1312 Increasing cottonseed utilization through breeding and genetic engineering to produce high levels of (+)-gossypol in seed

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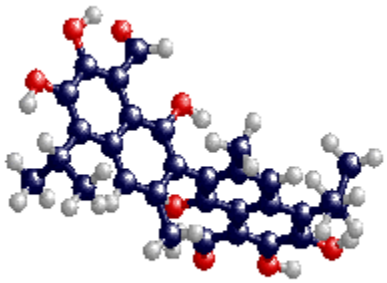
Cottonseed is composed of ~22.5% of high quality protein. The estimate of world cottonseed production for 2006 is over 42.5 million metric tons (National Agricultural Statistics Service, USDA, 2005), which translates to an estimated 9.56 million metric tons of protein available for use as a food/feed source. However, only a portion of cottonseed and thus its protein is actually utilized for food/feed. This under-utilization is due to the presence within most commercial cottonseed of a toxic compound called gossypol. Ruminants such as beef and dairy cattle, sheep and goats are capable of ingesting low levels of toxic gossypol without harmful effects but non-ruminants such as humans and chickens are susceptible to harm. The toxic metabolite, gossypol, occurs in the seed, foliage and roots of cotton where it provides protection against herbivorous insects and pathogens. Gossypol is biosynthesized by high-energy free radical coupling of two molecules of hemigossypol which yields two optically active enantiomers, (+)-gossypol and (-)-gossypol. In most commercial cottons (Gossypium hirsutum) grown in the U.S., the ratio of (+)- to (-)-gossypol is approximately 3:2. However, this ratio can be as high as 98:2 in some Moco cotton (G. hirsutum marie galante) accessions. Significantly, only (-)-gossypol is toxic to animals while toxicity toward insects and pathogens is independent of the (+)- to (-)-gossypol ratio. Therefore, if the ratio could be shifted predominately to (+)-gossypol in the seed, cottonseed could be used as a feed for non-ruminant animals. Advances in breeding cotton plants for high (+)-gossypol seed, and the protein that controls the biosynthesis of (+)- and (-)-gossypol are the subjects of this paper.

Researchers have worked extensively to unravel the sesquiterpenoid biosynthetic pathways operating in cotton. In addition to that presented herein, the proposed biosynthesis of gossypol is discussed elsewhere in this Proceedings (see Stipanovic et al.). Gossypol is biosynthesized by the free radical coupling of hemigossypol by a peroxidase (Benedict et al., 2006), and hemigossypol is derived *via* the cyclization of *E,E*-farnesyl diphosphate to give the sesquiterpene delta cadinene under the control of the enzyme, delta-cadinene synthase (D-CS). Rathore and colleagues (Sunilkumar, et al., 2006) have shown that a RNAi construct of D-CS, controlled by a seed-specific alpha-globulin B gene promoter from cotton, interfered with expression of the delta cadinene synthase gene during seed development. The authors report that the level of gossypol in seeds of these plants was reduced to as low as 0.1 µg/mg in a stable and heritable manner, but gossypol and related compounds that deter insect herbivory were not diminished in the foliage and in floral parts (discussed in this Proceedings, see Rathore et al.). This offers the opportunity to produce cottonseed that would be suitable as a feed for monogastric animals and that would be from a plant with a full compliment of defensive terpenoids.

An alternative strategy to produce cottonseed suitable as a feed for non-ruminant animals requires cottonseed with a preponderance of a specific non-toxic form of gossypol. Gossypol exists as two enantiomers due to hindered rotation around the binaphthyl bond. If polarized light is passed through a solution of each of these forms, the light will be rotated either to the right (clockwise) or to the left (counterclockwise). The form that rotates polarized light to the right is referred to as (+)-gossypol and is said to be the *S*-enantiomer, while the form which rotates polarized light to the left is referred to as (-)-gossypol and is said to be the *R* enantiomer (Jaroszewski et al., 1992).

