Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol

Ganesan Sunilkumar*, LeAnne M. Campbell*, Lorraine Puckhaber†, Robert D. Stipanovic‡, and Keerti S. Rathore*†§

*Institute for Plant Genomics and Biotechnology and †Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843; and ‡U.S. Department of Agriculture–Agricultural Research Station, Southern Plains Agricultural Research Center, College Station, TX 77845

Edited by Luis Herrera-Estrella, Center for Research and Advanced Studies, Guanajuato, Mexico, and approved October 4, 2006 (received for review June 27, 2006)

Global cottonseed production can potentially provide the protein requirements for half a billion people per year; however, it is woefully underutilized because of the presence of toxic gossypol within seed glands. Therefore, elimination of gossypol from cottonseed has been a long-standing goal of geneticists. Attempts were made to meet this objective by developing so-called “glandless cotton” in the 1950s by conventional breeding techniques; however, the glandless varieties were commercially unavailable 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 successfully used RNAi to disrupt gossypol biosynthesis in cottonseed tissue by interfering with the expression of the δ-cadinene synthase gene during seed development. We demonstrate that it is possible to significantly reduce cottonseed-gossypol levels in a stable and heritable manner. Results from enzyme activity and molecular analyses on developing transgenic embryos were consistent with the observed phenotype in the mature seeds. Most relevant, the levels of gossypol and related terpenoids in the foliage and floral parts were not diminished, and thus their potential function in plant defense against insects and diseases remained untouched. These results illustrate that a targeted genetic modification, applied to an underutilized agricultural byproduct, provides a mechanism to open up a new source of nutrition for hundreds of millions of people.


The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviation: dpa, days postanthesis.

© 2006 by The National Academy of Sciences of the USA
gossypol and other protective terpenoids. In this report, we provide evidence for spatial and temporal confinement of RNAi-mediated suppression of the δ-cadinene synthase gene in cottonseeds that contain the transgene. Our results clearly demonstrate the feasibility of a targeted RNAi-based approach to solve an age-old problem of cottonseed toxicity and provide an avenue to exploit the considerable quantities of protein and oil available in the global cottonseed output.

Results

Design of Silencing Vector and Screening for Low-Gossypol Lines. Although glandless cotton constitutively lacks δ-cadinene synthase activity in seed and foliage (20–22), all aspects of plant growth and development are normal. We therefore reasoned that disrupting the cadinane sesquiterpenoid biosynthesis exclusively in the seed at this point in the pathway would not have any inadvertent consequences. A 604-bp sequence from a δ-cadinene synthase cDNA clone obtained from a Gossypium hirsutum developing embryo library was chosen as the trigger sequence (Fig. 7, which is published as supporting information on the PNAS web site). The selected portion of the clone has 80.9–99.8% homology to several other published sequences of δ-cadinene synthase genes from the diploid (Gossypium arboreum) and tetraploid (G. hirsutum) cottons (refs. 23 and 24; see Table 1, which is published as supporting information on the PNAS web site). We expect this trigger sequence to target all members of the δ-cadinene synthase gene family, including Cad1-A, because it bears several stretches (20–35 bp) of perfect homology to the selected sequence. An intron-containing hairpin (ihp) transformation construct was made by using the pHANNIBAL/pART27 system (ref. 25; Fig. 8, which is published as supporting information on the PNAS web site). Importantly, the transcription of the ihpRNA sequence was under the control of a highly seed-specific α-globulin B gene promoter from cotton (26). Cotton (G. hirsutum, cv. Coker 312) was transformed by using the Agrobacterium tumefaciens method (27), and the transgenic T0 plants were grown to maturity in a greenhouse. A pooled sample of 30 T1 seeds from each of the 26 independent transgenic lines was analyzed by HPLC for gossypol (28), which is the predominant form of terpenoid in this tissue. Several of these lines produced seeds with significantly low levels of gossypol (Fig. 9, which is published as supporting information on the PNAS web site).

