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Ultra-low gossypol cottonseed: generational stability of the seed-specific, RNAi-mediated phenotype and resumption of terpenoid profile following seed germination

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Summary

Cottonseed, containing 22.5% protein, remains an under-utilized and under-valued resource because of the presence of toxic gossypol. RNAi-knockdown of δ-cadinene synthase gene(s) was used to engineer plants that produced ultra-low gossypol cottonseed (ULGCS). In the original study, we observed that RNAi plants, a month or older, maintain normal complement of gossypol and related terpenoids in the roots, foliage, floral organs, and young bolls. However, the terpenoid levels and profile of the RNAi lines during the early stages of germination, under normal conditions and in response to pathogen exposure, had not been examined. Results obtained in this study show that during the early stages of seed germination/seedling growth, in both non-transgenic and RNAi lines, the tissues derived directly from bulk of the seed kernel (cotyledon and hypocotyl) synthesize little, if any new terpenoids. However, the growing root tissue and the emerging true leaves of RNAi seedlings showed normal, wild-type terpenoid levels. Biochemical and molecular analyses showed that pathogen-challenged parts of RNAi seedlings are capable of launching a terpenoid-based defence response. Nine different RNAi lines were monitored for five generations. The results show that, unlike the unstable nature of antisense-mediated low seed-gossypol phenotype, the RNAi-mediated ULGCS trait exhibited multi-generational stability.

Introduction

Cotton, cultivated for its lint, is a major crop in many countries including the United States. For every kilogram of fibre, the cotton plants produce approximately 1.6 kg of seeds. Cottonseeds contain approximately 22.5% of a relatively good quality protein. Utilization of this, nearly 10 million metric tons (MMT) of protein resource locked up in the approximately 43 MMT (average annual, worldwide production from 2006/07 to 2009/10; FAS-USDA) of annual cottonseed output, is hampered by the presence of toxic gossypol in the seed. Glands within the seed contain mostly gossypol while those present in other parts of the plant contain gossypol and additional terpenoids that are derived from the same biosynthetic pathway. These compounds play an important role in defending the cotton plant against various insect pests. However, the presence of toxic gossypol in the seed prevents its direct use as food or even as feed for the non-ruminant animals. Thus, elimination of gossypol from the cottonseed holds the promise to make this by-product safe for use as feed for various monogastric animals and possibly even as food. Accomplishment of this long sought-after goal will be of tremendous value in meeting the growing, worldwide demand for food and feed. The constitutively present terpenoids not only serve a defensive function against insects, but these chemicals are also induced in cotton cells in response to pathogen attack (Bell, 1969; Abraham *et al.*, 1999; Bianchini *et al.*, 1999). Thus, these compounds constitute important components of the plant's basal defence mechanism, a characteristic unique to the tribe Gossypeae (Stipanovic *et al.*, 1999). Because it serves such an important defensive function, elimination of gossypol must be limited strictly to the seed so as not to weaken the ability of the plant to respond to biotic stresses.

We utilized RNAi technology to silence the *δ-cadinene synthase* (*dCS*) gene(s) that encodes an enzyme involved in gossypol biosynthesis (Sunilkumar *et al.*, 2006). By using a seed-specific, α-globulin promoter (Sunilkumar *et al.*, 2002), and transgene-mediated RNAi, we successfully obtained cotton plants that produced seeds with ultra-low levels of gossypol. Importantly, the levels of gossypol and related, protective terpenoids remained unchanged in other, non-seed (foliar, floral, and root) tissues of the plant. However, in this original study, the terpenoid analysis was conducted on plants that were 4 weeks or older. It is critically important to determine the levels of terpenoids during the very early stages of the growth of the RNAi

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seedling. As the seed-derived reservoir of gossypol present in the wild-type seedling is not available in a young RNAi seedling, it is imperative to ascertain if and when, and to what degree the defensive terpenoids can be induced in cotton seedling tissues in response to a biotic challenge. *Rhizoctonia solani* is one of the pathogens that cause cotton seedling disease. When challenged with this pathogen, the cotton seedling responds by increased production of gossypol and related terpenoids. *Trichoderma virens* elicits a stronger induction of terpenoids in cotton compared to *R. solani* (Howell *et al.*, 2000). We have used both the fungal species as elicitors to evaluate the terpenoid-mediated defence response in young RNAi seedlings. As opposed to traits developed through transgene over-expression, RNAi-mediated gene silencing is a relatively recent development. Therefore, little information is available on the long-term stability of RNAi-mediated traits in crop plants. In this study, we present results from biochemical and molecular analyses conducted to investigate the stability of the ultra-low gossypol cottonseed (ULGCS) trait and the ability of the RNAi lines to launch terpenoid-based defence response during the early stages of seedling development.

Results

Generational Stability of the ULGCS trait

Prior to the generation of ULGCS through the use of RNAi technology, we had conducted an extensive investigation targeting the same, δ-cadinene synthase gene for silencing through antisense technology. Two different types of antisense events were generated using two different promoters, i.e. a CaMV 35S promoter and the cotton α-globulin promoter that was also used to generate RNAi-ULGCS events. Examination of gossypol levels in T1 generation (pooled) seeds from the antisense events revealed several lines in each case that had substantially lower levels of seed gossypol compared to the wild-type counterparts (Figures S1 and S2). After elimination of null segregants, 6–12 plants each from selected, low seed-gossypol lines were grown in the greenhouse until maturity to obtain T2 seeds. Gossypol analysis on these seeds revealed that in a majority of cases, gossypol levels in the T2 generation were higher than the values observed in T1 generation seeds (Figure S3). These results indicated instability of the antisense-mediated low seed-gossypol trait. Therefore, this line of investigation was not pursued further.

The instability of the antisense-mediated, low seed-gossypol trait served as an impetus for examining the RNAi-ULGCS events in a more rigorous manner for trait stability. We therefore selected nine promising RNAi events exhibiting ULGCS phenotype (Sunilkumar *et al.*, 2006) and examined gossypol levels in the seeds obtained from plants grown in a greenhouse over five generations. The results presented in Figure 1 show clearly that as opposed to antisense technology, the RNAi-mediated ULGCS trait remains stable.