The biological activity of these enantiomers differs. For example, (-)-gossypol but not (+)-gossypol shows anti-HIV-1 activity in humans (Lin et al., 1989, 1993). (-)-Gossypol is also a more effective anti-amoebic agent (Gonzalez-Garza et al., 1993). Matlin et al. (1985), Lindberg et al. (1987), Wang et al. (1987), and Wu et al. (1986) showed that (-)-gossypol, but not (+)-gossypol, has male antifertility activity and is more toxic to animals. This last observation led to a chicken feeding study by Bailey et al. (2000) in which they showed that broiler chickens fed a diet containing 5% of ground, dehulled cottonseed with a (+)- to (-)-gossypol ratio of 83:17 gained weight at the same rate as a 100% soybean control diet and a control diet with 5% ground, dehulled glandless cottonseed. In contrast, broilers fed a diet containing 5% of a ground, dehulled cottonseed with a (+)- to (-)-gossypol ratio of 62:38 gained significantly less weight. Regression analysis also showed that cumulative weight gains of the chickens decreased ~126 g for each 100 mg increase in (-)-gossypol consumed, while the cumulative weight gains were not significantly affected by increased (+)-gossypol consumption. This latter study indicated that a broiler chicken diet that contained cottonseed with <95% (+)-gossypol would have no detrimental effects on the birds.



Absolute configuration of (*S*)-(+)-gossypol.

Cass et al. (1991) identified a source of high (+)-gossypol seed in some accessions of the *G. hirsutum marie galante*, otherwise referred to as Moco cotton. The suitability of such plants to resist insects and pathogens is suggested by the results of a number of investigations. First, studies have shown that plants that lack glands and, therefore, gossypol and related terpenoids are susceptible to attack by recognized cotton insect pests as well as by new pests (Lukefahr et al., 1966; Jenkins et al., 1966). Early laboratory insect feeding studies (Stipanovic et al., 1977) confirmed the importance of gossypol and related terpenoids in protecting the cotton from attack while a more recent *Helicoverpa zea* feeding study (Stipanovic et al., 2006) revealed that no significant differences occur in pupal weight, days-to-pupation and survival of 1st instar larvae fed racemic [i.e., a 1:1 mixture of (+)- and (-)-gossypol], (+)-gossypol, and (-)-gossypol diets. The effect of (+)- and (-)-gossypol on the growth (ED_{50}) and survival (LD_{100}) of the cotton seedling pathogen *Rhizoctonia solani* also has been studied with the result that (+)- and (-)-gossypol were found to be equally toxic to *R. solani*. In other fungal toxicity tests, gossypol was

established to be significantly less inhibitory than other terpenoids that also occur in the roots and stem (Puckhaber et al., 2002). Furthermore, Yildirim-Aksoy et al. (2004) found (+)-gossypol was a better bacteriostat than racemic or ( ) gossypol.

Finally, the (+)- to (-)-gossypol ratio and the total terpenoid in the foliage and roots of *Gossypium hirsutum marie galante* accessions that exhibited high levels of (+)-gossypol in the seed were studied. Neither the total terpenoids nor (+)- to (-)-gossypol ratios in these tissues correlated with the (+)- to (-)-gossypol ratio in the seed. Thus, regulation in these various tissues appears to be under separate genetic control. Significantly, the gossypol enantiomeric ratio was found to be reasonably constant in seed. Meredith and collaborators (Rayburn, et al., 2000) measured the levels of seed gossypol in comparative tests in a number of commercial *G. hirsutum* and *G. barbadense* cultivars growing at several locations across the U.S. As expected, since terpenoid synthesis can be activated in a host stress response, environment significantly affected the total amount of gossypol present within a specific cultivar. However, the ratio of (+)- to (-)-gossypol remained constant within each cultivar.

In summary, these data indicate that animal toxicity of gossypol is due to the (-)-enantiomer, while insect and pathogen resistance is not affected by the (+)- and (-)-gossypol ratio. Therefore, plants exhibiting a high (+)- to (-)-gossypol ratio in the seed will retain their natural defense capability while producing seeds that can be fed to non-ruminant animals. A traditional breeding program utilizing *G. hirsutum marie galante* could achieve the goal of high (+)-gossypol seed. An alternative approach could utilize genetic engineering to regulate and express the protein that controls the (+)- to (-)-ratio. We will discuss progress in the traditional breeding program first.

