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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 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 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.

food safety | gene silencing | RNAi | seed-specific promoter | terpenoids

Cotton has been cultivated for its fiber for >7,000 years. Despite the availability of synthetic alternatives, it continues to serve as the most important source of fiber for textiles. Cotton is grown in >80 countries and is a cash crop for >20 million farmers in developing countries in Asia and Africa, where malnutrition and starvation are rampant (1). An attribute of cotton not widely recognized is that for every 1 kg of fiber, the plant produces ≈1.65 kg of seed. This makes cotton the third largest field crop in terms of edible oilseed tonnage in the world. In addition to 21% oil, cottonseed is a source of relatively high-quality protein (23%). However, the ability to use this nutrient-rich resource for food is hampered by the presence of toxic gossypol that is unique to the tribe Gossypieae. This cardio- and hepatotoxic terpenoid, present in the glands, renders cottonseed unsafe for human and monogastric animal consumption (2). Unfortunately, this toxicity subjugates this abundant agricultural resource to the ranks of a feed for ruminant animals either as whole seeds or as meal after oil extraction. In fact, the 44 million metric tons (MT) of cottonseed (9.4 million MT of available protein) produced each year could provide the total protein requirements of half a billion people for 1 year (50 g/day rate) if the seed were safe for human consumption. Thus, gossypol-free cottonseed would significantly contribute to human nutrition and health, particularly in developing countries (3–5), and would help meet the requirements of the predicted 50% increase in the world population in the next 50 years.

Gossypol and related terpenoids are present throughout the cotton plant in the glands of foliage, floral organs, and bolls, as well as in the roots. In addition, these terpenoids are induced in response to microbial infections. These compounds protect the plant from both insects and pathogens (6, 7). After the discovery of a glandless mutant (8), several breeding programs were launched in the U.S., Africa, and Asia to transfer the glandless trait into commercial varieties to produce gossypol-free cottonseed (9–11). These programs provided cottonseed that could be fed to monogastric animals that use feed more efficiently and was even deemed safe for human consumption (5, 11). Cottonseed compared favorably as a source of protein to other traditional food sources in several human nutrition studies (3, 5, 11). However, these glandless cotton varieties were a commercial failure. Under field conditions, glandless plants were extraordinarily susceptible to attack by a host of insect pests, because they constitutively lacked protective terpenoids (12, 13) and were, therefore, rejected by farmers. Thus, the potential of cottonseed in contributing to human nutrition remains unfulfilled.

Gossypol and other sesquiterpenoids are derived from (+)-δ-cadinene. The enzyme δ-cadinene synthase catalyzes the first committed step involving the cyclization of farnesyl diphosphate to (+)-δ-cadinene (Fig. 6, which is published as supporting information on the PNAS web site). Thus, tissue-specific RNAi of δ-cadinene synthase expression to disrupt terpenoid biosynthesis offers a possible mechanism to eliminate gossypol from the seed while retaining a full complement of this and related terpenoids in the rest of the plant for maintaining its defensive capabilities against insects and diseases. However, in *Caenorhabditis elegans*, some insect species, and flatworm, the RNAi-mediated silencing is known to spread systemically (14). RNAi-(posttranscriptional gene silencing)-mediated systemic silencing of certain target genes has also been reported in plants (15–19). If such a systemic propagation from its point of origin (i.e., RNAi construct-expressing developing embryo) occurred in the RNAi transformants, the silencing of the target gene homologs in the foliage and floral tissues could reduce the levels of protective terpenoids in these nontarget organs of the cotton plant. Another possibility exists, in that once the ''components'' of the silencing mechanism are generated in the developing embryo, they will persist and, after seed germination, will spread and cause silencing in the resulting plant. Either scenario will result in an undesirable phenotype that will suffer from the same weakness as the glandless cotton, i.e., systemic reduction of

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Abbreviation: dpa, days postanthesis.

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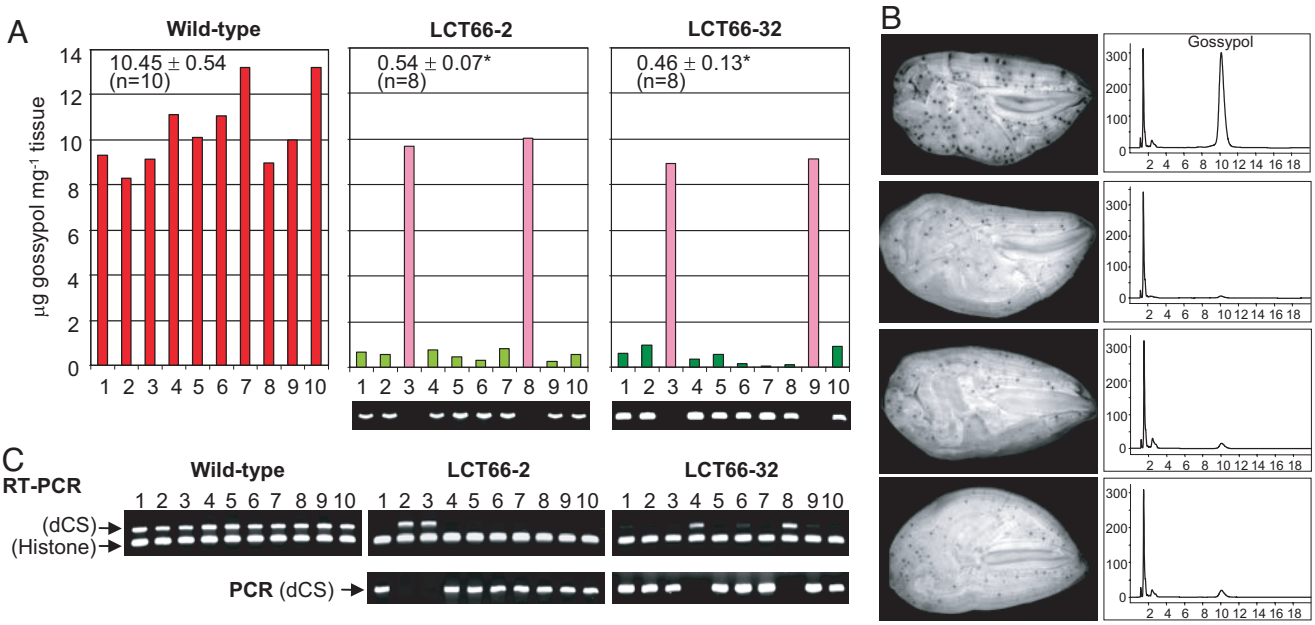