Terpenoid levels in young seedlings

In our earlier study, we had shown that RNAi-mediated silencing of δ-cadinene synthase gene in cotton using a seed-specific promoter produced ultra-low gossypol seeds without altering the gossypol/terpenoid levels in other parts of the plant (Sunilkumar *et al.*, 2006). However, in this original investigation, terpenoid analysis on the foliar, floral, and root tissues was conducted on plants that were 4 weeks or older. We were interested in examining the effect of ULGCS trait on the terpenoid levels and their profile during the early stages of seedling development. As the bulk of cottonseed is composed of cotyledonary and hypocotyl tissues, the gossypol levels in these two organs of a young seedling were significantly lower in the RNAi lines compared to the wild-type (Figure 2). Similar differences were observed for other terpenoids in the cotyledon and hypocotyl tissues of the two types of seedlings (Figure 2). Even in the case of wild-type seedlings, the levels of terpenoids in these tissue types decreased as the seedlings grew, suggesting a diluting effect caused by their growth on the seed-derived reservoir of these compounds and possible lack of new synthesis. In contrast to cotyledon and hypocotyl tissues, the terpenoid levels were comparable in the root and the newly formed leaves of the young seedlings (Figure 2 and Figure S4). Moreover, there was a general increase in the root terpenoid levels in both types of seedlings as these grew.

Sensitivity of RNAi seedlings to *Rhizoctonia solani*

An important question to ask is whether the RNAi seedlings have a weakened defence and if so are they more sensitive to pathogens. To address this question, we tested three different RNAi lines along with various controls against a seedling pathogen of cotton, *R. solani*. Seedlings were grown in pathogen-infested soil and the disease indices were scored as described in our earlier investigation (Kumar *et al.*, 2009). The results from these assays are presented in Figure 3 and Figure S5. The results show that both, non-transgenic controls as well as RNAi seedlings are equally susceptible to this pathogen. The RNAi lines do not exhibit a higher degree of susceptibility towards *R. solani* compared to the null segregant, glandless cotton, or the wild-type controls.

Induction of terpenoids in response to seedling pathogen

An obvious question that arises is whether the young RNAi seedlings are capable of launching a terpenoid-based defence response when challenged with a pathogen, such as *R. solani*. To answer this question, we exposed the seedling root to *R. solani* and measured terpenoid levels in various organs. Although *T. virens* is not a pathogen of cotton, it induces a strong defence response in cotton (Howell *et al.*, 2000). Therefore, we also examined the response of cotton seedlings to this fungal species.

An examination of terpenoid profile in young, uninfected wild-type cotton seedlings showed detectable levels of hemigossypolone (HGQ), desoxyhemigossypol (dHG), and gossypol (G), present mainly in the cotyledon and hypocotyl tissues. As expected, these compounds were present in the RNAi seedlings in minute quantities. The roots of both the wild-type as well as RNAi seedlings when exposed to *R. solani* showed visible yellow coloration, an indication of a terpenoid-based defence response (data not shown). This type of response has been observed in the cotton roots and documented in our earlier study (Kumar *et al.*, 2009). Eight hours following exposure of seedling root to *R. solani*, cotyledon, hypocotyl, and root tissues were harvested and their terpenoid profile determined. Figure 4 shows that the root tissue, which was directly exposed to the fungus, showed the highest level of terpenoid-induction in both wild-type and RNAi seedlings. Dramatic increases in the dHG and HG levels were observed in root and hypocotyl tissues following infection (Figure 4). However, terpenoid-induction in the RNAi lines was

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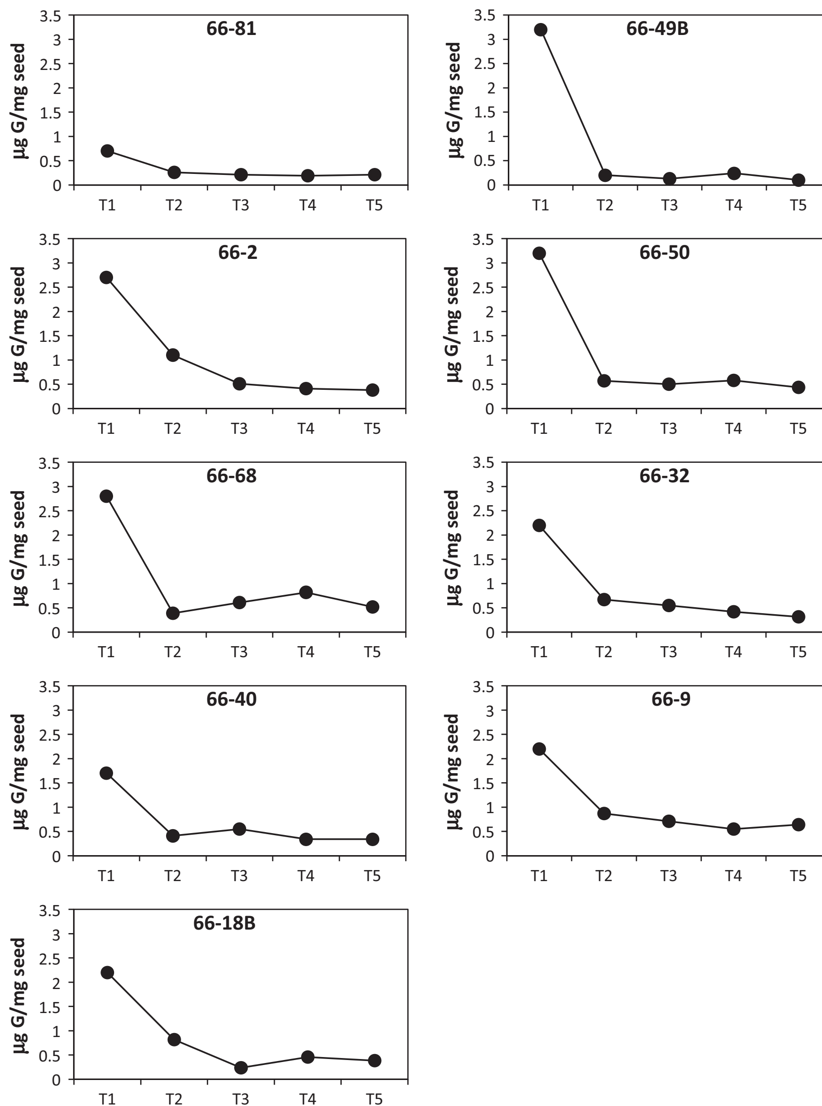

Figure 1 Levels of gossypol (G) in pooled, kernel samples obtained from 30 mature seeds each from nine independent transgenic lines over five generations (T1 through T5). Also note that the T1 seeds were segregating for the transgene, and therefore the gossypol levels in the pooled seeds presented here included the values from the contaminating null-segregant seeds. T2 and subsequent generation seeds were obtained from a homozygous parent in each case.

somewhat lower compared to the wild-type controls at the 8-h time point. In future, a more detailed analysis at later time-points will be conducted to ascertain if these differences wane with time. Unlike root and hypocotyl, the cotyledons did not show significant induction of terpenoids upon infection, at least up to eight hours following the exposure of root. The high, basal levels of terpenoids in the wild-type cotyledons possibly reflect a carry-over from the seed. Qualitatively and in terms of trend, similar results were obtained when the seedling roots were exposed to *T. virens* (Figure S6). Taken together, the data clearly suggest that the pathogen-induced terpenoid biosynthetic pathway remains functional, albeit at somewhat lower level in the RNAi seedlings.

