1811 RNAi-mediated, selective, and substantial reduction in gossypol levels from cottonseed to enhance its food and feed value

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Annual, worldwide cottonseed output can potentially provide the protein requirements of half a billion people if it could be used directly as food. However, the presence of gossypol within seed-glands renders cottonseed toxic to humans and monogastric animals. Therefore, elimination of gossypol from cottonseed has been a long-standing goal of geneticists. The "glandless cotton" developed by breeders in the 1950s to meet this objective was commercially unviable because of the increased susceptibility of the plant to insect pests due to the systemic absence of glands that contain gossypol and other protective terpenoids. Thus, the promise of cottonseed in contributing to the food requirements of the burgeoning world population remained unfulfilled. We have demonstrated that RNA interference (RNAi) can be employed to disrupt gossypol production in a tissue (seed)-specific manner in cotton. Targeted engineering of the gossypol biosynthetic pathway by inhibiting the expression of a key gene encoding δ-cadinene synthase during seed development resulted in a significant reduction in cottonseed-gossypol levels. This trait was heritable and stable. Results from molecular and enzyme analyses on developing, transgenic embryos were consistent with the observed phenotype in the mature seeds. Most importantly, the levels of gossypol and related terpenoids in the foliage and floral parts were not diminished and thus remain available for plant defense against insects and diseases. This overcomes the major weakness of "glandless cotton". The use of reduced gossypol cottonseed either directly as food or indirectly as feed for the more efficient monogastric animals has the potential to significantly impact global food security.

The terpenoid class of secondary metabolites produced by many plant species plays an important ecological role either as attractants (e.g. linalool) or as defense compounds (e.g. bitter triterpenoid cucurbitacins, pungent diterpenoid polygodial, gossypol and related compounds in cotton) (Aharoni et al., 2005; Langenheim, 1994; Stipanovic et al., 1999). However, the presence of some of the terpenoids or other types of defense compounds also renders the plants or their parts that produce them toxic to humans and animals. Over the course of human history, man has learned to either avoid consumption of toxic plants/their parts or to inactivate/neutralize the toxic compounds present before ingestion (i.e., cassava, kidney beans). In some cases, the plant product is used as feed for domestic animals with a rumen where the toxin can be inactivated or metabolized before digestion; thus the animal suffers little or no ill effect from the toxin. Cottonseed, produced in abundance as a byproduct of the fiber production, represents such a case.

With the world population at 6.5 billion and rising, little spare arable land remains for cultivation to increase the needed food supply. Moreover, new expectation from agriculture to supply our energy needs and meet the rising demand for meat and dairy products because of the increasing prosperity of the two most populous countries have placed additional pressure on agricultural resources. A few avenues available to satisfy these ever-growing needs are improving the crop yields in a sustainable manner and more efficient utilization of the agricultural output.

Cotton is grown for its fiber, however, for every kg of fiber, the plants also produce 1.65 kg of seed. In addition to 21% oil, cottonseed contains 23% protein that is of relatively good quality compared to that of other major oilseeds. Worldwide cotton cultivation yielded 23.5 million metric tons (MMT) of lint and over 40 MMT of seed in the year 2005. This amount of global cottonseed output could potentially provide the total protein requirements of almost half a billion people for a year (50 g/day rate) if the seed were safe for human consumption. Cereal grains serve as the staple food for majority of the people in developing countries. These cereal-based diets not only provide most of the calories but also are a source of most of their protein intake. However, cereals are a very poor source of protein. For example, rice contains about 7% while maize, millet, and wheat contain 9-10% protein (Bressani, 1965). Thus, protein deficiency is widespread in the developing countries. Cottonseed protein could play an important role in addressing this deficit.

