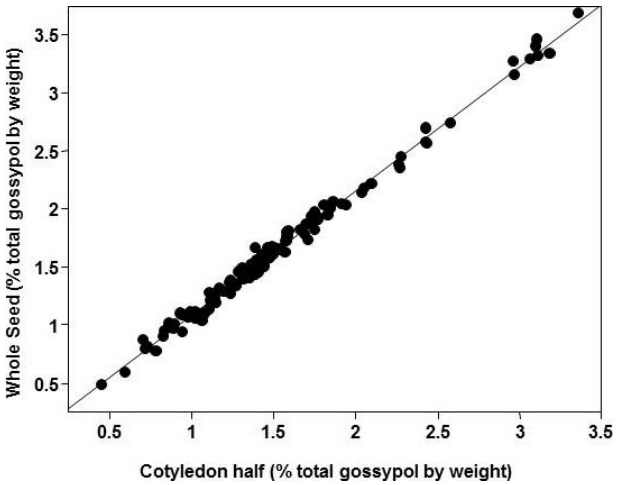

Figure 4. Comparison of percent gossypol as measured on half a seed versus on the whole seed. The Pearson R-value = +0.995 ($p < 0.0001$).

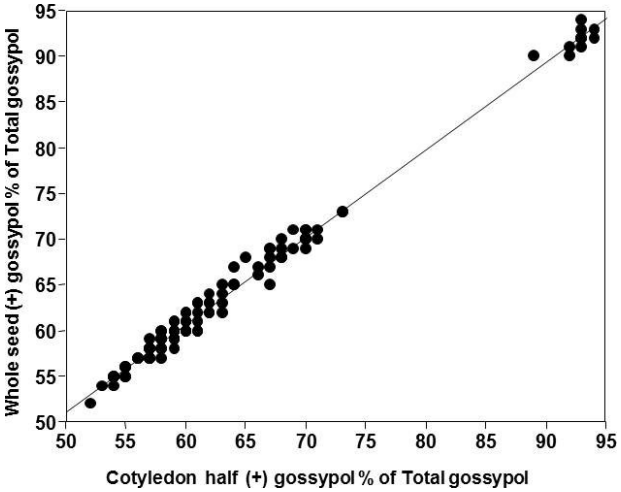

Figure 5. Comparison of percent (+) gossypol measured on whole seed versus the cotyledon half of the seed. Pearson R-value = +0.989 ($p < 0.0001$).

Testing the Reliability and Repeatability of Selecting in the F_2 Generation. A 70-seed population from the cross between H1220 and Mac7-0113 was evaluated to determine if it was possible to select for the highest percentage of the (+) enantiomer or for total gossypol based on F_2 seed analysis. Of the 70 half seeds planted, 81% survived and produced sufficient seed for HPLC analysis on a bulk seed sample, as well as to plant the F_3 generation in the field for further testing. The 10 with the highest proportion of (+) gossypol were the same from the half-seed analysis of F_2 seed or the 16-seed analysis of F_3 seed (Table 3). Correlation analysis of the surviving 57 plants showed reasonable agreement between F_2 and F_3 values for the percentage of the (+) enantiomer (Pearson R-value = +0.883, $p < 0.0001$, Fig. 6) and for total gossypol level (Pearson R-value = +0.774, $p < 0.0001$, Fig. 7). In a second cross between MD51ne and Mac7-1238, 44 of the 50 half seed planted produced sufficient seed to analyze and plant the F_3 generation in the field for further testing. Eight of the 10 selected with the highest proportion of (+) gossypol in the F_2 generation also were selected in the F_3 generation (Table 4). In a third cross between A1006 x Mac7-1238, 9 of the top 10 selected in the F_2 also were selected in the F_3, confirming the ability of the F_2 half-seed method to select individuals with the highest percentage of (+) gossypol. As illustrated in Figs. 6 and 7, although the overall Pearson R-values were less than 0.9, there was good agreement for the values within the target range for selection, with most of the variation in the middle range of values.

From these three populations, two elite lines were developed and released (Release Notice P 0001.13). GVS6 came from the cross between MD51ne and Mac7-1238 (Table 4) and GVS7 the cross between A1006 x Mac7-1238. Total seed gossypol for GVS6 averaged 22.5 µg/mg (2.25%) with 91% in the (+) form. Total seed gossypol for GVS7 averaged 12.2 µg/mg (1.22%) with 90% of the gossypol in the (+) form. Replicated field trials over two years, with the elite lines, their three parents, and a high yield check variety SG 747 (SureGrow 747) showed that the two elite lines generally yielded better than their parents and their fiber properties were comparable or better than their adapted parent or the check variety (Table 5).