Transgenic Cottonseed Exhibits a Significant Reduction in Gossypol Level. Ten mature T1 seeds each from eight of these selfed T0 lines, which were regenerated from the first batch of transformation experiments, were individually analyzed for gossypol. Results from two of these lines (LCT66-2 and -32), along with 10 wild-type control seeds, are shown in Fig. 1A. All transgene-containing mature seeds, identified by PCR analysis, showed a dramatic and significant reduction in the level of gossypol. The cosegregation of the reduced seed-gossypol trait with the presence of the transgene was unambiguous. The null segregant seeds did not show any reduction in gossypol levels. Also, the low gossypol phenotype is clearly noticeable in lighter-colored and smaller-sized glands in the transgenic seeds (Fig. 1B). Compared with an average gossypol value of 10 μg/g in wild-type seeds, individual transgenic seeds showed values as low as 0.1 μg/g, a 99% reduction. Genomic DNA from three lines that were characterized more extensively in this study were subjected to Southern blot analysis, and the results show integration of the
transgene in their genomes (Fig. 10, which is published as supporting information on the PNAS web site).

**Presence of Hairpin RNA-Encoding Transgene and the Level of Target Message in the Developing T1 Embryo.** Activity of the target δ-cadinene synthase gene is expected to be high in the developing cotton embryos ∼35 days postanthesis (dpa; ref. 21). We conducted RT-PCR analysis to determine the levels of δ-cadinene synthase transcripts during this stage in a separate set of developing embryos from wild-type control plants and the two transgenic lines. The presence of the transgene in the embryos from the transgenic lines was independently confirmed by PCR. The results show clearly the suppression of δ-cadinene synthase gene transcripts in the transgene-containing embryos from the two RNAi lines (Fig. 1C). Importantly, the transcript levels in the null segregant embryos were similar to control values, suggesting that they remained unaffected by the neighboring embryos that were undergoing RNAi-induced silencing. Thus, the molecular data support and confirm results of the biochemical analysis presented earlier.

**The Levels of Gossypol and Other Protective Terpenoids Are Not Reduced in Foliage, Floral Organs, and Roots.** The terpenoid present in cottonseed is almost exclusively gossypol, whereas in the leaf, hemigossypolone, and heliocides, H1, H2, H3, and H4 occur together with gossypol. These compounds are derived from the same biosynthetic pathway (Fig. 6), and their presence and induction in the aerial parts protect the cotton plant from insects and diseases (6, 7). The leaves from transgenic and control plants were examined for the levels of these protective compounds. A different batch of 10 seeds from each of the transgenic lines and 10 wild-type control seeds was germinated and grown in soil in a greenhouse, and leaf tissue from each was analyzed for terpenoids (29). The levels of gossypol, hemigossypolone, and heliocides in the foliage of control and T1 transgenic plants are presented in Fig. 2. Transgene-bearing plants were identified by PCR analysis. The data show clearly that the presence of the transgene, which results in a significant reduction in gossypol in the seed, did not diminish gossypol and related terpenoids in the leaves. Moreover, levels of the other protective terpenoids, hemigossypolone, and the heliocides were not reduced in the leaves of transgenic plants.

In addition to the leaves, other tissues that are targeted by insects as well as roots were also examined for terpenoid levels. The levels of the protective terpenoids were not reduced in the terminal buds, bracts (epicalyx), floral buds, petals, bolls, and roots in the progeny from the RNAi transgenic lines compared with the values observed in the wild-type plants (Fig. 3). Taken together, the results show that the low-gossypol phenotype is seed-specific, and therefore the terpenoid-dependent defensive capabilities should not be compromised in the transgenic lines. Thus, by using modern molecular tools, we have overcome the major shortcoming of the glandless cotton previously developed by conventional breeding.

**Developing T2 Embryos from Transgenic Plants Show Significant Reductions in the Message for the Target Gene(s) and Target Enzyme Activity.** Homozygous T2 progeny from transgenic lines LCT66-2 and -32 and null segregant plants of the same generation were identified and grown in the greenhouse. Developing embryos (35 dpa) from these plants and wild-type control plants were examined for the δ-cadinene synthase transcripts and enzyme activities. The data show significant reductions for both, the target message and enzyme activity (Fig. 4), thus confirming the results of RT-PCR analyses presented earlier and lending support to the notion that the low-gossypol cottonseed phenotype is because of targeted knockdown of the δ-cadinene synthase gene.