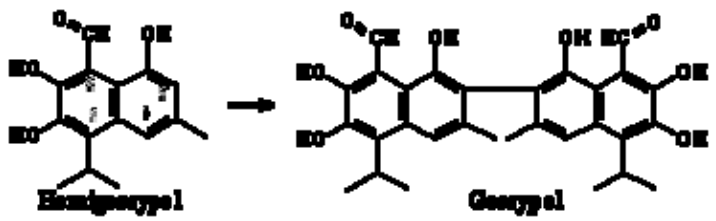
Breeding for high (+)-gossypol seed – Although several wild cotton species exhibit the high (+)-gossypol seed trait (Stipanovic et al., 2005), *G. hirsutum marie galante* appeared to be the most amenable even though it would be necessary to remove its photoperiodicity trait and the trait that delays bloom and boll set until the second year after planting.

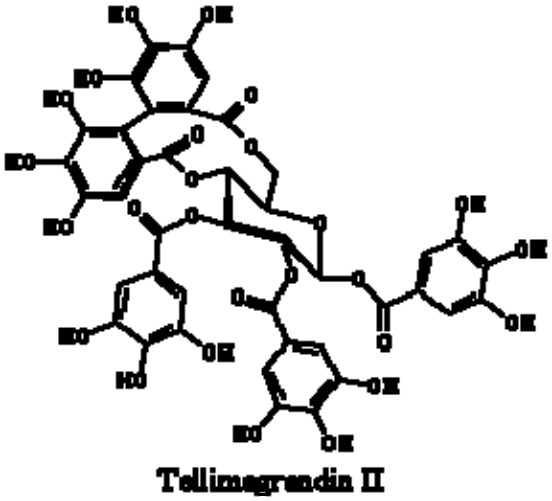
Moco cottons that contain 87% to 97% (+)-gossypol were selected and crossed with the commercial cultivar 'Tamcot CAMD E' (*G. hirsutum*) (Bell et al., 2000). The percentages of the (+)-enantiomer in F1 plants from 19 crosses ranged from 66% to 86%. In each cross, the ratio was intermediate between the two parents, a characteristic associated with incomplete dominance or quantitative inheritance with additive effects. Eight F1 hybrids were selected for a backcross genetic study to elaborate the genetic basis of high (+)-gossypol using Tamcot CAMD E as the recurrent parent.

Flower petals and seed were analyzed in the BC_1F_1, BC_2F_1, BC_3F_1, BC_3S_1 and BC_3S_2 generations. Elevated (+)-gossypol concentrations were due to two major genes designated G_1+ and G_2+. The gene G_2+ was dominant and expressed mostly in flower petals, whereas G_1+ was mostly recessive and expressed equally in flower petals and seed. Plants with homozygous G_1+ contained about 90% (+) gossypol in seed. Adding G_2+ in the homozygous condition further increased the percentage to about 95%. Four homozygous stocks (BC_3S_2) with 93-95% (+) gossypol in seed have been developed.

Additionally, the ratio of (+)- and (-)-gossypol remained constant for a particular plant regardless of boll location on the plant or whether the seed came from bolls that were produced early or late in the season.

Engineering cottonseed with a high (+)- to (-)-gossypol ratio – Before discussing the alternative genetic engineering approach, it is essential to have some background knowledge of the biosynthesis of gossypol. Early steps in its biosynthesis are discussed elsewhere in this Proceedings (see Stipanovic et al.). Herein, the focus will be on the last step, which is the free radical coupling of hemigossypol by a peroxidase (Benedict et al., 2006). Free radical coupling reactions are characteristically high energy reactions in which the reaction product (i.e., gossypol) is formed in a random fashion. However, this coupling reaction in cotton produces an excess of one enantiomer in preference to the other. For example, in some Moco cotton accessions the (+)-gossypol to (-)-gossypol ratio can be as high as 49:1 (Stipanovic et al., 2005) while in some *G. barbadense* accessions the (+)- to (-)-ratio may be the reverse, i. e., ~2:3 (Percy et al., 1996; Cass et al., 1991). If gossypol is synthesized *in vitro* using commercially available peroxidase or laccase and hemigossypol, the product is a racemic mixture (i.e., equal parts of (+)- and (-)-gossypol) (Benedict et al., 2006**)**. In cotton itself, some additional factor (i.e., a protein or enzyme) must be involved in controlling the stereochemical outcome of the reaction. Precedents in the literature point to two prospective mechanisms to account for the cultivar-specific ratios observed in cotton.