Fig. 1. Reductions in gossypol levels and target transcripts in the transgenic cottonseeds and developing embryos, respectively, from two RNAi lines. (*A*) Gossypol levels in 10 individual mature seeds each from wild-type control plants (red) and two independent RNAi transgenic lines, LCT66-2 (light green) and LCT66-32 (dark green). The results from PCR analysis on DNA from the same individual seeds from RNAi lines are depicted under the respective graphs. Note that the gossypol levels in the null segregant seeds (pink) are similar to control values. Mean (±SEM) gossypol values for control ($n = 10$) and the transgene-bearing seeds ($n = 8$) from each of the transgenic lines are shown with the respective graphs. *, The value for the transgenic line is significantly different from wild-type control value at $P < 0.001$. (*B*) Photomicrographs of sections of four mature T_1 seeds obtained from the transgenic line LCT66-32 (*Left*). The seed at the top was a null segregant, whereas the others were transgenic seeds. HPLC chromatograms (*Right*) show the gossypol levels in the extracts from the same four seeds. *y* axis, absorbance at 272 nm; *x* axis, elution time (min). Note the correlation between visible phenotype and gossypol level in the seed. (*C*) RT-PCR analysis of δ-cadinene synthase (dCS) expression in a separate set of 10 individual, developing embryos (35 dpa) each from a wild-type control plant and the two RNAi transgenic lines. Transcripts from histone 3 gene of cotton were amplified as internal controls in the duplex RT-PCR analyses. The results from PCR analysis on DNA from the same individual embryos from the RNAi lines are also shown to illustrate a correlation between reduced dCS transcripts and presence of the transgene.

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 *Cad*1-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 T_0 plants were grown to maturity in a greenhouse. A pooled sample of 30 T_1 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 T_1 seeds each from eight of these selfed T_0 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. 1*A*. 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. 1*B*). Compared with an average gossypol value of 10 μg/mg in wild-type seeds, individual transgenic seeds showed values as low as 0.1 μg/mg, 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 T_1 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. 1*C*). 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, H_1, H_2, H_3, and H_4 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 T_1 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 T_2 Embryos from Transgenic Plants Show Significant Reductions in the Message for the Target Gene(s) and Target Enzyme Activity. Homozygous T_1 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.

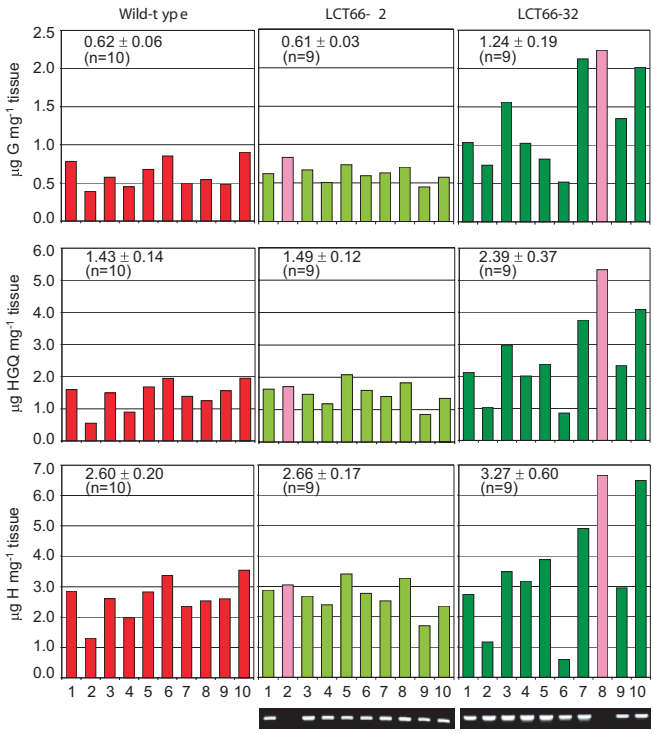

Fig. 2. The levels of gossypol and related terpenoids in the leaves of transgenic progeny from RNAi lines are not reduced. The levels of gossypol (G), hemigossypolone (HGQ), and total heliocides (H) in leaf tissues from 10 individual wild-type control plants and the T_1 progeny of the two RNAi transgenic lines. The results from PCR analysis on DNA from the same individual progeny plants from the RNAi lines are depicted under the respective graphs. Mean (±SEM) values for terpenoid levels in the leaf tissue of control plants (n = 10) and the transgene-bearing T_1 plants (n = 9) from each of the transgenic lines are shown with the respective graphs. The key to bar colors is consistent with Fig. 1*A*.

The Low-Gossypol Cottonseed Trait Is Stable and Successfully Transmitted to Progeny. To confirm the stability of the transgenic trait, homozygous T_1 progeny from transgenic lines LCT66-2 and -32 were grown to maturity in the greenhouse, and 50 individual T_2 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. T_2 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 μg/mg (600 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