Nutritional Value of Cottonseed in Feed and Food. During the1960s to 1980s, several investigators conducted extensive studies to evaluate the cottonseed meal (CSM) as a protein source for monogastric animals as well as food for human beings (Lusas and Jividen, 1987). These investigations used CSM either from glandless cottonseeds or from processed glanded cottonseeds (discussed later). Jonston and Watts (1961; 1964) examined feeding of chicks with glandless CSM and glanded CSM that had been processed in a certain manner. They concluded that processed glandless CSM was of equal nutritional value to soybean meal (SBM) in supporting chick growth. CSM from a glandless Acala variety was evaluated in broiler rations (Waldroup et al., 1968). Based on the results from this study, the authors summarized that glandless CSM can be used to replace part or all of SBM in practical diets for broilers. Roberson (1970) compared the effects of glandless CSM with SBM, supplemented with lysine and methionine, on the performance of laying hens and egg characteristics. The results from this study suggested that the protein of glandless CSM was about equal to SBM in sustaining the performance of the laying hens. A comparison of glandless CSM with regular glanded CSM was carried out using 5, 10, and 15% of each in the diet of laying hens for 336 days (Reid et al., 1984). They found that egg production rates with diets containing up to 10% glandless CSM were comparable to those of birds fed on a SBM based diet; egg mass output was not inhibited by any of the three dietary treatments. LaRue et al. (1985) conducted growth trials with 28-day-old, weaner pigs (7.5 kg) and growing-finishing pigs (19 to 97 kg). In these trials, glandless CSM was substituted in 20% increments for supplemental protein provided by SBM in corn-soybean based diets. Lysine was added to all glandless CSM diets to make them equal to the control corn-soybean diets. Pigs that were fed up to 40% supplemental glandless CSM protein showed similar performance to those on control, corn-soybean diet. The authors concluded that glandless CSM could be effectively used in the diets of starter, grower and finisher pigs when used in limited amounts with the addition of supplemental lysine.

Food grade cottonseed flour (CSF) was produced in the United States under the name Proflo* in the 1930s (Frank, 1987). Although no longer in use today, at the time it was used as an ingredient in bread, biscuits, crackers, and doughnuts in the U.S. and Canada without any adverse effects. In the early 1950s, a cooperative international institute (INCAP) was established by Central American countries and Panama to study nutrition-related problems in the area and to find practical solutions for them (Bressani, 1965; Frank, 1987; Lambou et al., 1966). This institute developed a product known as Incaparina containing 38% CSF as the principal source of protein. Human nutrition trials were conducted in many Central and Latin American countries and in India. In one of the trial conducted with 1-5 year-old children, Incaparina found favorable acceptability and no adverse effects even though CSF furnished 50 to 80% of the dietary protein (Lambou et al., 1966). Blends of CSF with millet flour were evaluated in a different set of trials in several West African countries. These blends were reported to provide adequate protein for normal as well as malnourished

infants and children (Alford et al., 1996; Frank, 1987). Clinical studies conducted at Texas Woman's University (TWU) showed that cottonseed protein provided in the form of CSM was safe and nutritious for children (Alford et al., 1996). The effects of cottonseed protein diets on the nitrogen balance in young, adult women were also investigated at TWU. Cottonseed protein in the form of liquid diet or when incorporated into baked products during 5-6 week-long studies maintained nitrogen balance with no change in nutritional status (Alford et al., 1996). Glandless CSF has also been evaluated as a substitute for wheat flour in bread, biscuits, cake, chapattis, noodles, spaghetti, etc. In addition, a program was initiated in 1968 by Oilseed Products Research Center at Texas A&M University to test roasted, salted glandless cottonseed as snack food under the name 'TAMUNUTS' (Lawhon et al., 1970). Consumer acceptance tests showed a favorable response for these nuts as snack food. Detailed accounts of various human nutrition studies conducted in different parts of the world have been provided in several reviews (Alford et al., 1996; Bressani, 1965; Frank, 1987; Lambou et al., 1966).