Induction of genes involved in terpenoid biosynthesis pathway

To confirm the results of biochemical analysis, we conducted molecular analysis and examined the induction of two genes, the RNAi-targeted *dCS* gene and the untargeted *δ-cadinene-8-hydroxylase* (*dCH*) gene. Both these genes encode enzymes that are involved in catalyzing the early steps of gossypol biosynthesis and are known to be induced in response to pathogen infection in cotton (Benedict *et al.*, 1995; Davis and Essenberg, 1995; Bianchini *et al.*, 1999; Luo *et al.*, 2001). We investigated, at the molecular level, the effect of exposing the seedling root to *R. solani*. Northern blot analysis with *dCS* probe revealed relatively low levels of *dCS* transcripts in the cotyledon tissues of RNAi line, in both infected and uninfected samples (Figure 5). In the cotyledons of infected wild-type seedlings, a small induction of *dCS* transcripts was observed. In hypocotyl tissue, an increase in *dCS* gene activity was observed in both types of seedlings, but with somewhat lower induction in the RNAi seedlings compared to the non-transgenic controls. In case of roots, a significant elevation in the *dCS* transcripts was observed in both types of seedlings upon exposure to the pathogen. Gene *dCH* encodes a cytochrome P-450 mono-oxygenase. The dCH enzyme, involved in the step following the dCS-catalysed cyclization of farnesyl diphosphate to δ-cadinene, is also induced in response to pathogen challenge and therefore, the gene encoding it served as a convenient non-target control. As opposed to *dCS*, that was fairly active in non-transgenic seedlings, *dCH* gene showed a low-level basal activity in all tissue types in both kinds of seedlings. Exposure of the root tissue to *R. solani* did not cause a major induction of *dCH* activity in cotyledon and

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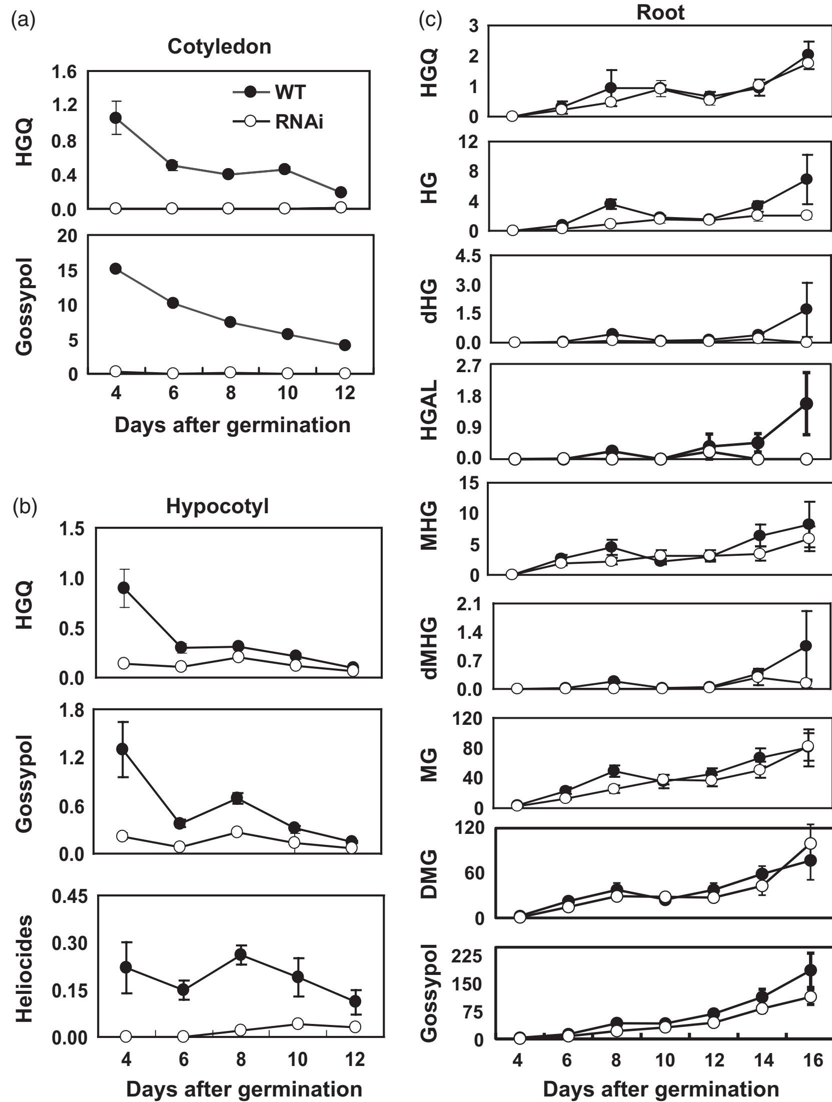

Figure 2 Gossypol and related terpenoids in cotyledon (a), hypocotyl (b), and root tissues (c) of an RNAi line (66–81) and wild-type seedlings. The results shown represent mean (±SE) terpenoid values in tissue samples taken from three individual plants. The values are presented as μg terpenoid/mg dry wt. for cotyledon and hypocotyl tissues, and as μg terpenoid/root system. The terpenoids detected were HG: Hemigossypol; Gossypol; Heliocides; dHG: Desoxyhemigossypol; HGQ: Hemigossypolone; DMG: Gossypol-6,6′-dimethyl ether; dMHG- desoxyhemigossypol-6,6′-methyl ether; MHG: Hemigossypol-6-methyl ether; MG: Methyl gossypol; and HGAL: hemigossylic acid lactone.

hypocotyl tissues of either type of seedlings, however, it resulted in a dramatic increase in the transcript levels in the roots of both the RNAi lines and the wild-type seedlings (Figure 5). The Northern blot for *dCS* had shown some low-level transcripts in various organs of RNAi seedlings in the absence of infection. Real-time PCR analysis for the expression of *cad*1-C subfamily on RNA samples from root, hypocotyl, and cotyledon tissues derived from uninduced seedlings confirmed these results (Figure S7).