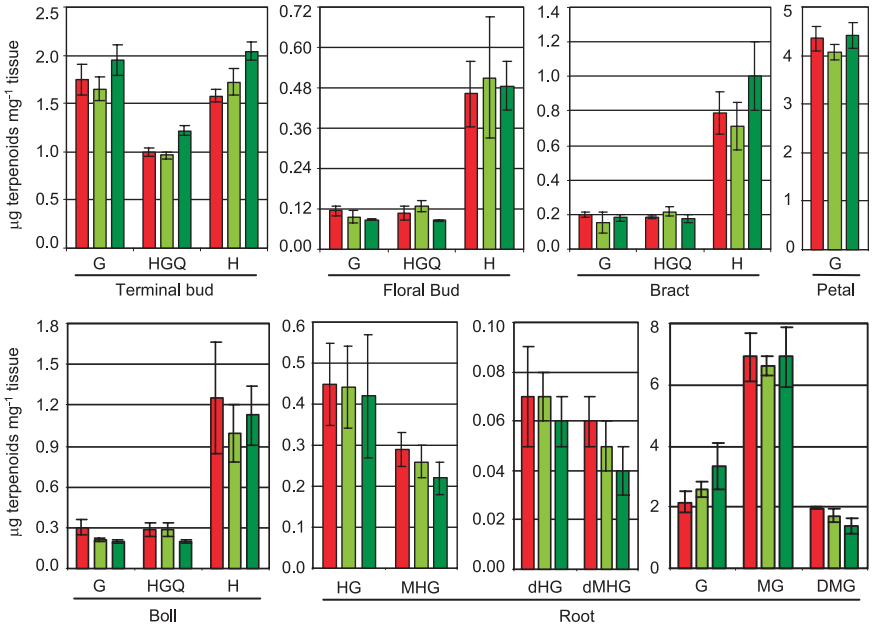

Fig. 3. The levels of gossypol and related terpenoids in terminal buds, bracts, floral organs, bolls, and roots of transgenic progeny from RNAi lines are not reduced. The levels of terpenoids in various organs of wild-type control plants (red), T_1 transgenic progeny from RNAi line LCT66-2 (light green), and T_1 transgenic progeny from RNAi line LCT66-32 (dark green). The results shown are mean (±SEM) terpenoid values in tissue samples taken from three individual plants in each category. Note that in petals, gossypol was the only terpenoid detected and in the root tissue, the terpenoids detected were: gossypol (G), gossypol-6-methyl ether (MG), gossypol-6,6′-dimethyl ether (DMG), hemigossypol (HG), desoxyhemigossypol (dHG), hemigossypol-6-methyl ether (MHG), and desoxyhemigossypol-6,6′-methyl ether (dMHG).

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. 1*C*). 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 plasmodesmatal 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-

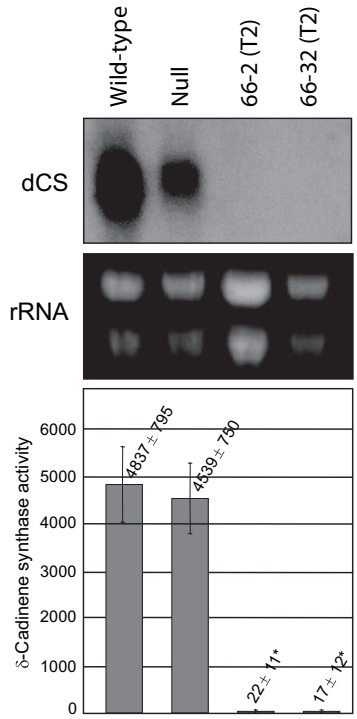

Fig. 4. δ-Cadinene synthase transcripts and enzyme activity are significantly reduced in developing embryos from the RNAi lines. Separate sets of embryos (35 dpa) isolated from wild-type plants, null segregant plants, and homozygous T_1 plants from lines LCT66-2 and -32 were used for each type of analysis. (*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 $min^{-1} \cdot mg^{-1}$ 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 $P < 0.004$.

zation and World Health Organization. Thus, cotton, which has served the clothing needs of humanity for millennia, has the potential to make a significant contribution to its nutritional requirements. This research opens up a new frontier in the use of genetic manipulation to enhance global food supply. It raises the possibility of using a similar approach to eliminate harmful compounds from other potential food sources, such as *Lathyrus sativus*, a hardy tropical/subtropical legume plant that could 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 paraparesis, 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 cad*1-C1 (XC1) gene. Sequencing confirmed that our clone belonged to the δ-cadinene synthase C subfamily. A 604-bp-long internal fragment amplified from our 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 *npt*II 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 T_1 plants from the two

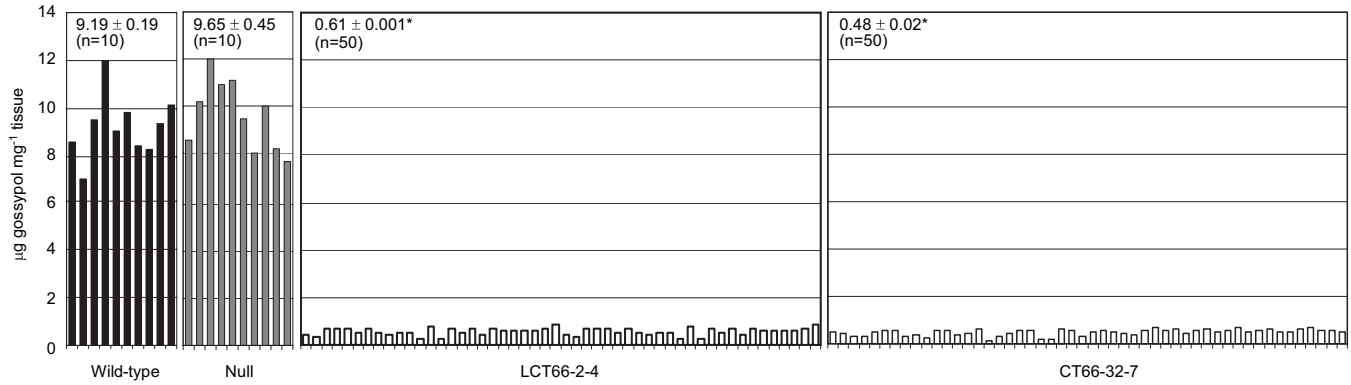

Fig. 5. The low-seed-gossypol trait is successfully transmitted to T_2-generation seeds in the transgenic RNAi lines. Gossypol levels in 10 individual seeds each from wild-type control plant and a null segregant plant and 50 individual T_2 seeds each from homozygous T_1 plants that were derived from 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 T_1 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 T_1 transformants 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*. A 50-μl fraction of the extract was analyzed on HPLC, as described earlier.

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.

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Table 1. Homology of the 604 bp dCS trigger sequence to various isoforms of δ-cadinene synthase gene from cotton

δ-cadinene synthase gene	Genbank accession no.	Plant source	Homology with the trigger sequence (%)
Cad1-C14 (XC14)	U23205	*G. arboreum*	99.8
Cdn1-C4	AF270425	*G. hirsutum*	98.8
Cdn1	U88318	*G. hirsutum*	98.5
Cad1-C2	Y16432	*G. arboreum*	96.4
Cad1-C3	AF174294	*G. arboreum*	96.2
Cad1-C1 (XC1)	U23206	*G. arboreum*	96.0
Cad1-B	X95323	*G. arboreum*	92.9
Cdn1-D1	AY800107	*G. hirsutum*	90.9
Cad1-A	X96429	*G. arboreum*	80.9

Supporting Text

RNA Isolation. RNA was isolated from one-half of the 35 days postanthesis (dpa) embryo that was stored in RNAlater solution. The embryo was ground in 550 μl of RNA isolation buffer (4 M guanidine isothiocyanate, 30 mM disodium citrate (Ambion; catalogue no. 7020; 30 mM 2-mercaptoethanol) with Proteinase K (1.5 mg per sample) using mortar and pestle. The extract was then processed using the RNeasy Plant Mini Kit (Qiagen, Cat no. 74904) for RNA isolation.