Table 3. Comparison of the best 10 F_2 plants selected for plus gossypol by half-seed analysis with the best 10 selected using a 16-seed bulk sample of F_3 seed

		F_2	F_2	F^2	F_3	F_3	F_3
	ID Nr.	Rank	% (+) Isomer of Total Gossypol	Total % Gossypol	Rank	% (+) Isomer of Total Gossypol	Total % Gossypol
1	204	1	89.6	1.8	8	89.0	1.8
2	214	2	89.5	2.4	5	90.6	1.5
3	232	3	89.2	1.5	4	90.8	1.6
4	110	4	89.1	1.6	1	91.1	1.4
5	212	5	88.7	2	3	90.9	1.6
6	215	6	88.5	2.8	6	89.3	2.0
7	208	7	88.2	1.1	7	89.3	1.5
8	115	8	87.4	1.4	9	88.4	0.9
9	216	9	87.1	0.9	10	87.2	0.4
10	116	10	85.5	0.9	2	91.0	0.5
Mean and Range[Z]			70.4 82.7–58.8			74.4 85.0–61.7	
Parent	Mac7-0113		88.6	1.7		89.3	1.9
Parent	H1220		68.7	1.8		70.1	1.6

Population size = 70, survival rate = 81%

[Z] Mean and range for % (+) isomer of total gossypol of the 70 not selected.

Table 4. Comparison of the best 10 F_2 plants selected for plus gossypol by half-seed analysis with the best 10 selected using a 16-seed bulk sample of F_3 seed

		F_2	F_2	F_2	F_3	F_3	F_3
	ID Nr.	Rank	% (+) Isomer of Total Gossypol	Total % Gossypol	Rank	% (+) Isomer of Total Gossypol	Total % Gossypol
1	301	1	93.1	1.8	2	92.1	1.9
2	282	2	92.9	2.2	4	92.0	2.4
3	281	3	92.8	2.0	8	91.3	2.2
4	324	4	92.1	2.3	6	91.5	2.3
5	292	5	91.9	2.1	7	91.4	2.1
6	313	6	91.6	2.1	11	90.8	2.4
7	312	7	91.2	2.1	5	91.6	2.1
8	296	8	90.4	1.9	3	92.1	1.8
9	304	9	89.8	1.9	1	92.2	1.8
10	289	10	88.7	1.9	13	90.1	1.9
11	328	11	88.2	1.9	10	91.2	1.8
12	319	12	87.6	1.8	9	91.3	1.7
13	318	13	87.4	1.8	12	90.1	1.7
Mean and Range[Z]			71.3 88.2–64.4			70.1 90.8–63.1	
Parent	Mac7-1238		93.4	2.0		92.9	2.2
Parent	MD51ne		65.3	1.6		64.2	1.8

Population size = 50, survival rate = 88%

[Z] Mean and range for % (+) isomer of total gossypol of the 44 not selected.

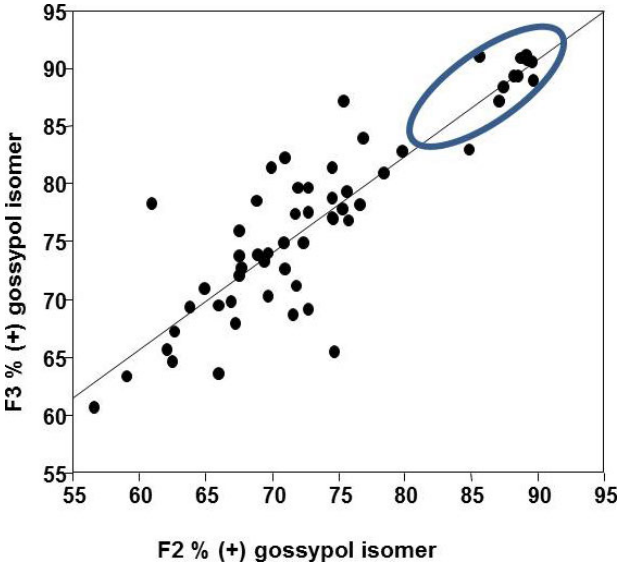

Figure 6. Percent (+) gossypol measured in the F_2 generation compared to the F_3 generation. The Pearson R-value = +0.883 ($p < 0.0001$).