The dCS enzyme is encoded by a multigene family, with at least two subfamilies *cad*1-A and *cad*1-C (Tan *et al.*, 2000). There is some indication from this study that *cad*1-A transcripts are induced in cotton in response to a cotton pathogen, *Verticillium dahliae*. To examine whether the member(s) of this subfamily are also the ones that are induced by *R. solani*, we conducted an RT-PCR analysis on the same RNA samples that were used for Northern blot analysis. The results from a duplex RT-PCR using primers specific for *cad*1-A and cotton histone gene are shown in Figure 6. Little or no activity of this gene was seen in various organs in uninduced seedlings. However, following exposure of the seedling root to *R. solani*, a dramatic induction in the *cad*1-A transcript level was observed in the

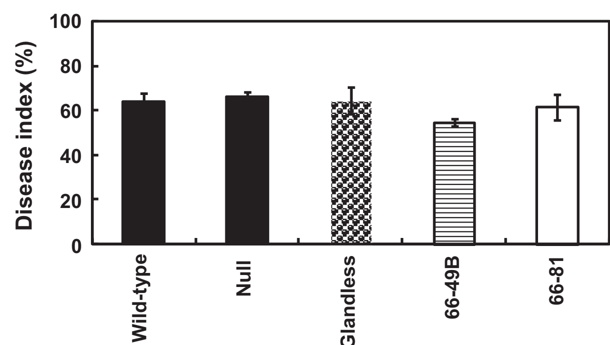

Figure 3 Disease index for cotton seedlings following growth on *Rhizoctonia solani*-infested soil. After 5 days of growth on *R. solani*-infested soil, disease severity (mean ± SE) was scored. Mean disease severity values were obtained by averaging results from three independent experiments, each consisting of 12 wild-type, null segregant, glandless or RNAi seedlings (66–49B and 66–81). Glandless cotton seedlings were used as control for comparative purpose as these lack constitutive terpenoids, but are known to induce terpenoids when challenged with a pathogen.

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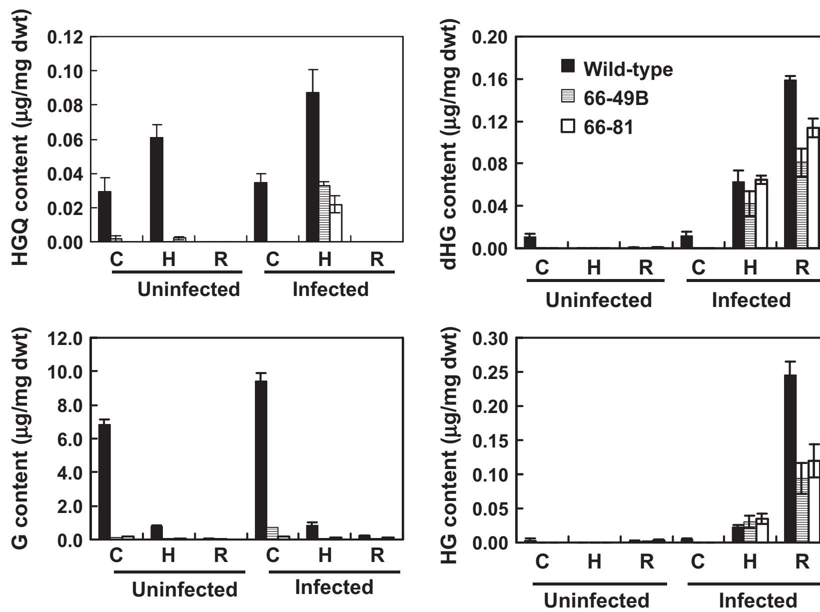

Figure 4 The levels (mean±SE) of gossypol and related terpenoids in the cotyledon (C), hypocotyl (H), and root (R) tissues of wild-type and two RNAi lines (66–49B and 66–81), 8 h following exposure of seedling root to *Rhizoctonia solani*. The terpenoids detected were HG: Hemigossypol; G: Gossypol; DHG: Desoxyhemigossypol; HGQ: Hemigossypolone.

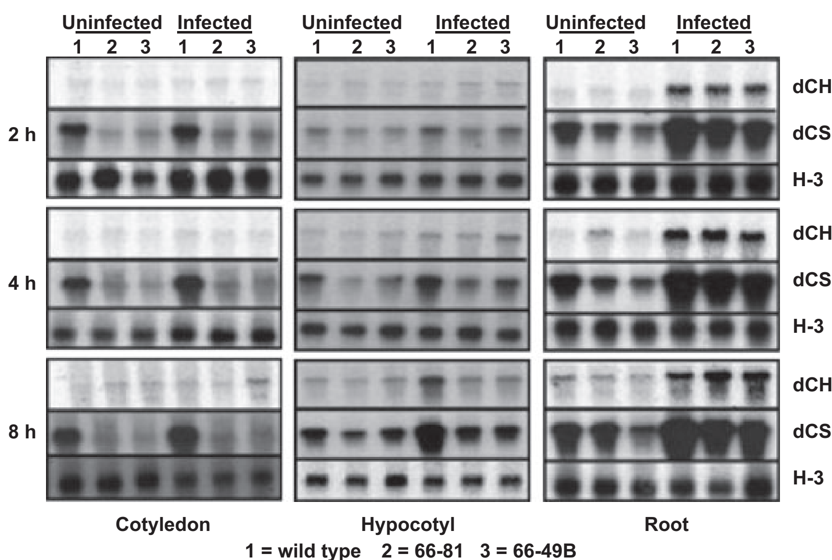

Figure 5 δ-cadinene synthase (*dCS*) and δ-cadinene-8-hydroxylase (*dCH*) transcript levels in the cotyledon, hypocotyl, and root tissues of 2-day-old seedlings of wild-type Coker 312 (1), RNAi line #66-81 (2), and RNAi line #66-49B (3) after 2, 4 and 8 h exposure of the seedling root to *Rhizoctonia solani*. H-3: cotton histone-3 was used as an internal loading control.

roots of both, RNAi and the wild-type seedlings, but not in the hypocotyl or cotyledons (Figure 6).