**The Low-Gossypol Cottonseed Trait Is Stable and Successfully Transmitted to Progeny.** To confirm the stability of the transgenic trait, homozygous T2 progeny from transgenic lines LCT66-2 and -32 were grown to maturity in the greenhouse, and 50 individual T2 seeds obtained from these plants were analyzed for gossypol levels. The results from these analyses show clearly that the low-seed-gossypol trait is successfully inherited and stably maintained in both RNAi lines (Fig. 5). In addition to these two lines that were selected from the first batch of transformants, we identified more low-seed-gossypol lines that were recovered from the second batch of transformation experiments. T2 seeds from one of these new lines (LCT66-81) showed an average gossypol value of 0.19 ± 0.013 μg/mg (mean ± SEM; see Fig. 11, which is published as supporting information on the PNAS web site). The United Nations Food and Agriculture Organization and World Health Organization permit up to 0.6 ppm) free gossypol in edible cottonseed products (11). The levels of gossypol in the seeds from the RNAi lines fall within these safety limits.

**Discussion**

Extensive efforts in several laboratories over the last decade to eliminate gossypol from cottonseed by using the antisense method have proved unsuccessful (24), have resulted in a small reduction in seed gossypol (unpublished results from our laboratory), or have provided ambiguous results (30, 31). Here, we show that by using the RNAi approach coupled with a tissue-specific promoter, it is possible to significantly and selectively reduce the toxic terpenoid, gossypol, from cottonseed without...
diminishing the levels of this and related defensive terpenoids in parts of the plant usually attacked by insects. Comparative studies involving antisense and RNAi have shown that the silencing of the target gene by the latter method is more efficient and more pronounced (25, 32, 33). The differences in the underlying mechanisms involved in each case (34, 35) may explain the relative weakness of the antisense technology. Several lines of evidence suggest that RNAi-mediated silencing remains confined to the tissues that express the hairpin RNA-encoding transgene in cotton. The null segregant embryos that are developing within the same ovary as the transgene-bearing silenced embryos remain unaffected in their levels of the transcripts corresponding to the target gene (Fig. 1C). Furthermore, gossypol levels in the mature null segregant seeds were not reduced (Fig. 1 A and B). The results suggest that the silenced status of transgenic embryos does not spread to the neighboring null segregant embryos. The strict isolation of the reduced-gossypol trait in the seeds that are expressing the hairpin RNA-encoding transgene is further supported by results obtained from some unrelated research conducted in our laboratory that involved the RNAi-mediated silencing of GFP in cotton (Fig. 12, which is published as supporting information on the PNAS web site). In these lines, the null segregant seeds that grew within the silenced maternal tissue among silenced embryos continued to exhibit green fluorescence. This observation suggests that individual embryos develop in seclusion and are not influenced by the RNAi-induced silenced status of the neighboring embryos or even the maternal tissue. The absence of direct vascular and plasmodesmal connections between a developing embryo and the maternal tissue may account for the strict isolation of this new sporophyte (36–39). Taken together, our results suggest that the silencing signal from the developing δ-cadinene synthase-suppressed cotton embryo is unlikely to spread and reduce the levels of terpenoids in nontarget tissues, such as the foliage, roots, etc. As mentioned earlier, another possibility that can result in an undesirable phenotype is that, once initiated in the developing seed, the silenced state will persist and spread throughout the plant after germination. However, the fact that the vegetative and floral tissues from the plants that originate from the silenced seeds do not show any reductions in terpenoid levels (Figs. 2 and 3) suggests that the RNAi-mediated silencing phenomenon is developmentally confined. It is possible that the double-stranded RNA and small-interfering RNA components, generated during the development of transgenic embryo, no longer survive in the mature seed and, if they do, silencing does not spread from its point of origin in cotton. To directly determine whether cotton plants exhibit RNAi spreading, a different set of experiments involving reciprocal grafting between GFP-expressing plants and GFP-suppressed RNAi plants were conducted. We did not observe the transmission of the GFP-silencing signal across the graft junction in any of these grafts (Fig. 13, which is published as supporting information on the PNAS web site). The results suggest that the RNAi-mediated silencing signal against GFP does not propagate systemically in cotton. It is, therefore, possible that the strict tissue specificity of the low-seed-gossypol trait observed in cotton may, in part, be due to the fact that silencing does not spread in cotton tissues. A similar tissue-specific confinement of silencing has been observed in Arabidopsis and oilseed rape in experiments involving conversion of petals into sepals through RNAi (40). A lack of systemic silencing or a highly restricted spread of silencing has also been noted in several other plant systems (41, 42). Taken together, these results suggest that, although systemic silencing can occur in some plants in some specific situations (15–18), RNAi is not always associated with spreading.