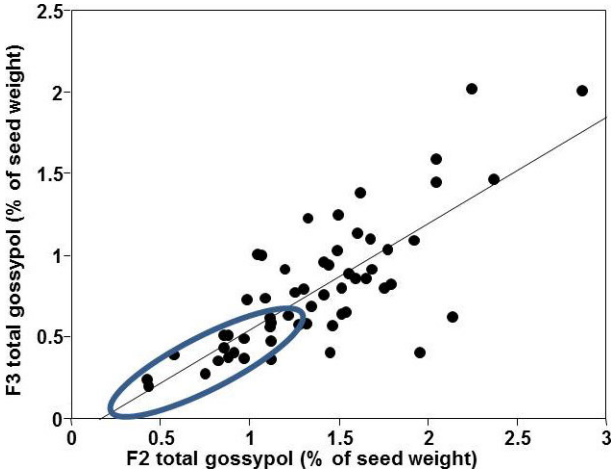

Figure 7. Percent total gossypol measured in the F_2 generation compared to the F_3 generation. The Pearson R-value = +0.774 ($p < 0.0001$).

Table 5. Lint yield and fiber data for the two released lines compared to their parents and a high yield check SG 747. Means averaged over two replications in each of 2 yrs

Entry	Fiber Yield (kg/ha)	Length HVI-UHM (mm)	Strength (kN m/kg)	Micronaire
SG 747	1373	27	298	4.5
A1006	1050	32	331	4.8
MD51ne	1131	28	334	5.1
Mac7	927	22	327	4.8
GVS6	1196	28	329	5.3
GVS7	1002	30	327	5.1
LSD	238	1.8	22	0.7

CONCLUSION

The half-seed preparation and plant propagation methods combined with a reduced-scale HPLC analysis, provided a simple, cost-effective, and successful procedure to breed for modified gossypol content and enantiomer profile. These methods have subsequently been incorporated into a breeding program to produce elite lines with modified gossypol properties. The techniques reduce the number of lines that need to be advanced to later generations and decrease development time, saving time and labor (Table 6). Other testing and trait evaluation methods can be combined with this method. For example, marker-assisted selection with simple sequence repeats (SSR) or single nucleotide polymorphism (SNP) DNA markers can be used for traits of interest including nematode or Fusarium wilt resistance or the nectariless trait. A single 1-cm diameter leaf can be collected from each F_2 plant and genotyped with DNA markers for multiple traits, again allowing plants to be tested and a majority of the lines discarded in early generations. The half-seed propagation technique also can be used to assay for protein and oil and is being evaluated for use with cottonseed fatty acid analysis.

Table 6. Cost comparison per sample for a 100-mg (16-seed) sample versus a half seed sample analyzed for total gossypol and amount of each enantiomer. Only direct costs were considered and were based on analysis of a set of 40 samples

	100-mg sample Total and (+)/(−) gossypol		Half-seed sample Total and (+)/(−) gossypol	
Supplies	Cost per sample	Percent of total cost	Cost per Sample	Percent of total cost
Chemicals[z]	$1.56	71.0	$1.08	62.4
2-ml microfuge tube	$0.07	3.2	$0.07	4.0
Autoinjector vial with cap	$0.17	7.6	$0.17	9.8
Inertsil column (1 per 2000 samples)	$0.25	11.0	$0.25	14.5
Guard Column (1 per 500 samples)	$0.16	7.2	$0.16	9.3
Total cost per sample	$2.21	100	$1.73	100

[z] Chemicals included (R)-2-amino-propanol (2-AP), acetic acid (acoh), dimethyl formamide (DMF), acetonitrile (MeCN)

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Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

REFERENCES

Adams, R., T.A. Geissman, and J.D. Edwards. 1960. Gossypol, a pigment of cottonseed. Chem. Rev. 60:555–574.

Bell, A.A., and R.D. Stipanovic. 1977. The chemical composition, biological activity, and genetics of pigment glands in cotton. p. 244–258 *In* Proc. Beltwide Cotton Conf., Atlanta, GA. 10-12 Jan.1977. Natl. Cotton Counc. Am., Memphis, TN.

Bell, A.A., L. Puckhaber, H. Kim, R. Stipanovic, and E. Percival. 2000. Genetic approaches for increasing percentages of (+)-gossypol levels in cotton. p. 218–230 *In* Proceedings of the Genetic Control of Cotton Fiber and Seed Quality Workshop, San Antonio, TX. 5-6 Dec. 2000. Cotton Inc., Cary, NC.