Duplex RT-PCR Analysis. Total RNA (400 ng) was reverse-transcribed with oligo (dT) primers using the Taqman Reverse Transcription Reagents (Applied Biosystems, Cat no. N808-0234) in a 10-μl reaction. The reaction conditions were per the manufacturer's instructions. Two microliters of the synthesized first-strand cDNA was used for PCR amplification of δ-cadinene synthase cDNA and an internal control, histone 3 (GenBank accession no. AF024716) cDNA in the same reaction. The following primers were used: dCS1: 5'-ATG CCG AGA ACG ACC TCT ACA-3'; dCS2: 5'-ACT TTT GTC AAC ATC TTT CTA CCA AG-3'; His3F: 5'-GAA GCC TCA TCG ATA CCG TC-3'; His3R: 5'-CTA CCA CTA CCA TCA TGG C-3'. The PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. Primers dCS1/dCS2 amplify a 580-bp fragment from δ-cadinene synthase cDNA. Primers His3F/His3R amplify a 412-bp fragment from histone 3 cDNA. PCR products were analyzed by gel electrophoresis on a 1.6% agarose gel in TBE buffer.

Northern Hybridization Analysis. Total RNA was extracted from five pooled 35-dpa T_2 embryos. Denatured total RNA (18 μg) was separated by electrophoresis on a 1.5% agarose gel containing formaldehyde and transferred onto Hybond N^+ (Amersham Pharmacia Cat No. RPN303B) membrane as described by Sambrook and Russell (1). Radio-labeled (^{32}P dCTP) 416-bp DNA fragment PCR amplified from the 3' end of δ-cadinene synthase by using the primers: 5'-CAT AGG AGA GAA GAC GAT TGC TCA GC-3' and 5'-GGA AAT GAA TAC AAA GAC AG-3' was used as a probe. Hybridization was performed at 60°C for 16 h in a solution containing 0.5 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 7% SDS, and 1% BSA. Blots were washed for 10 min at room temperature with 2 × SSC and 0.1% SDS solution followed by two washes at 60°C for 10 min each with 0.5 × SSC and 0.1% SDS solution.

DNA Isolation from Immature Embryo and Mature Cottonseed Kernel. Developing embryos were collected from wild-type and T_0 transgenic plants from the greenhouse at 35 dpa, sliced along the axis into two halves, and stored in RNAlater solution at -80°C. One-half was used for DNA isolation, whereas the other half was saved for RNA isolation. The immature embryo half or ≈20 mg of the kernel powder from mature seed was transferred to a 1.5-ml microfuge tube and further ground with a pellet pestle (Fischer Scientific Cat no. K749520-0000) in 350 μl of extraction buffer [200 mM Tris-HCl (pH 8.0), 25 mM EDTA, 250 mM NaCl, 0.5% SDS]. An additional 350 μl of extraction buffer was added to the tube, and the sample was mixed well. This mixture was then centrifuged at 13,000 rpm (Biofuge pico, Heraeus) at room temperature for 5

min. The supernatant was transferred to a fresh tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) followed by centrifugation at 5,000 rpm. After an additional extraction with chloroform:isoamyl alcohol (24:1), the DNA from the aqueous phase was precipitated with equal volume of cold isopropanol. The DNA precipitate was lifted out with a Pasteur pipette and transferred to a fresh microfuge tube containing 1 ml of 70% ethanol. After centrifugation at 13,000 rpm for 5 min, the pellet obtained was air-dried and dissolved in 0.1 × TE buffer. The sample was treated with DNase-free RNase at a final concentration of 20 μg/ml for 15 min at 37°C. The DNA was then precipitated with 1/10 the volume of 3 M sodium acetate, pH 5.2, and two volumes of 100% ethanol and centrifuged at 13,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, air-dried, and dissolved in water.

DNA Isolation from Cotton Leaf. Approximately 200 mg of leaf tissue from a newly opened cotton leaf was ground in 500 μl of extraction buffer [0.35 M glucose, 0.1 M Tris-HCl (pH 8.0), 0.005 M EDTA, 2% PVP-40; just before use, the following were added to a final concentration of: 1 mg/ml ascorbic acid and 2 μl/ml 2-mercaptoethanol] in a microfuge tube using a pellet pestle. The sample was centrifuged at 13,000 rpm at 4°C for 20 min. The pellet was resuspended in 400 μl of lysis buffer (0.14 M sorbitol, 0.22 M Tris-HCl (pH 8.0), 0.8 M NaCl, 0.22 M EDTA, 1% PVP-40; just before use, the following were added to a final concentration of: 0.8% CTAB, 1 mg/ml ascorbic acid, 2 μl/ml 2-mercaptoethanol, 10 mg/ml *N*-lauroylsarcosine, and 5 μg/ml Proteinase K) and incubated at 65°C for 30 min with gentle mixing every 10 min. Chloroform:isoamyl alcohol (480 μl; 24:1) was mixed thoroughly with the lysate followed by centrifugation at 5,000 rpm at room temperature for 20 min. The upper aqueous phase was transferred to a fresh microfuge tube, and the DNA was precipitated with an equal volume of cold isopropanol. The DNA precipitate was lifted out with a Pasteur pipette, transferred to a fresh microfuge tube, and washed with 1 ml of 70% ethanol. After centrifugation at 13,000 rpm for 5 min, the pellet obtained was air-dried, dissolved in 500 μl of 0.1 × TE, and treated with DNase-free RNase at a final concentration of 20 μg/ml for 15 min at 37°C. The DNA was then precipitated with 1/10 the volume of 3 M sodium acetate (pH 5.2) and two volumes of 100% ethanol and centrifuged at 13,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, air-dried, and dissolved in water.

PCR Analysis to Detect the Intron-Containing Hairpin (ihp)-dCS Transgene. Genomic DNA (100 ng) from mature seed, immature embryo, or leaf tissue was used for PCR analysis. The following primers were used: dCS3: 5'-TCT ACA ATA GAA GCC ATT GC-3'; OCS: 5'-GCG ATC ATA GGC GTC TCG-3'. The PCR conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. The OCS/dCS3 primers amplify a 653-bp fragment from the genomic DNA from transgenic tissues. PCR products were analyzed by gel electrophoresis on a 1.2% agarose gel in TBE buffer.