Tellimagrandin II

One example comes from research on *Tellima grandiflora*. (S) Tellimagrandin II is synthesized in the leaves of *T. grandiflora* by a free radical phenol coupling of 1,2,3,4,6-penta-*O*-galloyl-Beta D-glucopyranose that is catalyzed by a laccase (Niemetz and Gross, 2003). A similar type of laccase or peroxidase could control the dimerization of hemigossypol in cotton.

Alternatively, a "dirigent" protein may be involved. In a variety of plant species, *E*-coniferyl alcohol is dimerized by a free radical mechanism that exhibits both regio- and stereospecificity giving either (+)- or (-)-pinoresinol. Lewis' group (Davin et al., 1997) has elegantly demonstrated the intervention of what they call a dirigent protein. The dirigent protein, which was isolated from *Forsythia suspensa*, by itself has no catalytic activity. However, when the dirigent protein is combined with *E*-coniferyl alcohol and laccase (previously isolated from *F. intermedia*) only (+)-pinoresinol is formed. If the dirigent protein is missing from this reaction, a mixture of racemic products is formed. Boiling deactivates the protein (Hall and Lewis, 2002). Davin and Lewis (2000) proposed that the dirigent protein captures the *E*-coniferyl alcohol-derived free radical and directs stereoselective coupling. The native protein was shown to have a molecular weight of 78 kDa arising from three presumably identical 26 kDa subunits. Two clones were identified (*psd-Fi1* and *psd-Fi2*) that code for nearly identical proteins with a molecular weight of ~18kDa (Gang et al., 1999). The assumption that glycosylation accounts for the 8 kDa required to reach the 26 kDa mass of the native subunits was verified by expressing clone *psbFi1* in an insect cell culture system (*Spodoptera frugiperda*/baculovirus) that permits glycosylation (O'Reilly et al., 1992). When *psd-Fi1* was introduced under control of the

Autographa californica polyhedrin promoter, different degrees of glycosylation by *S. frugiperda* gave three major glycoproteins in the size range of ~22 kDa to ~26 kDa. Most critically, the heterologously expressed protein showed the same substrate specificity in the presence of laccase as the native protein. Furthermore, Gang et al. (1999) used PCR and Southern blotting to detect homologous DNA sequences in both gymnosperms and dicotyledonous angiosperms. They concluded that dirigent proteins involved in the synthesis of lignin are widely distributed and perhaps ubiquitous throughout vascular plants. More recently, Davin and Lewis (2000) showed that a different dirigent protein found in flaxseed produces the corresponding (-)-antipode. Thus, non-catalytic proteins can steer the regio- and stereospecific dimerization of free-radical species.

We developed an assay to guide the identification of a protein or enzyme that controls the sterospecific coupling of hemigossypol (J. Liu unpublished). The assays utilized hemigossypol as the substrate and crude flower petal extracts, either alone or supplemented with laccase or H_2O_2. In each case, controls included: 1) with/without fresh petal extract; 2) with/without boiled petal extract; 3) with/without enzyme; and 4) with/without exogenous hemigossypol. The assay reaction was extracted with an organic solvent and the organic layer was reacted with D-alaninol and the (+)- and (-)-enantiomers of gossypol were qualitatively and quantitatively analyzed using HPLC (Kim et al., 1996). Our first goal was to determine if the stereospecificity was controlled by an enzyme or a dirigent type protein.