Blackstaffe, L., M.D. Shelley, and R.G. Fish. 1997. Cytotoxicity of gossypol enantiomers and its quinone metabolite gossypolone in melanoma cell lines. Melanoma Res. 7:364–372.

Conte, L.S., O. Leoni, S. Palmieri, P. Capella, and G. Lercker. 1989. Half-seed analysis: rapid chromatographic determination of the main fatty acids of sunflower seed. Plant Breeding 102:158–165.

Dierig, D.A., A.M. Salywon, P.M. Tomasi, G.H. Dahlquist, and T.A. Isbell. 2006. Variation of seed oil composition in parent and S1 generations of *Lesquerella fendleri* (Brassicaceae). Industrial Crops Prod. 24:274–279.

Dowd, M.K., and S.M. Pelitire. 2001. Recovery of gossypol acetic acid from cottonseed soapstock. Industrial Crops Prod. 14:113–123.

Downey, R.K., and B.L. Harvey. 1962. Methods of breeding for oil quality in rape. Can. J. Plant Sci. 43:271–275.

Eisele, G.R. 1986. A perspective on gossypol ingestion in swine. Veterinary and Human Toxicology 28:118–122.

Getinet, A., G. Rakow, J.P. Raney, and R.K. Downey. 1994. Development of zero erucic acid Ethiopian mustard through an interspecific cross with zero erucic acid Oriental mustard. Can. J. Plant Sci. 74:793–795.

Kim, H.L., M.C. Calhoun, and R.D. Stipanovic. 1996. Accumulation of gossypol enantiomers in ovine tissues. Comp. Biochem. Physiol. 113:417–420.

Leahy, J. 1948. Structure of the cottonseed. p. 105–116 *In* A.E. Bailey (ed.) Cottonseed and Cottonseed Products: Their Chemistry and Chemical Technology. Interscience Publishers, New York, NY.

Liu, S.L., S.K. Kulp, Y. Sugimoto, J. Jiang, H-L. Chang, M.K. Dowd, P. Wan, Y.C. Lin. 2002. The (−)-enantiomer of gossypol possesses higher anticancer potency than racemic gossypol in human breast cancer. Anticancer Res. 22:33–38.

Lordelo, M.M., A.J. Davis, M.C. Calhoun, M.K. Dowd, and N.M. Dale. 2005. Relative toxicity of gossypol enantiomers in broilers. Poultry Sci. 84:1376–1382.

Lusas, E.W., and G.M. Jividen. 1987. Glandless cottonseed: a review of the first 25 years of processing and utilization research. Amer. Oil Chem. Soc. 64**:** 839–854.

McCarty, J.C. Jr, P.A. Hedin, and R.D. Stipanovic. 1996. Cotton *Gossypium* spp. plant gossypol contents of selected GL_2 and GL_3 alleles. J. Agric. Food Chem. 44:613–616.

Pettigrew, W.T., and M.K. Dowd. 2011. Varying plant dates or irrigation regimes alters cottonseed composition. Crop Sci. 51:2155–2164.

Santos, J.E., M. Villaseňor, P.H. Robinson, E.J. DePeters, and C.A. Holmberg. 2003. Type of cottonseed and level of gossypol in diets of lactating dairy cows: plasma gossypol, health, and reproductive performance. J. Dairy Sci. 86:892–905.

Scheffler, J.A., and G.B. Romano. 2008. Modifying gossypol in cotton (*Gossypium hirsutum* L.): A cost effective method for small seed samples. J. Cotton Sci. 12:202–209.

Stipanovic, R.D., L.S. Puckhaber, A.A. Bell, A.E. Percival, and J. Jacobs. 2005. Occurrence of (+)- and (−)-gossypol in wild species of cotton and in *Gossypium hirsutum* Var. *marie-galante* (Watt) Hutchinson. J. Agric. Food Chem. 53:6266–6271.

Tahmasebi-Enferadi, S., Z. Rabiei, M. Turi, M. Baldini, and G.P. Vannozzi. 2004. Half-seed analysis for comparing linolenic acid synthesis between high and low oleic acid sunflower inbred lines. Helia 27:63–72.

Velasco, L., and C. Möllers. 2002. Nondestructive assessment of protein content in single seeds of rapeseed by spectroscopy. Euphytica 123:89–93.

Wang, S., K. Kanamaru, W. Li, J. Abe, T. Yamada, and K. Kitamura. 2007. Simultaneous accumulation of high contents of alpha-tocopherol and lutein is possible in seeds of soybean. Breeding Sci. 57:297–304.