Presence of Gossypol Limits Efficient Utilization of Cottonseed. The protein content of CSF is 55-68% and its protein efficiency ratio is higher than that of a number of other major vegetable proteins. Nutrition studies with CSF showed that it compared favorably to other animal and vegetable sources of protein (Alford et al., 1996). However, full utilization of the nutrient-rich cottonseed for food is hampered by the presence of toxic gossypol that occurs naturally within the seed-glands. This cardio- and hepatotoxic terpenoid, present in the glands of all aerial parts of the plant, renders cottonseed unsafe for human and monogastric animal consumption (Risco and Chase, 1997). Unfortunately, this toxicity relegates an abundant agricultural resource to the ranks of feed for ruminant cattle either as whole seeds or as CSM following oil extraction. Because of the presence of the reactive aldehyde groups in gossypol, gossypol binds to the free ε-amino groups, such as lysine residues (Cater and Lyman, 1969; Jaroszewski, 1998; Lyman et al., 1959; Reiser and Fu, 1962). Older screw-press method of extracting oil involved a moist heating step that increased protein binding, thus converting free gossypol (toxic) to the bound (non-toxic) form (Frank, 1987). Although this process reduces the toxic form of gossypol in the cottonseed meal, it decreases protein solubility and lysine bioavailability. In fact, the toxicity of gossypol molecule itself is largely due to its property of binding to proteins. Thus, the presence of gossypol in CSM not only accounts for its toxicity, it also diminishes protein quality by chemical modifications. The current, solvent-based methods for oil extraction do not use the heating step and as a result, the amount of free gossypol in the CSM is substantially higher.

Mature, ruminant animals are able to detoxify gossypol in the rumen by binding it to soluble proteins or by dilution and reduced absorption (Reiser and Fu, 1962; Risco et al., 1992). Thus, these animals can tolerate a small level of cottonseed intake. Whole "gin-run" cottonseed is a good source of energy, protein, and fiber for lactating dairy cows. The apparent digestibility of cottonseed by dairy cows is well within an acceptable range of about 70% (Smith et. al., 1981). The advantages of including cottonseed in the diets of lactating dairy cows are well documented (Coppock et al., 1987). Even though cottonseed does find such indirect use in human nutrition in the form of feed for cattle, these ruminant animals are highly inefficient in terms of feed conversion. For example, the cow is rather wasteful at converting feed nutrients into edible milk components. Bernard and Calhoun (1997) reported that cows consuming diets containing daily intakes of 3.66 kg of crude protein produced only 0.83 kg of milk protein per day. Also, the feed:weight gain ratio (feed conversion ratio, FCR) for beef cattle is 5.8; thus, cattle must consume 5.8 kg of grain for each 1 kg of weight gain (Klopfenstein et al., 1991). For the non-ruminant pig, the feed:weight gain ratio is lower at 3.3 (Cromwell, 1991), while this ratio is even more favorable for fowl and farmed aquatic animals (Table 1). Fernandez (1987) evaluated the

use of CSM as a replacement for shrimp and fish meals in the diets fed to three species of shrimp. The results indicated that cottonseed meal protein is an acceptable source of protein for shrimp; however, the author concluded that "naturally occurring, growth-inhibiting substances" such as gossypol in cottonseed meal warrants caution while the use of meal from a low gossypol cotton variety could prove more acceptable. Thus, the ability to feed cottonseed to non-ruminant animals would provide a more efficient means of converting this nutritious resource into edible animal products. As discussed earlier, gossypol-free cottonseed can even be used directly as food to improve human nutrition, especially in poor countries that do grow substantial amounts of cotton and also suffer from problems of hunger and malnutrition. Thus, the potential of cottonseed in contributing to the food requirements of the burgeoning global population provides a great impetus for eliminating gossypol from cottonseed.