Southern Hybridization Analysis. Fifteen micrograms of genomic DNA, isolated following the protocol described by Chaudhry *et al.* (2), was digested with EcoRI and separated on 1% agarose gel in TAE buffer. Blotting was carried out as described by Sambrook and Russell (1). DNA fragments specific to the *npt*II gene or octopine synthase

terminator were used as probes. Labeling, hybridization, and posthybridization washing conditions were same as for Northern hybridization analysis.

δ-Cadinene Synthase Enzyme Assay. Enzyme extract was prepared by grinding 1 g of 35-dpa embryos frozen in liquid nitrogen following the procedure described by Martin *et al.* (3). The enzyme assay was performed in a 300-μl reaction mixture containing 255 μl of enzyme extract, 27 mM potassium fluoride, 1 mM magnesium chloride, 200 μM (1*RS*)-[1-^{2}H]-(*E,E*)-farnesyl diphosphate (FDP) at 30°C for 20 min. The reaction mix was then extracted with 300 μl of hexane:ethyl acetate (3:1). One microliter of the organic phase was analyzed for deuterated δ-cadinene by a GC-MS instrument fitted with an AGE BP1 (25 × 0.25-mm) column. The sample was run in a splitless mode with an injector temperature of 250°C. The initial temperature of the instrument was 40°C, and the temperature was increased at a rate of 10°C min^{-1} until 180°C, followed by 20°C

min^{-1} up to 270°C (1-min hold). The flow of helium was constant at 1 ml min^{-1}. The area of the peak (total ions) corresponding to δ-cadinene was used as a measure of enzyme activity.

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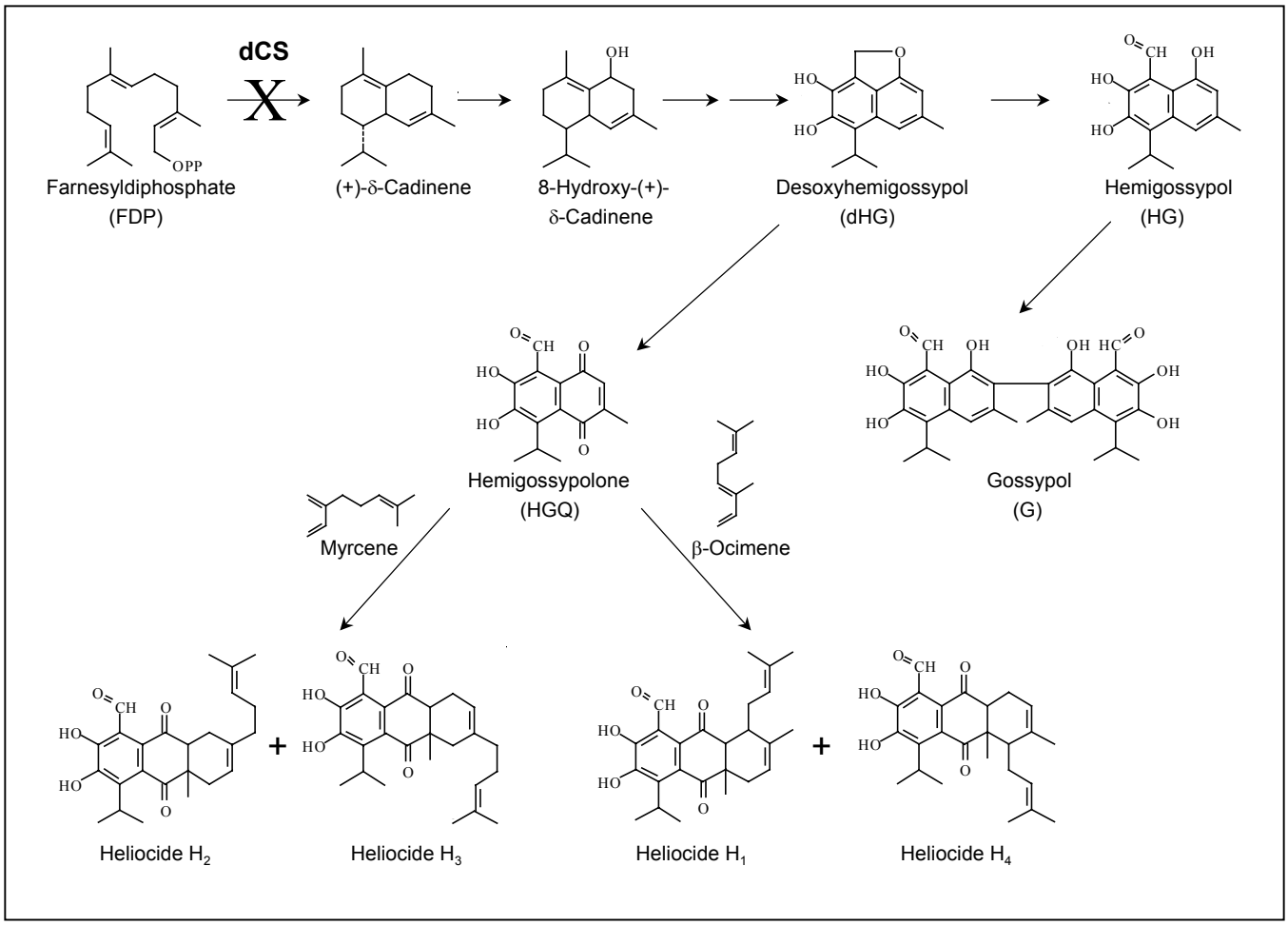

Fig. 6. Structures and proposed biosynthetic pathway of gossypol and other terpenoids in cotton plants. δ-Cadinene synthase (dCS) enzyme was targeted in the seed through RNAi to interfere with gossypol biosynthesis.

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  1 atgccgagaa cgacctctac accacatccc ttcgattccg attactccga gagcatggat
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361 cccataataa ggttttgttg gagtttgcta agatcgattt caacatggta caacttttgc
421 ataggaaaga gctaagtgag atttctaggt ggtggaagga tttagacttt caaagaaagt
481 tgccatacgc aagagataga gtggttgaag gctatttttg gatctcagga gtgtactttg
541 agccccaata ttctcttggt agaaagatgt tgacaaaagt gatagcaatg gcttctattg
601 taga
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Fig. 7. A 604-bp-long internal fragment of δ-cadinene synthase gene used as the trigger sequence in the ihpRNA vector.