Dimerization of hemigossypol with peroxidase, laccase or ammonium persulfate (a free radical generating oxidizing reagent) gave a racemic (i.e., 1:1) mixture of gossypol. In contrast, when hemigossypol is added to a crude enzyme preparation of Moco flower petals, (+)-gossypol is preferentially formed. The crude protein preparation must contain endogenous peroxidase as evidenced by an increase in the synthesis of gossypol when H_2O_2 was added. In one experiment the ratio of (+)- to (-)-gossypol was 81:19. When hemigossypol and laccase were added to a boiled crude enzyme preparation of Moco flower petals, racemic gossypol was produced. Gossypol was not produced when hemigossypol was added to a boiled crude enzyme preparation of Moco flower petals without laccase. These experiments show that a protein/enzyme controls the enantiomeric ratio and that its activity can be monitored in our assay.

To further address the question of the involvement of a dirigent type protein versus an enzyme, additional experiments were conducted. In these, hemigossypol was added under aerobic conditions to an aliquot of a crude enzyme preparation or a boiled crude enzyme preparation of Moco flower petals with and without laccase or H_2O_2 (Table 1, Experiment 1). The reaction with the crude enzyme preparation alone gave a 74:26 ratio of (+)- and (-)-gossypol. However, when the boiled crude enzyme preparation was used, a racemic mixture was produced. Again, this confirmed that the crude enzyme preparation contained the protein or enzyme that was controlling the sterospecific coupling. In the two experiments using the crude enzyme preparation with either H_2O_2 or laccase, the % (+)-gossypol remained the same compared to the crude preparation alone (Table 1, Experiment 1). If a stereospecific enzyme (i.e., laccase) in the extract was responsible for controlling the (+)- to (-)-gossypol ratio, the % (+)-gossypol should decrease when commercial laccase was added because laccase competes for the same substrate (i.e., hemigossypol) and laccase alone yields racemic gossypol. Thus, this result does not support the presence of a stereospecific laccase or peroxidase that leads to stereospecific coupling. When H_2O_2 is added, significantly more total (+)-gossypol was produced indicating that significant quantities of peroxidase are present in the crude preparation. To further hone in on the

nature of the protein or enzyme that controls the stereospecific coupling, it was necessary to separate the protein that controls stereospecificity from endogenous cotton peroxidase in the crude enzyme preparation.

To accomplish this, the crude enzyme preparation was subjected to chromatography on a CM-Sepharose FF column. The protein concentration of the individual fractions collected from the column was determined (Figure 1). Selected fractions were reacted with laccase and the % (+)-gossypol was determined. Excess (+)-gossypol was produced by fractions 28 to 34. Fraction 32 was selected for further study because it showed the highest activity when laccase was added (Table 1, Experiment 2). An aliquot of the original crude enzyme preparation that also gave approximately 65% (+)-gossypol when laccase was added was also selected. A concentration of ~10 pmole of gossypol is at the detection limit of the HPLC system. Thus, values close to 10 pmole indicate no or very little gossypol formation. In Experiment 2, when the assay utilizing the crude enzyme preparation with hemigossypol and H_2O_2 was compared to the assay using the purified fraction, over 50 times more total gossypol was produced in the crude preparation as compared to the purified fraction. This indicates that the column successfully separated the protein or enzyme that controls the (+)- to (-)-gossypol ratio from the endogenous peroxidase that is present in the crude preparation. Significantly, when hemigossypol was reacted with the crude preparation, 99 pmole gossypol/reaction/hr was produced, but no gossypol was produced when hemigossypol was added to Fraction 32. However, when hemigossypol was reacted with Fraction 32 in the presence of laccase, 2,912 pmole of gossypol were produced of which 65% was (+)-gossypol. Thus, the protein in Fraction 32 is incapable of converting hemigossypol into gossypol by itself. However, when an oxidative enzyme such as laccase was added, it readily produced significant quantity of gossypol and the (+)- to (-)-gossypol ratio was almost 2:1. These results are consistent with the presence of a dirigent type protein, and notably inconsistent with the intervention of a stereospecific enzyme.

CONCLUSION

The genetic makeup of some Moco cottons, whose seed have a (+)- to (-)-gossypol ratios above 95:5, offer a unique opportunity to provide commercial cotton plants with a seed suitable for consumption by monogastric animals but with the normal complement of protective terpenoids in the foliage. Utilizing this germplasm, we have identified the $+G_1$ gene that has somewhat greater effect on the percent (+)-gossypol in the seed, and the $+G_2$ gene that has somewhat greater effect on the percent (+)-gossypol in the flower petals. Using traditional breeding techniques, we have developed lines that provide >95% (+)-gossypol in the field. On the enzyme level, we have demonstrated that a "dirigent like" protein controls the (+)- to (-)-gossypol ratio in Moco cotton. Identification of this enzyme and the regulator(s) that control its expression, could facilitate the generation of high (+)-gossypol seed plants both through marker assisted-breeding and a genetic engineering approach.