Classical Breeding Methods to Remove Gossypol from Cottonseed. Gossypol occurs in pigment glands in the foliage and the seed. Thus, the discovery of glandless trait in a mutant cotton variety (Hopi Moencopi) generated a great deal of excitement among the plant breeders as it provided them with a tool to eliminate glands and therefore gossypol from the seed (McMichael, 1954; 1959; 1960). Several national and international programs were launched to transfer this useful trait into commercial varieties to produce gossypol-free cottonseed (Lusas and Jividen, 1987; Miravalle, 1972). These programs provided cottonseed that could be fed to the more efficient feed-utilizing, monogastric animals and was even deemed safe for human consumption (Lusas and Jividen, 1987). Cottonseed compared favorably as a source of protein to other traditional food sources in several human nutritional studies (Lusas and Jividen, 1987).

Glandless cottonseed proved safe and nutritious, however, the glandless cotton varieties were a commercial failure. Under field conditions, glandless plants were more susceptible to several insect pests compared to their glanded counterparts (Bottger et al., 1964; Jenkins et al., 1966; Lukefahr et al., 1966; Maxwell et al., 1965). Gossypol and other biosynthetically-related terpenoids produced by the cotton plant have been shown to be toxic to various insect pests (Hedin et al., 1992; Lukefahr and Houghtaling, 1969; Lukefahr and Martin, 1966; Stipanovic et al., 1977; Stipanovic et al., 1978a; Stipanovic et al., 1978b). Because glandless cotton constitutively lacked glands, it was also devoid of the contents including the protective terpenoids and thus more susceptible to insect attack. In addition to being more vulnerable to traditional pests of cotton, the glandless plant was attacked by insect species that usually do not feed on cotton (Maxwell et al., 1965). Because of their weakened resistance to insects, the glandless cotton plants were not accepted by the farmers with the result that no large-scale planting of glandless cotton occurs today.

Although glandless cotton eventually was a commercial failure due to its weakened insect resistance, its availability and its potential as a source of food and feed created a great deal of excitement. As discussed earlier, several nutritional studies were launched that yielded important results related to the usefulness of CSM either as food or feed for the monogastric animals.

A few wild, diploid *Gossypium* species from Australia exhibit glandless-seed and glanded-plant phenotype (Brubaker et al., 1996). Plant breeders have been attempting to introgress this highly desirable trait into tetraploid cottons (Altman et al., 1987; Dilday, 1986; Kulkarni et al., 2002; Vroh Bi et al., 1999; Zhu et al., 2005). However, because of the genetic barriers imposed by genome differences between the wild species and commercially

important cottons, these efforts have had limited success (Townsend and Llewellyn, 2007). Consequently, the potential of cottonseed in contributing to human nutrition was not realized.

Physical and Chemical Means to Remove Gossypol from Cottonseed Products. As discussed earlier, human and animal nutrition studies utilized glandless CSM or CSM from processed glanded cotton. The glanded cottonseed can be processed by physical means such as the Air Classification Process (ACP) or Liquid Cyclone Process (LCP) to remove gossypol-bearing glands or by binding (heating, iron salts, etc.) most of the gossypol and leaving only a small amount of free (toxic) gossypol in the meal (Frank, 1987, Lusas and Jividen, 1987). Physical separation of the gossypol containing pigment glands was studied extensively during the late 1970s and early 1980s. Several patents have been issued on the production of edible cottonseed flour using physical/mechanical processing. These are: "Process for Producing Cottonseed Protein Concentrate" (3,615,657); "Process for Producing an Edible Cottonseed Protein Concentrate" (3,972,861); "Process for Treating Cottonseed Meats" (4,139,646); and "Process for Producing a Low Gossypol Protein Product from Glanded Cottonseed" (4,201,709). Gardner et al. (1973) reported that the LCP was capable of producing edible CSF that contained 0.04% or less free gossypol and more than 65% protein. The CSF produced by the LCP was approved as a food additive by the Food and Drug Administration on July 13, 1972, and a processing plant to produce deglanded, high-protein edible grade cottonseed flour began commercial production at Plains Cooperative Oil Mill, Lubbock, Texas, on August 15, 1973 (Gardner et al. 1973). This facility was unable to remain financially viable and ceased its operation within a short period of time.