The results described herein demonstrate that targeted gene silencing can be used to modulate biosynthetic pathways in a specific tissue to obtain a desired phenotype that is not possible by traditional breeding. Gossypol values in the seeds from some of the lines are well below the limit deemed safe for human consumption by United Nations Food and Agriculture Organi-
serve as an important source of nutrition-rich food if it were not for the presence of the neurotoxin β-N-oxalylamino-L-alanine (43). Beans from this so-called “famine crop” are regularly consumed by poor people in many Asian countries and parts of Africa who, as a result, suffer from a form of spastic paralysis, lathyrism. In addition, traditional foods such as cassava and fava beans could also be made safer for consumption by eliminating cyanogenic and fava glycosides, respectively (43, 44). Thus, an approach based on the removal of naturally occurring toxic compounds from the edible portion of the plant not only improves food safety but also provides an additional and potentially extraordinary means to meet the nutritional requirements of the growing world population without having to increase either crop yields or acreage planted.

Materials and Methods

Hairpin RNA Construct and Cotton Transformation. A clone of the δ-cadinene synthase gene was obtained by probing a cDNA library prepared from staged-embryo mRNA from G. hirsutum (cv. Coker 312) with the G. arboreum cad1-C1 (XC1) gene. Sequencing confirmed that our clone belonged to the δ-cadinene synthase C subfamily. A 604-bp-long internal fragment amplified from cDNA clone was used as the trigger sequence (Fig. 7). This sequence was used to make an intron-containing hairpin (ihp) construct with the pHANNIBAL/pART27 system (25). The seed-specific promoter from the cotton α-globulin B gene (26) was used to control the expression of the ihpRNA sequence. The final hairpin vector pAGP-iHP-dCS (Fig. 8), which harbors nptII as the plant-selectable marker gene, was introduced into Agrobacterium strain LBA4404, which was then used to transform G. hirsutum cv. Coker 312 as described (27).

Determination of Gossypol and Related Terpenoids. Levels of gossypol and related terpenoids in cottonseed and other tissues were determined by using HPLC-based methods, as described (28, 29). The kernel from individual mature cottonseed (dry weight ranged from 70 to 95 mg) was ground to a fine powder by using agate mortar and pestle. Approximately 20 mg of kernel powder from each seed was saved for DNA extraction. The remaining portion was weighed and mixed with 5 ml of solvent-containing ethanol:ether:water:glacial acetic acid (59:17:24:0.2) by vortexing. The suspension was vortexed every 10 min for the next 1-h incubation at room temperature. The sample was then centrifuged for 5 min at 2,800 × g. A 50-μl fraction of the extract was analyzed on a Hewlett-Packard (Palo Alto, CA) 1090 liquid chromatograph, as described (28). A fully expanded third leaf from either a wild-type or each of the 10 T1 plants from the two

Fig. 4. δ-Cadinene synthase transcripts and enzyme activity are significantly reduced in developing embryos from the RNAi lines. Separate sets of embryos (35 dpa) isolated from wild-type plants, null segregant plants, and homozygous T1 plants from lines LCT66-2 and -32 were used for each type of analysis. (Top) The hybridization band (dCS) on a Northern blot; (Middle) ethidium bromide-stained RNA gel before blotting; (Bottom) δ-cadinene synthase activities. The enzyme activity is presented as total ion peak area of δ-cadinene generated min⁻¹ mg⁻¹ embryo. Enzyme activity results are mean (±SEM) of values obtained from three separate sets of embryo samples from each type of plant. *, The value for the transgenic line is significantly different from the control (wild-type and null segregant) value at \( P < 0.004 \).