Detection of siRNA in developing embryo, mature seed, and germinating seedling

We investigated whether the remnants of *dCS* siRNAs generated during embryo development persist in the mature RNAi seed and whether these can be detected in the germinating seedlings. This was examined by siRNA gel-blot analysis on 35-day-old embryo, mature seed, and 3-day-old seedling using the *dCS* trigger region as a probe. Results showed the presence of *dCS*-specific small RNAs in all these three stages of development in the RNAi line, but not in the wild type (Figure 7a). The presence of *dCS* siRNA in the RNAi seedlings was further investigated by separately examining the cotyledon, hypocotyl and root tissues. Among these tissues, the *dCS*-specific small RNAs were detected only in the cotyledon tissue (Figure 7b). To rule out the possibility of a leaky activity of the seed-specific, α-globulin promoter in the cotyledons of a germinating seedling, we examined various seedling organs for the presence of *Flavaria trinervia* pyruvate orthophosphate dikinase intron. This intron is present in the original pHANNIBAL vector used to assemble the intron-containing *dCS* hairpin construct. Northern blot analysis using the *Flavaria* intron as a probe showed bands only in the embryo, but not in the mature seed, or the cotyledon, hypocotyl and root of an RNAi seedling (Figure 8). The results suggest that the siRNAs detected in the seedling organs represent residual molecules derived from the seed and not due to leaky promoter activity in seedling organs.

Discussion

Elimination of the terpenoid, gossypol, from cottonseed while retaining this and related compounds in the rest of the plant to provide defensive capabilities has been a long-standing goal of

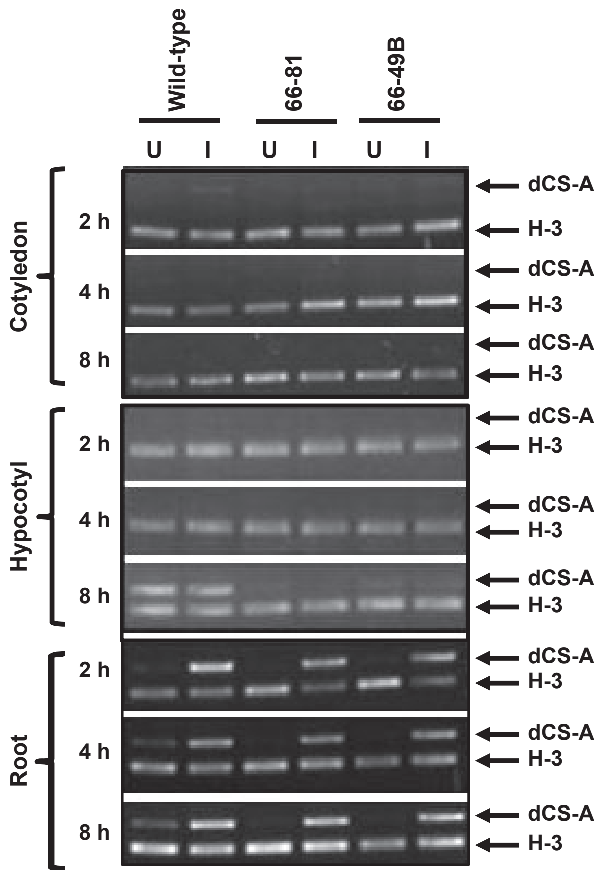

Figure 6 RT-PCR analysis for δ-cadinene synthase-A (*dCS*-A) transcript levels in the cotyledon, hypocotyl, and root tissues of 2-day-old seedlings of wild-type Coker 312, RNAi line #66–81, and RNAi line #66–49B after 2, 4 and 8 h exposure of the seedling root to *Rhizoctonia solani*. U: Uninduced; I: Induced. H-3: histone-3 used as an internal control.

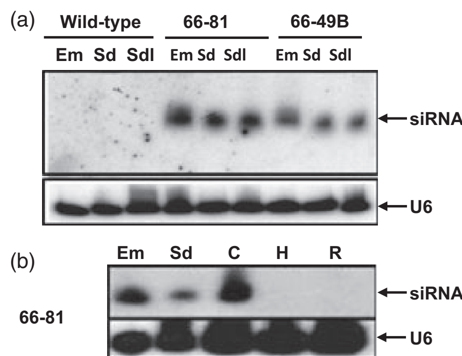

Figure 7 RNA gel-blot analysis to detect *dCS*-siRNA accumulation; (a) in developing embryo, mature seed, and germinating seedling of two RNAi lines (66–81 and 66–49B), (b) in developing embryo, mature seed, and seedling organs of RNAi line #66–81. Em: 35-day-old embryo, Sd: mature seed, Sdl: 2-day-old seedling, C: 2-day-old seedling cotyledon, H: 2-day-old seedling hypocotyl, and R: 2-day-old seedling root. For RNA loading control, the membrane was rehybridized with U6-specific probe after striping the *dCS* probe.

cotton geneticists. Mutation of a native gene, either by traditional means or through gene knockout technology (such as homologous recombination or zinc finger nuclease), that

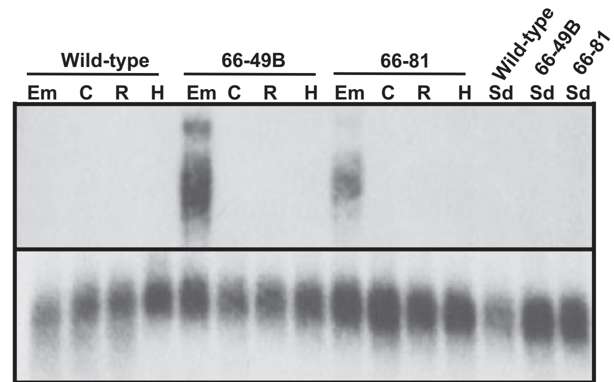

Figure 8 Northern blot analysis to detect *Flaveria trinervia* intron transcripts in various tissues of RNAi lines (66–49B and 66–81). Em: 35-day-old embryo, Sd: mature seed, C: 2-day-old seedling cotyledon, H: 2-day-old seedling hypocotyl, and R: 2-day-old seedling root. Blot was probed with *F. trinervia* intron (top), stripped and then reprobed with cotton histone gene (bottom).