The ACP was also designed to reduce the gossypol content of cottonseed flour by physically removing the pigment glands. It was developed as an improvement to the LCP technology. A cost analysis conducted by Decossas et al. (1982) showed that this process has merit and should have been financially viable. Unfortunately, the failure of the LCP commercial venture caused widespread skepticism in the cottonseed industry and the ACP has never been attempted on a commercial scale in the U.S.

Various chemical additives have been used over the years to bind gossypol, thereby reducing its toxicity and increasing the level of cottonseed meal usage in livestock rations. By far, the most commonly used substance is iron in the form of ferrous sulfate. Gossypol is bound by iron and its bioavailability is greatly reduced when iron is added to the diet of pigs and chickens at a weight ratio of 1:1 (iron:free gossypol). It is postulated that the iron:gossypol complex is strong enough to resist absorption in the intestinal tract and is excreted from the animal without being absorbed. Wedegaertner (1981) presents the steps to follow for the addition of iron to livestock diets. Unfortunately, many factors such as potential liability, the high fiber content of CSM, and low lysine availability have prevented widespread use of gossypol containing CSM in feeds for monogastric animals.

Biochemical and Molecular Aspects Related to Gossypol Synthesis. As mentioned above, cotton plants constitutively contain several sesquiterpenes, including gossypol in the sub-epidermal glands of the above-ground parts and in the root epidermal cells (Bell, 1986). Their presence in the aerial parts serves a protective function against various insect pests. In addition, many of these terpenoids are also induced in response to fungal or bacterial infection and serve as phytoalexins. Various cotton tissues that were induced by *Xanthomonas campestris* pv. *malvacearum* or *Verticillium dahliae* were used to isolate genes as well as study the enzymatic steps involved in gossypol biosynthesis.

A number of groups have been involved in deciphering the biochemical aspects related to synthesis of gossypol and associated terpenoids. Two of the early and critical steps are well characterized. In the 1990s, several reports were published showing that (+)-delta-cadinene synthase, a cyclase enzyme, catalyzes the conversion of farnesyl diphosphate to (+)-delta-cadinene, the first committed step in the biosynthesis of sesquiterpenoids, including gossypol (Benedict et al., 1995; Bianchini et al., 1999; Chen et al., 1995; Davis et al., 1996; Davis and Essenberg, 1995; Liu et al., 1999). Thus, the sesquiterpene skeleton, (+)-delta-cadinene, is the early enzymatic intermediate in the synthesis of sesquiterpenoids. The next step in the biosynthesis involves hydroxylation of (+)-delta-cadinene to 8-hydroxy-(+)-delta-cadinene that is catalyzed by (+)-delta-cadinene-8-hydroxylase, a cytochrome P450 mono-oxygenase (Luo et al., 2001). The remaining biochemical steps related to the formation of hemigossypol, gossypol, and heliocides have been outlined in several publications (Benedict et al., 2004; Benedict et al., 2006; Martin et al., 2003; Stipanovic, 1992; Wang et al., 2003).