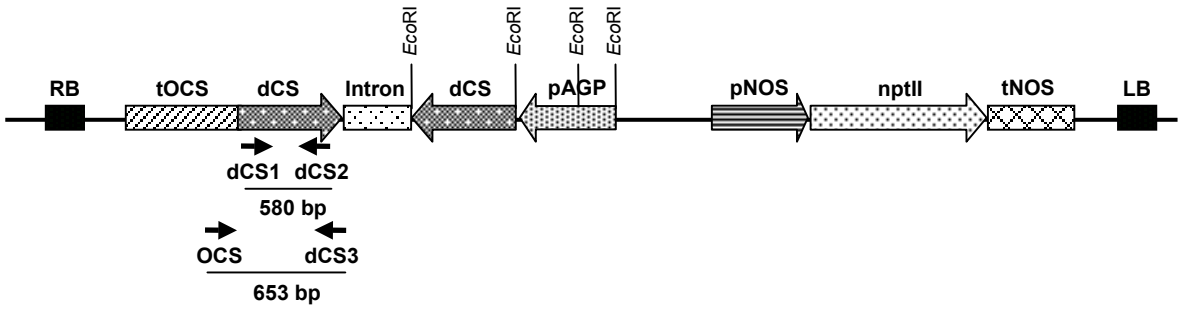


Fig. 8. The T-DNA region of the binary vector pAGP-iHP-dCS. Arrows indicate the primers used in the PCR analyses. RB-right T-DNA border, tOCS: octopine synthase terminator, dCS: 604-bp δ-cadinene synthase sequence, pAGP: cotton α-globulin promoter, pNOS: nopaline synthase promoter, *npt*II: neomycin phosphotransferase II, tNOS: nopaline synthase terminator, LB: left T-DNA border.

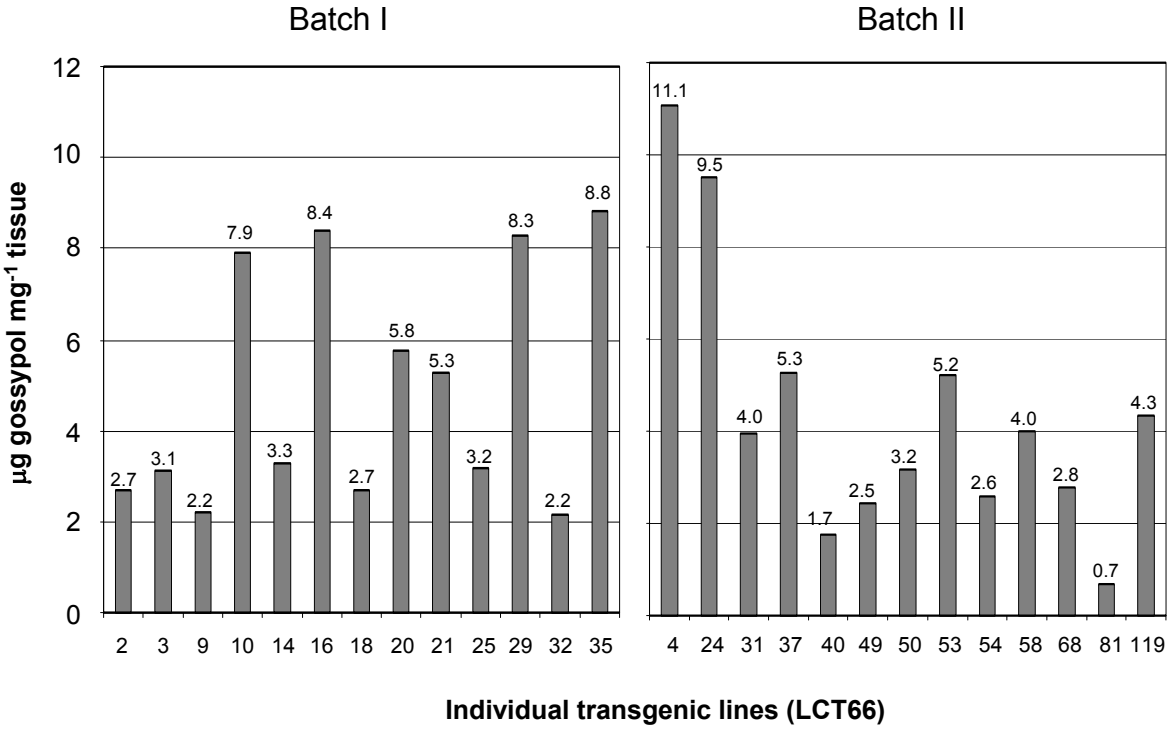


Fig. 9. Levels of gossypol in pooled samples of 30 mature T_1 seeds from 26 independent transgenic lines. The results presented are for two separate batches of transgenic plants recovered at different stages of the project. Note that the T_1 seeds will be segregating for the transgene, and therefore the gossypol levels in the pooled seeds presented here will also include the values from the contaminating null segregant seeds.