directly encodes or whose encoded enzyme activity enables the production of a toxin/allergen would be the most appropriate means to eliminate the undesirable compound from a crop plant. However, given the facts that the dCS enzyme is encoded by a multigene family, the products of dCS activity provide important defensive function, and the commercial cotton plant is a tetraploid, it is nearly impossible that either mutations or gene knockout technologies can be used to eliminate gossypol from the cottonseed. Considering these factors, our earlier report on the seed-specific, RNAi-based gene silencing approach (Sunilkumar *et al.*, 2006) provided a more realistic means to selectively eliminate gossypol from cottonseed. However, before the ULGCS can become a commercial reality, it is important to thoroughly examine the stability and robustness of RNAi-based technology. Although there are a large number of publications describing the efficacy of RNAi to significantly knockdown gene function, there are only a few studies examining its generational stability and temporal/spatial confinement of the resultant phenotype (Stoutjesdijk *et al.*, 2002; Davuluri *et al.*, 2005; Nakamura *et al.*, 2006; Houmard *et al.*, 2007; Zouhar *et al.*, 2009). This study represents the first of its kind involving a detailed characterization of RNAi lines that were generated to eliminate gossypol from cottonseed by tissue-specific silencing of δ-cadinene synthase gene(s).

As described in the Results section, our efforts to suppress the activity of *dCS* gene using antisense constructs had limited success. Using either the seed-specific, cotton α-globulin promoter or the constitutive, CaMV 35S promoter, we were able to obtain some antisense lines that showed reduced gossypol in the T1 seeds. However, T2 seeds from selected T1 plants of the promising lines showed reversal of the low-gossypol phenotype. These results were surprising because, in an earlier study, we have been able to utilize antisense technology to knockdown Δ-12 desaturase activity to obtain higher oleic acid cottonseed phenotype that remains stable (Sunilkumar *et al.*, 2005). The generational instability of the antisense-mediated low-gossypol cottonseed phenotype that we encountered does not represent a unique situation. Similar cases of unstable, 'antisense' traits have been observed in other systems. Examples include the low Rubisco phenotype in wheat (Mitchell

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et al., 2004) and the colourless phenotype in petunia flowers (Tsuda *et al.*, 2004), both of which were unstable. Attempts in other laboratories to utilize an antisense approach to eliminate gossypol from cottonseed also proved unsuccessful (Benedict *et al.*, 2004; Townsend *et al.*, 2005). Although the reasons for the failure of antisense strategy to reduce gossypol in the cottonseed are unclear, the results cast doubt on the usefulness of antisense technology to obtain silencing of a particular gene to obtain a desirable phenotype of value in crop plants. Heritability and generational stability of a transgene-mediated trait is critical, if the resulting phenotype is to be exploited commercially. Given the unstable nature of the antisense-based silencing, it was critically important to evaluate the generational stability of the RNAi-based ULGCS phenotype. In our original study (Sunilkumar *et al.*, 2006), we had some indication that in contrast to our antisense lines, two of the RNAi lines investigated did maintain the ULGCS phenotype up to T2 generation. Given the potential value of the ULGCS trait, it was imperative that the stability of the RNAi-based phenotype be tested in a very thorough manner. We, therefore, extended our earlier study by including seven additional lines and examined the seeds for gossypol levels over five generations. The results presented in this report show clearly that unlike the case with the antisense lines, the RNAi-mediated ULGCS trait is stable in cotton.

There are some other studies that have also addressed the question of generational stability of RNAi-mediated gene silencing. RNAi-induced silencing of a native gene, *DET1*, in tomato resulted in fruit-specific increase in flavonoids and carotenoids (Davuluri *et al.*, 2005). Stability of this phenotype was examined over three generations and was found to be stable. Similarly, in Arabidopsis, RNAi silencing of *AtVPS*45 was evaluated over five generations and was also found to be heritable and stable (Zouhar *et al.*, 2009). Stoutjesdijk *et al.* (2002) targeted the *FAD2* gene in Arabidopsis using hairpin (HP) RNA, intron-spliced HP (iHP) and co-suppression constructs. One of the silenced iHP line, evaluated over five generations, showed no reversion or diminution of the extent of the silencing. Li *et al.* (2008) examined RNAi-suppression of a resident *bar* transgene in perennial poplar trees over 2 years and found the phenotype to be stable even under field conditions. Although not directly relevant to RNAi-mediated ULGCS, it is noteworthy that viral resistance due to the expression of inverted repeat constructs is stable compared to the unstable nature of this trait obtained by the use of traditional antisense constructs (Jørgensen and Albrechtsen, 2007). Taken together, these results indicate that expression of RNAi-inducing sequences in plants can create a phenotype that is heritable and stable.

In addition to the doubts regarding the stability and heritability of an RNAi-based trait, there were also questions about the feasibility of this new gene silencing approach to suppress the activity of an enzyme that is encoded by a multigene family in commercial cotton, a tetraploid species. However, the results suggest that RNAi is an efficient and effective tool to overcome functional gene redundancy attributed to multiple gene family and/or homeologous genes in polyploids. Studies conducted by Travella *et al.* (2006) with hexaploid wheat, Liu *et al.* (2002) with *Gossypium hirsutum*, and Kusaba *et al.* (2003) with rice support our findings.

Examination of terpenoid levels in various organs of young seedlings provided some interesting results. In a cottonseed kernel (cv. Coker 312), the proportionate dry weights of cotyledon, hypocotyl, and radicle are 95.08% (64.11 mg), 4.12% (2.78 mg), and 0.8% (0.53 mg), respectively (average dry weight values were derived from 15 seeds obtained from greenhouse-grown Coker 312 plants). Thus, cotyledons and hypocotyl constitute the bulk of cottonseed embryo. Interestingly, in the germinated, young seedling, these two organs of an RNAi line exhibited little, if any, increase in the terpenoid levels. In fact, these two organs, even in the case of wild type, show a gradual decline in the levels of terpenoids over time as the seedling grows. Taken together with the data from the RNAi seedlings, the results suggest that the bulk of the terpenoids present in these two organs largely represents the 'carry-over' from the dry seed kernel and little, if any, new biosynthesis occurs in cotyledon or hypocotyl. In contrast to cotyledon and hypocotyl tissues, the radicle represents a minor portion of a cottonseed kernel. However, as the seedling germinates, it grows relatively rapidly giving rise to new root tissue. In comparison to cotyledon and the hypocotyl, the relative growth rate of root is substantially higher (Figure S8). Biochemical analysis shows that the growing root portion of a germinating seedling produces fresh terpenoids in both WT and RNAi seedlings. Similarly, newly formed leaves of both types of seedlings do produce terpenoids. Thus, the new growths of a young RNAi seeding are capable of producing terpenoids.