(+)-delta-Cadinene is a critical intermediate in gossypol biosynthesis, therefore, the gene(s) encoding (+)-delta-cadinene synthase were important targets for molecular cloning. Chen et al., (1995) at Purdue University isolated and characterized two cDNA clones encoding (+)-delta-cadinene synthase from *V. dahliae*-induced *Gossypium arboreum* suspension cultures [*cad*1-C1 (U23206) and *cad*1-C14 (U23205) belonging to cdn1-C subfamily]. An additional cDNA clone (*cad*1-A, Y18484) belonging to subfamily cdn1-A was obtained by the same group in a similar way (Chen et al., 1996). Davis et al. (1998) isolated and sequenced a cDNA clone of a (+)-delta-cadinene synthase gene, *cdn*1 (U88318), from *X. campestris*-induced cotyledons of *G. hirsutum*. Sequence comparison suggests that this clone belongs to cdn1-C subfamily. Another member of the same subfamily was isolated from *V. dahliae*-induced *G. arboreum* suspension cultures by Meng et al. (1999). Expression analyses using RT-PCR for this *cad*1-C2 (Y16432) gene and the previously isolated genes *cad*1-C1, *cad*1-C14, *cdn*1, *cad*1-A were performed in developing seeds of *G. hirsutum*. A common set of primers were used for *cad*1-C1, *cad*1-C14, *cdn*1 and separate sets of primers were used each for *cad*1-C2 and *cad*1-A. With each of the three sets of primers, different levels of gene expression were observed in the developing cottonseeds from a glanded cultivar of *G. hirsutum*. Importantly, neither the transcripts nor the activity for (+)-delta-Cadinene synthase enzyme were detected in a glandless cultivar of *G. hirsutum*. The same group isolated a genomic clone, *cad*1-C3 (AF174294), from *G. arboreum* that is part of the cdn1-C subfamily (Tan et al., 2000). More recently, using previously published sequence information, Townsend et al. (2005) isolated a new clone (*cdn*1-C4, AF270425) from *G. hirsutum* developing embryo cDNA library. This clone was further used as a probe to screen a genomic library resulting in the identification of five different genomic clones. One of the clones named *cdn*1-C5 (AY800106) is the genomic counterpart of *cdn*1-C4. Another genomic clone (*cdn*1-D1, AY800107) had significant sequence differences from the other two subfamilies and was categorized to a new, cdn1-D subfamily while the remaining three clones turned out to be pseudogenes [*cdn*1-C6 (AY800006), *cdn*1-C7 (AY800007), and *cdn*1-C8 (AY800008)]. A different genomic clone (*cad*1-B) isolated by the Purdue group has been assigned to cdn1-B subfamily (X95323; Townsend et al., 2005). Thus, (+)-delta-cadinene synthase enzyme is encoded by a multigene family in cotton where individual members are expressed in a developmental manner and/or are induced as a result of pathogen infection.

The cDNA clone for (+)-delta-cadinene-8-hydroxylase enzyme that catalyzes the hydroxylation of (+)-delta-cadinene was isolated from *G. arboreum* by Luo et al. (2001). Recombinant enzyme produced from expression of this cDNA in yeast cells was shown to catalyze the hydroxylation of (+)-delta-cadinene to 8-hydroxy-(+)-delta-cadinene. Further characterization of this gene in *G. arboreum* showed that it is expressed during embryo

development and can be also induced by treating suspension cultures with *V. dahliae*. In the diploid genome of *G. arboreum*, Southern blotting detected a single copy of (+)-delta-cadinene-8-hydroxylase. Availability of the sequences for this and various members of (+)-delta-cadinene synthase gene family makes it possible to selectively silence target gene(s) to eliminate or substantially reduce gossypol from cottonseed.

Genetic Engineering of Cotton Plant to Reduce Gossypol in Cottonseed by Silencing the Genes Encoding (+)-delta-Cadinene Synthase. Blocking the branch-point step, involving the cyclization of farnesyl diphosphate to (+)-delta-cadinene, in a seed-specific manner offers the possibility to obtain seeds with reduced gossypol levels while maintaining wild-type levels of gossypol and other defensive terpenoids in the rest of the plant tissues. Attempts to use the antisense gene suppression mechanism targeting (+)-(delta)-cadinene synthase to eliminate gossypol from cottonseed were unsuccessful, or resulted in a small gossypol reduction, or provided ambiguous results (Martin et al., 2003; Sunilkumar et al., 2006; Townsend et al., 2005). It took a more powerful gene silencing mechanism and a strong, highly seed-specific promoter to achieve a meaningful reduction in seed-gossypol that had frustrated the efforts of many scientists around the globe for over a decade. Sunilkumar et al. (2006) used RNA interference (RNAi)-mediated suppression of the (+)-delta-cadinene synthase gene in conjunction with a cotton seed promoter to significantly reduce the (+)-delta-cadinene synthase activity in the seed which resulted in a seed-specific reduction of toxic gossypol.