Fig. 5. The low-seed-gossypol trait is successfully transmitted to T2-generation seeds in the transgenic RNAi lines. Gossypol levels in 10 individual seeds each from wild-type control plant and a null segregant plant and 50 individual T2 seeds each from homozygous T1 plants that were derived from their respective parental transgenic lines, LCT66-2 and -32. Mean (±SEM) gossypol values for control (n = 10) and transgenic seeds (n = 50) are shown with the respective graphs. *, The value for the transgenic line is significantly different from the control (wild-type and null segregant) value at \( P < 0.001 \).
RNAi transgenic lines was used for terpenoid aldehyde analysis. Terminal bud, floral bud (5–7 mm diameter), petals (0 dpa), bracts (0 dpa), boll (1 dpa), and root tissues were collected from three replicate PCR-positive transgenic T1 plants each from lines LCT66-2 and -32. Corresponding tissues collected from three wild-type plants, grown under the same conditions at the same time as the T1 transplants in the greenhouse, served as controls. The tissue samples were dried in a lyophilizer and ground to a fine powder. The powder (dry weight ranged from 50 to 100 mg) was extracted with 5 ml of solvent containing acetonitrile:water:phosphoric acid (80:20:0.1) by ultrasonification for 3 min. The sample was centrifuged for 5 min at 2,800 g.

50 to 100 mg was extracted with 5 ml of solvent containing acetonitrile:water:phosphoric acid (80:20:0.1) by ultrasonification for 3 min. The sample was centrifuged for 5 min at 2,800 g.

The sample was centrifuged for 5 min at 2,800 g. The supernatant was then filtered through 0.22 µm filters and used for the analysis.

Molecular and Enzymatic Analyses. The protocols used for total RNA extraction, RT-PCR, Northern analysis, genomic DNA isolation, PCR, Southern analysis, and enzyme assays are described in Supporting Text, which is published as supporting information on the PNAS web site.

We thank Dr. Jinggao Liu (U.S. Department of Agriculture–Agricultural Research Station, Southern Plains Agricultural Research Center) for providing the deuterated (1RS)-[1-2H]-farnesyl diphosphate (FDP) samples and for valuable advice on the enzyme assays. We thank Dr. Robert Creelman for screening the cDNA library to isolate the δ-cadinene synthase clone and John Landua for performing some of the grafting experiments. This research was supported by funds from the Texas Cotton Biotechnology Initiative (TxCOT), Cotton Inc., and Texas Agriculture Experiment Station.
atgcgcgagaa cgacctctac accacatccc ttgattccg attactccga gagcatggat
tcaatgttttc atgcgagcta ttcaacaagt ttaagacgta gcaagggaat ttcaagtcgt
ccgtagcaag cgatgttcca ggatgttgg aacctttaa cgctttctat ttgaggttcc
atgggaaga tatattgtat gaagcaaaat ttttcaccac caaccatatta agctttgcag
tagcatctttt ggactatccg ttatccgaag aagtttcaac tggcttgaaa caatcaattc
gaagaggttt gccaaggttt gaggcagac actatctttc agtataccaa gatattgagt
cccataataa gtttttgttg gagtgttctg aagtgcattt caacatgta caacatttgc
taggaagaag ccataagtga atttctagggt ggtggaagga tttagagctt ccaaggaagt
tgccatacgc aagagataga ggtggtgag actatattttg gatctcaggga gtgtactttg
agcccaataa ttctctctgt gaagagatgt tgacaaaggt gtagcaattg gcttctatttg
taga
Individual transgenic lines (LCT66)
Probe: nptII

Probe: OCS terminator
A. T2 seeds from line LCT66-81 (T2)

B. Seeds from wild-type plant
A. CaMV 35S promoter::GFP-RNAi

B. Wild-type

C. CaMV 35S promoter::GFP
A. (RNAi stock; GFP scion)

B. (GFP stock; RNAi scion)