As the seedlings germinate under field conditions, they encounter a variety of biotic stresses. Therefore, it is critical to examine whether the seedlings originating from ULGCS show heightened sensitivity to pathogens. In this regard, it is interesting to note that while the constitutive antisense cotton transformants targeting the *cdn*1-C4 member of the *dCS* family did not show lower seed-gossypol values or any reduction in *dCS* expression in response to *V. dahliae* infection, these same plants did show complete blockage of *dCS* transcripts and protein in response to bacterial blight infection (Townsend *et al.*, 2005). Their results suggested that various members of the gene family were responding to pathogen challenge independently. In light of these results, it was important to examine young seedlings for their sensitivity and response to pathogens. We germinated some RNAi lines and various types of control seeds in soil infested with *R. solani*. Coker 312 is usually sensitive to this pathogen and the development of disease is seen in wild-type and null-segregant controls. As expected RNAi lines were also infected, however, the degree of susceptibility in these lines was no greater than their non-transgenic counterparts. *R. solani* was also able to induce defensive terpenoids in the root and hypocotyl tissues of both WT and RNAi seedlings when the roots were exposed to this pathogen. The results from molecular analysis further support those that were based on the biochemical analysis. The non-target *dCH* and the RNAi-targeted *dCS* genes, usually activated by pathogens such as *R. solani*, are also induced in the roots of RNAi seedlings upon fungal exposure. Taken together, the results suggest that the RNAi seedlings are not impaired in their usual response to a pathogen and therefore, should respond in a manner similar to the WT seedlings under the biotic pressures present under field conditions. It should be noted that terpenoid-mediated defence is only one component of the overall defence portfolio of the cotton plant and the remaining defensive mechanisms are expected to remain functional in the RNAi lines.

We have shown previously that the α-globulin promoter used to drive the transcription of *dCS* hairpin sequences is active during embryo development (Sunilkumar *et al.*, 2002). It is to be expected that some siRNAs that are generated during

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this phase will be retained in the kernel of the mature seed. Upon seed germination, these are expected to degrade gradually. Results from experiments conducted to answer these questions, presented in Figure 7, show the presence of *dCS*-siRNAs in embryos, dry seed kernel, and young seedling. Interestingly, a more detailed examination of various organs showed these siRNAs in the cotyledon, but not in the hypocotyl or the root. To rule out the possibility that the promoter has leaky activity in the young germinating seedling, we examined RNA from developing embryos and various organs of 2-day-old seedlings. Northern blot analysis using the *F. trinervia* intron as the probe showed bands only in the RNA lanes for the embryos, thus discounting the possibility that the siRNAs detected in the seedling were due to the leaky activity of the α-globulin promoter. Taken together, the results indicate that the *dCS*-siRNAs detected in the dry seed kernel and young seedling of an RNAi line represent carry-over from the embryo.

The results described in this report show the robustness of the RNAi-mediated gene silencing approach, both in terms of its generational stability and its temporal/spatial specificity, at least with regards to the modification of cottonseed. The evidence presented in this report related to the confinement of the low-gossypol trait in the seed will ensure that the plants grown in the field have the ability to maintain their intrinsic defences against pests and pathogens. This research opens up the doors for the possible commercialization of the ULGCS trait that would otherwise remain simply an academic curiosity.

Experimental procedures

Plant material

Transgenic cotton (*G. hirsutum*, cv. Coker 312) lines expressing the intron-containing *dCS* hairpin RNA sequence in a seed-specific manner to silence the δ-cadinene synthase gene(s) (Sunilkumar *et al.*, 2006) were used in this study. The lines used to conduct generational studies on RNAi and antisense plants are shown in the respective figures. Other experiments were performed using T5 generation seeds obtained from homozygous plants from two individual RNAi lines, 66–81 and 66–49B. A summary of various RNAi lines used in different kinds of analyses is presented in Table S1. Wild-type seeds or seeds obtained from a null segregant (derived from line 66–32) and in some cases glandless cotton (GL3) were used as controls as per the need of experimental design.

Seeds soaked in water overnight were treated with hot tap water for 30 s to obtain uniform germination. These seeds were sown in moistened soil (Metro mix 300; Sun Gro Horticulture Ltd., Bellevue, WA) in a plastic tray and allowed to germinate in a growth chamber (maintained at 25 °C and 14:10 photoperiod; Conviron, ND) for studies involving pathogen inoculation. For terpenoid analysis experiments, the seeds were sown in small plastic pots and grown in a greenhouse (temperature in the greenhouse was maintained using the evaporative cooling system). Terpenoid analyses were carried out on tissue samples collected from three replicate seedlings. Samples were collected from germinating seedlings at 2-day interval up to 12 days after germination for cotyledon and hypocotyl tissues and up to 16 days after germination for roots. Leaves were sampled at 2-day interval from day 10 to day 14 after germination.

Seedling infection assay in soil

Preparation of the *R. solani* inoculum and infection assay was performed as detailed in Kumar *et al.* (2009). Three independent experiments were conducted and 12 replicate seedlings were used for each type of plants in an experiment. Disease index (infection severity) was calculated as described by Powell *et al.* (1971).

Plate-based infection assay

Inoculum, consisting of a single grain of *R. solani*-infested pearl millet, was placed in a Petri plate containing half-strength MS salts, solidified with 0.25% phytagel and allowed to grow for 24 h at 25 °C. Uniformly pre-germinated, 2-day-old seedlings, grown at 28 °C, were placed on the medium with their roots directly in contact with the actively growing mycelia. After inoculation, the plates were maintained at 25 °C in the dark. At 2, 4 and 8 h of root contact with mycelia, the root, hypocotyl, and cotyledons were harvested and either lyophilized for terpenoid analysis or frozen in liquid N_2 for RNA isolation. Control seedlings were manipulated in the same manner but without exposure to the pathogen before harvesting various organs.

Determination of gossypol and related terpenoids

Levels of gossypol and related terpenoids in cotton seedlings were determined by HPLC methods as described (Stipanovic *et al.*,1988; Benson *et al.*, 2001; Sunilkumar *et al.*, 2006).