To this end, a seed-specific, alpha-globulin promoter was isolated and characterized. Its efficacy, strength, and tissue specificity were examined using the reporter, beta-glucuronidase gene (Sunilkumar et al., 2002). This promoter becomes active at 15 days post-anthesis (dpa) in the developing cotton embryos. Initially, the activity is low and localized in the middle portion of the zygotic embryo. However, around 20 dpa, it begins to rise rapidly and spreads throughout the embryo. The promoter remains active until maturity. On the other hand, the (+)-delta-cadinene synthase transcripts are first detected at 23 dpa and reach steady-state level from 30 to 50 dpa (Martin et al., 2003). Thus, the alpha-globulin promoter is active for quite some time prior to the appearance of (+)-delta-cadinene synthase transcripts in the developing embryo and continues to be active until embryo maturity. This promoter was therefore used to generate hairpin transcripts targeting the (+)-delta-cadinene synthase gene(s) in cotton.

An efficient means to induce heritable, RNAi in plants is by stable integration and expression of a transgene construct encoding self-complementary transcripts that can fold back to generate double-stranded RNA molecules ('hairpin' RNA or hpRNA). The efficiency with which a target gene is silenced is significantly improved when the loop portion of these hpRNAs consists of a functional intron (ihpRNA; Smith et al., 2000; Wesley et al., 2001). To generate ihpRNA transcripts, a 604 bp sequence from a (+)-delta-cadinene synthase cDNA clone obtained from *G. hirsutum* was used as the trigger sequence to make the transformation construct. This sequence bore a high degree of homology to several previously published sequences of (+)-delta-cadinene synthase genes from both the diploid and tetraploid cottons and was expected to silence most members of the multigene family (Sunilkumar et al., 2006; Tan et al., 2000; Townsend et al., 2005). Following *Agrobacterium*-mediated transformation, several lines were obtained that produced seeds containing significantly reduced levels of gossypol (Sunilkumar et al., 2006). Analyses on individual seeds/developing embryos from some lines demonstrated that the reduced seed-gossypol phenotype was correlated with the presence of the transgene as well as with substantial reduction in the levels of the target transcripts. The low gossypol phenotype was clearly noticeable in the lighter-colored glands in transgenic seeds. Furthermore, using T2

generation developing embryos, we showed that both the transcript levels and the target enzyme activity were substantially reduced in the RNAi embryos. Most relevant, the levels of gossypol and related terpenoids involved in defense against insects and diseases were not lowered in the leaves, terminal buds, floral buds, bracts, petals, bolls, and roots of transgenic plants compared to the wild-type controls. Three different transgenic lines have been monitored to the T2 generation, and the reduced seed-gossypol trait was found to be stable and heritable. One of the lines produced seeds with gossypol levels as low as 0.2 µg/mg, showing approximately a 98% reduction in the level of the toxin. The United States Food and Drug Administration and United Nations Food and Agriculture Organization and World Health Organization permit up to 0.45 µg/mg (450 ppm) and 0.6 µg/mg (600 ppm), respectively, of free gossypol in edible cottonseed products (Lusas and Jividen, 1987). Thus, by using the tools of modern molecular biology, gossypol was reduced in cottonseed to a level considered safe for human consumption.