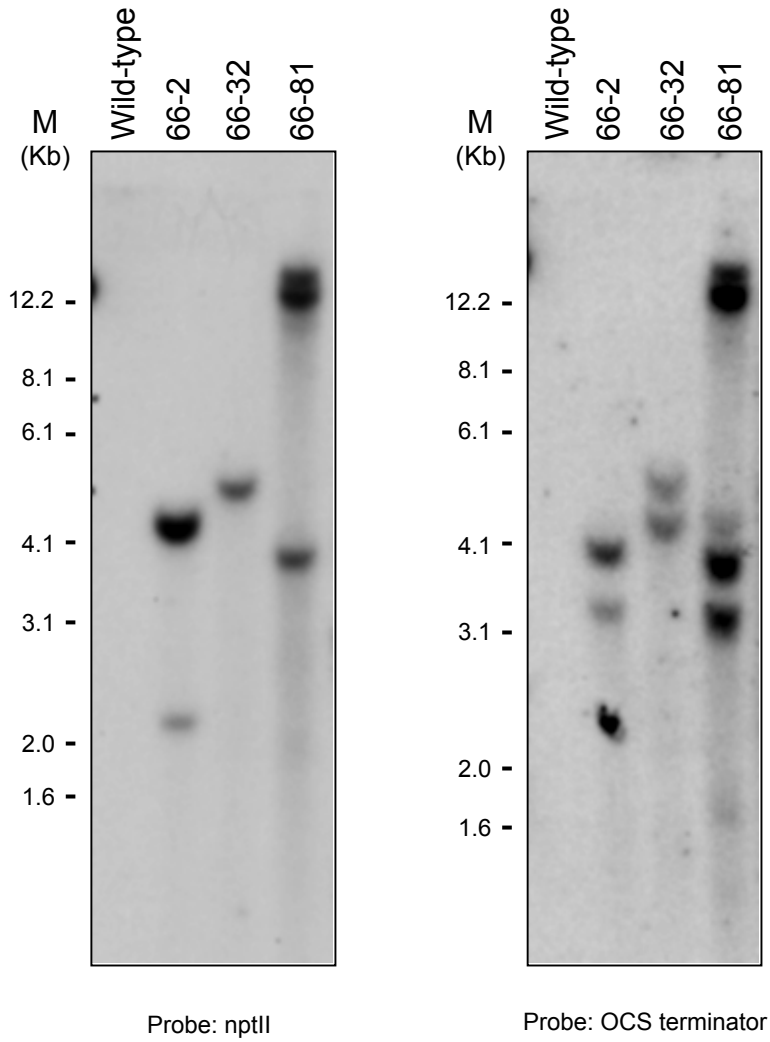
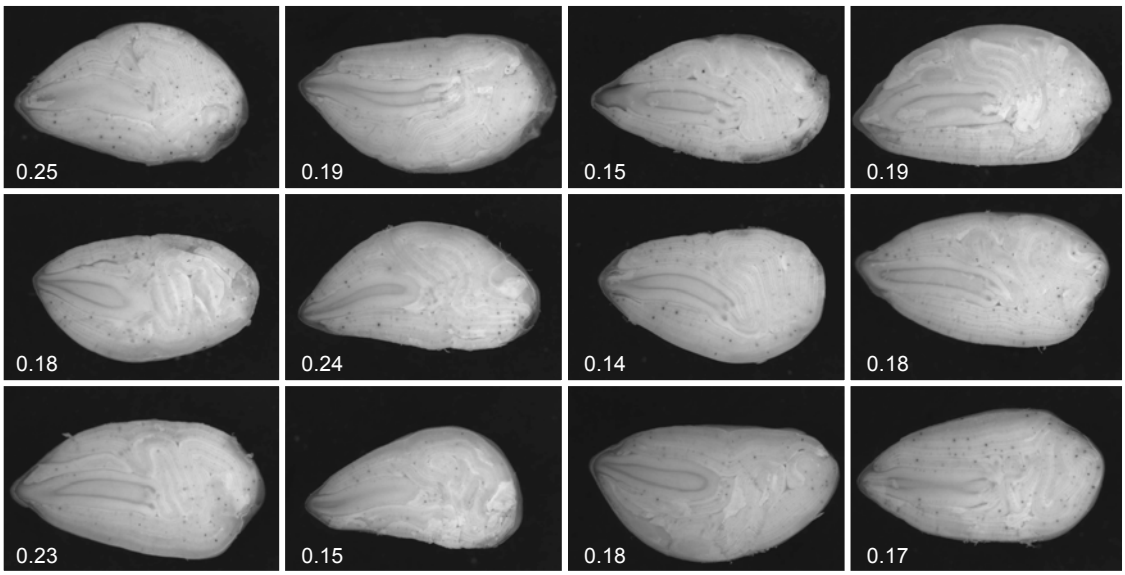


Fig. 10. Southern hybridization analyses on three low-seed-gossypol lines (LCT66-2, -32, and -81) used for various studies in this investigation. Genomic DNA (15 μg) was digested with EcoRI, and the blots were probed with either *npt*II gene (*Left*) or OCS terminator (*Right*). Because the low-seed-gossypol phenotype and PCR results for lines #LCT66-2 and -32 showed a strict 3:1 segregation, we believe that the transgene copies were integrated at a single locus. Line #LCT66-81 did not show a 3:1 segregation for the low-seed-gossypol phenotype, thus reflecting transgene integration in multiple loci.

A. T2 seeds from line LCT66-81-5



B. Seeds from wild-type plant

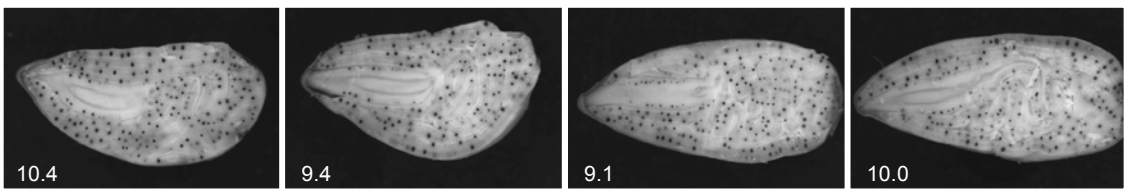


Fig. 11. Photomicrographs of sections of mature T_2 seeds (*A*) obtained from a T_1 plant from the RNAi transgenic line, LCT66-81. The seeds from the wild-type control plant (*B*) are shown for comparison. The levels of gossypol (μg/mg seed) for each individual seed are presented with the respective image. The mean gossypol levels for 12 T_2 seeds and four control seeds are 0.19 ± 0.013 μg/mg (mean ± SEM.) and 9.71 ± 0.295 μg/mg (mean ± SEM), respectively. Note the correlation between visible phenotype and gossypol level in the seed.