Gene expression analysis

Northern blot

Total RNA was extracted using Spectrum™ plant total RNA isolation kit (Sigma Aldrich, St. Louis, MO) from cotyledon, hypocotyl, and root tissues. Total RNA (20 μg) was denatured and run on 1.2% agarose gel containing 2.2 M formaldehyde as previously described (Sambrook and Russell, 2001). After electrophoresis the gel was rinsed with DEPC-H_2O, equilibrated with 10 × SSC, and then RNA was transferred to the Hybond-N^+ membrane (Amersham Biosciences, Arlington Heights, IL). Hybridization was performed at 50 °C with *dCS* probe. The same blot was then stripped, and reprobed successively with *dCH* probe and histone probe (*H-3*). These probes were prepared by PCR-amplification of the desired gene followed by radio-labelling with ^{32}P dCTP. In a separate analysis to detect the expression of the hairpin construct (pAGP-iHP-dCS) in various tissues, a PCR-generated, radio-labelled (^{32}P dCTP), *F. trinervia* pyruvate orthophosphate dikinase intron fragment was used as the probe.

RT-PCR

Total RNA extracted as described above was used for the synthesis of first-strand cDNA. Four hundred nanograms of total RNA was reverse transcribed using an oligo poly-T primer and MultiScribe™ Reverse Transcriptase (Taqman RT kit; Applied Biosystem, Foster City, CA) and the cDNA was used for both duplex PCR and real-time PCR. For duplex PCR, 2 μL of the synthesized, first-strand cDNA was used as template for amplification with δ-*cadinene synthase*-A (Genbank accession no. X96429.1) primers and an internal control, histone 3 (Genbank accession no. AF024716) primers in the same reaction. The primers used were: dCS-A1: 5′-CATTTCATCCGAAAATCGAC-3′;

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dCS-A2: 5′-CACAAGAAATATCGAAG-3′; His3-F: 5′-TGGAACTG TTGCTCTTCGTG-3′ His3-R: 5′-GATGTCCTTGGGCATAAT-3′. The PCR conditions were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 7 min. Primers dCS-A1/dCS-A2, that are highly specific for the δ-cadinene synthase-A, generate a 350 bp amplicon. Primers His3F/His3R amplify a 244 bp fragment for the histone 3 from cotton cDNA. PCR products were analysed by gel electrophoresis on a 1.6% agarose gel in TBE buffer.

For real-time PCR analysis, the SYBR Green PCR Master Mix (Applied Biosystem) and ABI PRISM 7700 sequence detector (Applied Biosystem) were used according to the manufacturer's instructions. The PCR conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 1 min. For each reaction, 40 ng of cDNA was used as template for PCR-amplification with primers specific for δ-cadinene synthase-C gene (Genbank accession no. AF270425.1; RT-dCS-F: 5′-ATAAGGATGAAATGCGTCC-3′; RT-dCS-R1: 5′-CG TTTTTGAGTTTCAGCATC-3′) and an internal control gene, cotton actin-1 (Genbank accession no. FJ560483.1; Ghact-2F: 5′-CTCTCAACCCCAAGGCCAA-3′ and Ghact-2R: 5′-CTGTTGTAC GACCACTGGCATAC-3′).

Gel-blot analysis for siRNA

Small interfering RNA (siRNA) of 21–25 nucleotide size were detected by resolving the low molecular weight RNA on a polyacrylamide-urea gel using the modified protocol described by Johansen and Carrington (2001). Total RNA was isolated using Trizol reagent (Sigma). Twenty five micrograms RNA was separated on 0.5 × Tris-borate/EDTA/7M urea/15% PAGE and electro-transferred on to Hybond-N$^+$ nylon membrane (Amersham Biosciences). Radio-labelled (^{32}P dCTP) δ-Cadinene synthase trigger sequence (Sunilkumar *et al.*, 2006) was used as a probe to detect siRNA. Arabidopsis U6snRNA (5′-AGGGGCCATGCTA ATC-3′; Pouch-Pelissier *et al.*, 2008) was included as RNA loading control. This probe was prepared by end labelling of DNA oligonucleotides with ATP [γ-^{32}P] using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Levels of gossypol (G) in pooled, kernel samples obtained from 30 mature seeds each from 48 T0 plants. The transformation construct used to generate these lines had an antisense δ-*cadinene synthase* gene sequence under the control of cotton α-globulin promoter.

Figure S2 Levels of gossypol (G) in pooled, kernel samples obtained from 30 mature seeds each from 42 T0 plants. The transformation construct used to generate these lines had an antisense δ-*cadinene synthase* gene sequence under the control of CaMV 35S promoter.

Figure S3 Levels of gossypol (G) in pooled, kernel samples obtained from 30 mature T1 or T2 seeds obtained from either α-globulin promoter or CaMV 35S promoter-driven antisense events. The T2 seeds used in this study were from transgene-containing T1 parent. Black bar: wild-type seeds, grey bar: T1 seeds, and white bar: T2 seeds.

Figure S4 Gossypol and related terpenoids in pooled leaves of an RNAi line (66–81) and wild-type seedlings. The results shown are mean (±SE) terpenoid values in tissue samples taken from three individual plants. The values are presented as μg terpenoid/mg dry wt. of leaf tissue. The terpenoids detected were Hemigossypolone (HGQ); Gossypol and Heliocides.

Figure S5 Image showing infected cotton seedlings after 5 days of growth on *Rhizoctonia solani* infested soil. After 5 days of growth on *R. solani* infested soil, disease severity was scored. Wild-type, null segregants (Null; derived from line 66–32), glandless cotton (Glandless), and three RNAi lines (66–49B, 66–81 and 66–32) were used in this study.

Figure S6 The levels of gossypol and related terpenoids in the cotyledon (C), hypocotyl (H) and root (R) tissues of wild-type and two RNAi lines, 8 h following exposure of root to *Trichoderma virens*. The results shown are mean (±SE) terpenoid values in tissue samples taken from three individual plants. The terpenoids detected were HG: Hemigossypol; G: Gossypol; DHG: Desoxyhemigossypol; HGQ: Hemigossypolone.

Figure S7 Real–time PCR analysis for mRNA levels of the δ-*cadinene synthase-C* gene in 4-d-old, uninduced seedlings. The values were first normalized to actin control and then calibrated to δ-*cadinene synthase-C* values in the wild-type plants.

Figure S8 Increase in dry weight of cotyledons, hypocotyl, and root tissues following germination of a cotton seedling.

Table S1 RNAi lines used for different kinds of analyses.

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