An intriguing property of RNAi is that it can spread from cell-to-cell and over a long-distance from its point of origin. This phenomenon initially observed in *C. elegans* is also believed to occur in plant systems. On the basis of these observations, doubts have been cast about the feasibility of achieving tissue- or cell-specific silencing of desired gene(s) in plants (Wang and Waterhouse, 2002). If the RNAi-induced silencing signal were to spread out of the developing embryos or if the components of the silencing mechanism remaining in the mature seed spread to the plant upon its germination, the seed (kernel)-specificity offered by the alpha-globulin promoter would be lost, and the plant would suffer from the same weakened resistance that was observed in the glandless cotton. However, the results presented in the report show strict confinement of the silencing to the seeds (Sunilkumar et al., 2006). Single seed analysis showed that null-segregant seeds that developed side-by-side with RNAi-silenced embryos in the boll of a T0 transgenic line had normal levels of gossypol. Thus, they were not affected by the silenced status of the adjacent embryos. In addition, the leaves, floral organs, and roots of transgenic plants, grown from the silenced seeds, exhibited wild-type levels of terpenoids. This observation suggests that spreading of the RNAi-induced silencing signal does not occur in cotton, and consequently, gossypol reduction remains confined to the cottonseed. The study also provided additional evidence showing that RNAi-mediated silencing signal targeting the expression of GFP in cotton does not spread and is limited to the tissues expressing the hairpin transcripts. These results suggest that RNAi-mediated gossypol reduction will remain confined to the developing embryo and the kernel in mature seed. Thus, the defensive capabilities offered to the remaining plant organs by gossypol and related terpenoids should not be compromised in the RNAi lines.

Conclusions. The availability of reduced-gossypol cottonseed potentially offers the possibility that the cotton plant, in addition to providing fiber, can also become a source of nutrition either directly as food in the form of seed/seed products or indirectly as feed for the more efficient feed-utilizing, monogastric animals. For developing countries where nearly 80% of the world's cotton is currently produced, the ability to feed cottonseed to non-ruminants would be extremely valuable in reducing protein malnutrition. As discussed earlier, this hinges on the feed:weight gain ratio, which is more favorable for non-ruminant animals as compared to ruminants. Farmers in these nations already know how to grow cotton and the infrastructure to process the fiber is in place. Transportation costs would be minimal since utilization of the seed as a feed source would be close to the site of production. Thus, the potential impact of this technology is huge, especially in poor, cotton-producing countries. Of course, the RNAi lines will have to withstand testing under the rigors of field conditions with the associated biotic stresses. In addition, the patent issues must be resolved and the transgenic lines will have to undergo the regulatory approval processes of various adopting countries before this technology becomes accessible.

Plant breeding has and will continue to help satisfy many of the demands of humanity for food, fiber, and fuel. The results from the RNAi study suggest that tools of modern molecular biology offer an alternative means to address the problems that are difficult or perhaps impossible to solve using the traditional breeding methods. This research also demonstrates that biotechnology, in addition to improving the productivity of the agricultural crops, can facilitate more efficient utilization of agricultural products by either modifying their nutritional composition or eliminating harmful compounds from the edible plant organs.

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Table 1. Feed Conversion Ratio (FCR) for various animals

Animal	FCR	Reference
Beef	5.8[a]	Klopfenstein et al., 1991
Pig	3.3[a]	Losinger, 1998; Cromwell, 1991
Duck	2.705[b]	Shalev and Pasternak, 1989
Turkey	2.102[b]	Shalev and Pasternak, 1989
Chicken	2.047[b]	Shalev and Pasternak, 1989
Shrimp	2.0[c]	Tacon, 2002
Tilapia	1.5[d]	McGinty and Rakocy, 1989; Boyd et al., 2005
Salmon	1.1[d]	Villamar, 2002
Channel Catfish	1.0[d]	Lovell, 1990

[a]Feed grain/weight gain ratio

[b]Total feed utilization of live weight for seven week-old broilers

[c]Dry aquafeed/weight gain ratio

[d]Feed/weight gain ratio