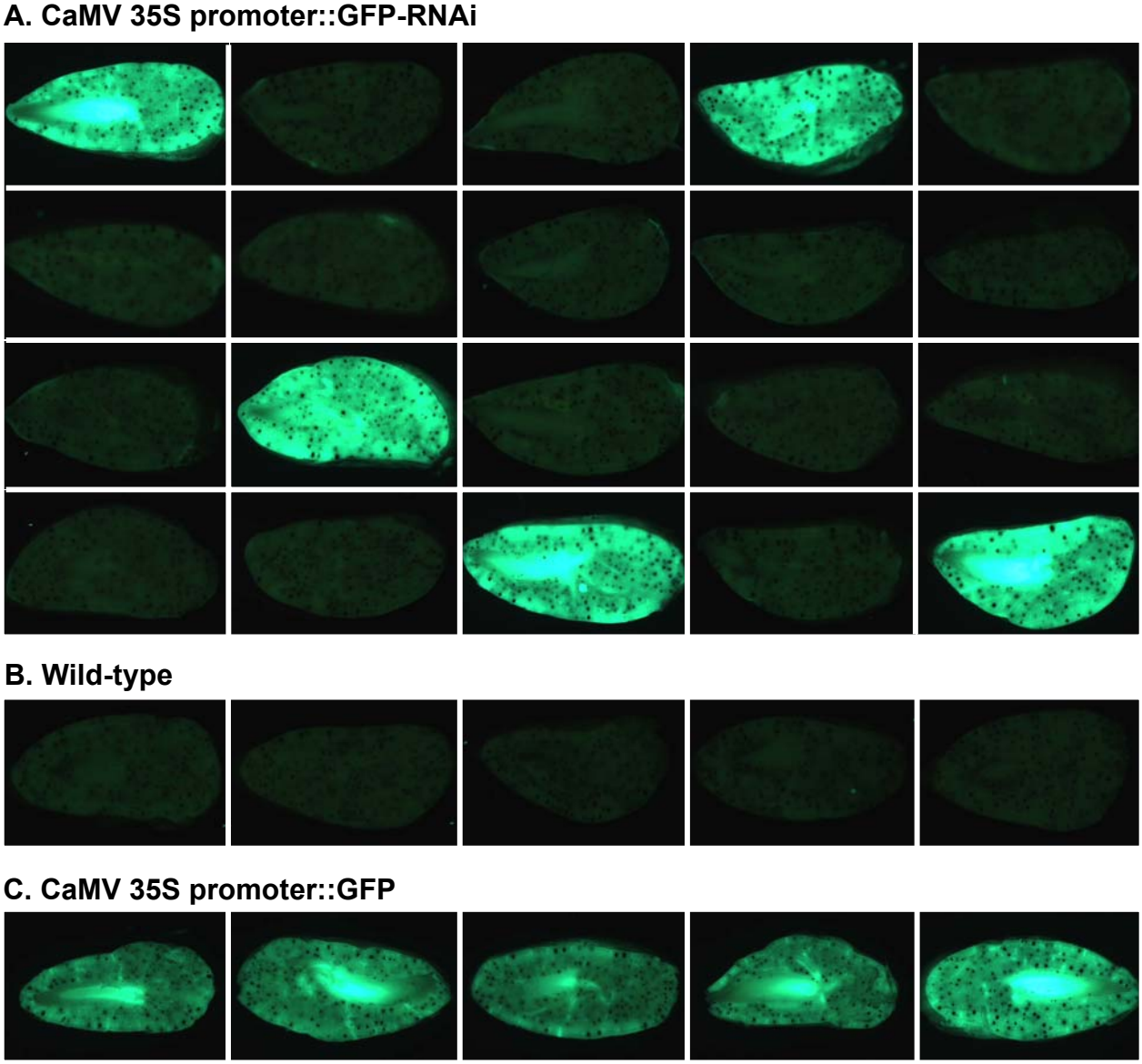


Fig. 12. Gene expression in a developing null segregant seed remains unaffected by RNAi-induced silenced status of the neighboring seeds and the maternal tissue. GFP-expressing (under the control of the constitutive CaMV 35S promoter) line of cotton was retransformed with an RNAi construct (again using the constitutive CaMV 35S promoter) to silence the GFP. (*A*) Seeds obtained from the boll of a silenced line were sliced and photographed by using a Zeiss M2BIO Fluorescence Combination Zoom Stereo/Compound microscope equipped with a GFP filter set comprising an exciter filter (BP 470/40 nm), a dichromatic beam splitter (495 nm), and a barrier filter (LP 500 nm). Wild-type cottonseeds (*B*) and seeds from the original GFP-expressing line (*C*) are shown for comparison. Note that the GFP expression in the null segregant seed that is growing within the silenced maternal tissue and surrounded by silenced seeds is not diminished (*A*).

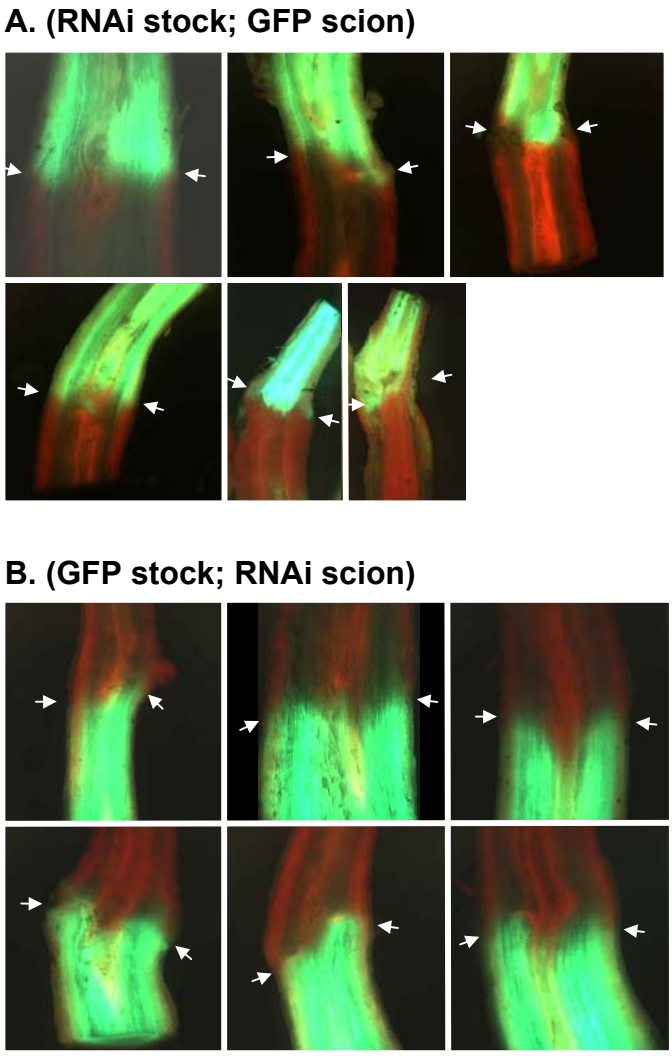


Fig. 13. The spread of RNAi silencing (targeting GFP expression) is not seen in experiments involving grafting in cotton. The same GFP-expressing transgenic cotton line and the retransformed RNAi line to silence the GFP were used for grafting. Stocks were prepared by cutting a 10-day-old seedling below the cotyledonary junction. Scions consisted of shoot apex containing 4-6 leaf primordia from a similar-aged seedling. Either scions from GFP expressing plants were grafted onto stocks from RNAi-GFP plants (*A*), or scions from RNAi-GFP plants were grafted onto stocks from GFP-expressing plants (*B*). Only the successful grafts showing the vigorous growth of the scion, ≈2 months after the grafting, were used for imaging. Longitudinal sections of the stem through the graft junctions were photographed by using the microscope settings described earlier. Arrows indicate the graft junctions. Note that GFP expression has not diminished across the graft junction.