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Table 1. Formation of (+)- and (-)-gossypol from hemigossypol (HG) under various protocols.

Experiment 1						
Assay						nmole
Type	Substrate		Reactant	Extract	% (+)-Gossypol	(+) Gossypol/hr
Oxidase	O_2	+	HG	Crude Enzyme	→ 74%	2.2
Peroxidase	H_2O_2	+	HG	Crude Enzyme	→ 79%	11.4
Dirigent	Laccase/ O_2	+	HG	Crude Enzyme	→ 78%	22.3
Control	Laccase/ O_2		HG	Boiled Crude Enzyme	→ 51%	19.6
Experiment 2						
Assay						pmol Total Gossypol/
Type	Substrate		Reactant	Extract	% (+)-Gossypol	Reaction/hr
Dirigent	Laccase/O_2	+	HG	Crude Enzyme	→ 62%	1,550
Peroxidase	H_2O_2	+	HG	Crude Enzyme	→ 67%	520
Oxidase	O_2	+	HG	Crude Enzyme	→ 65%	99
Dirigent	Laccase/ O_2	+	HG	Fraction # 32	→ 65%	2,912
Peroxidase	H_2O_2	+	HG	Fraction # 32	→ 68%	10
Oxidase	O_2	+	HG	Fraction # 32	→ 59%	11

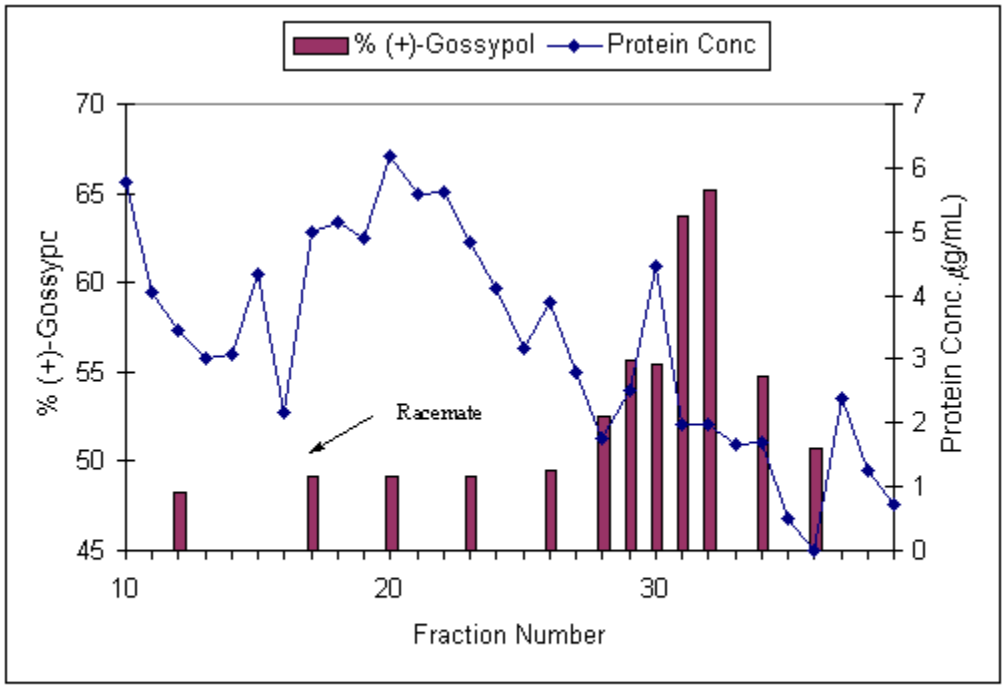

Figure 1. Protein concentration and % (+)-gossypol in fractions from CM-Sepharose FF column with hemigossypol and laccase.