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Terpenoid aldehyde formation and lysigenous gland storage sites in cotton: variant with mature glands but suppressed levels of terpenoid aldehydes

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Abstract

A new cotton variant with reduced levels of terpenoid aldehydes (sesquiterpenoids and sesterterpenoids (heliocides)) was isolated from the progeny of hemizygous cotton (*Gossypium hirsutum* cv. Coker 312) transformed with antisense (+)-δ-cadinene synthase cDNA. Southern analysis of leaf DNA digested with *Hin*dIII, *Pst* or *Kpn*I restriction endonucleases did not detect any antisense *cdn1-C1* DNA in the genome of the variant. The gossypol content in the seed of the variant was markedly lower than in the seed of T1 antisense plants. Eighty-nine percent of the variant seed had a 71.1% reduction in gossypol and the foliage of the variant plants showed a 70% reduction in gossypol and a 31% reduction in heliocides. Compared to non-transformed plants there was no reduction in the number of lysigenous glands in the seed of the variant. The cotton variant shows uncoupling of terpenoid aldehyde synthesis and gland formation. The cotton variant may have resulted from somaclonal variation occurring in the callus tissue during the transformation–regeneration process.

Keywords: *Gossypium hirsutum*; Malvaceae; Cotton variant; Low gossypol; Lysigenous glands

1. Introduction

Lysigenous glands in cotton plants contain terpenoid aldehydes (sesquiterpenoids and sesterterpenoids) (Bell, 1969; Stipanovic et al., 1977). Proposed pathways for the biosynthesis of these terpenoid aldehydes from sesquiterpene precursor (**1**–**4**) are shown in Fig. 1. The glands in cottonseed contain predominantly gossypol (**5**) with traces of desoxyhemigossypol (**3**), while glands in the foliage contain **5**, hemigossypolone (**6**), and heliocides 1–4 (**7**–**10**).

The presence of **5** makes cottonseed toxic to non-ruminants, preventing the commercialization of cottonseed protein for human consumption. In 1959, a glandless cotton was produced that lacked both lysigenous glands nad terpenoid aldehydes in plants homozygous for recessive genes gl_2, and gl_3 (McMichael, 1960). Food science research with glandless cottonseed protein opened new vistas of eliminating world hunger and protein shortage with glandless cotton (Lusas and Jividen, 1987). However, production of glandless cotton has been limited since the plants are extensively

Abbreviations: Terpenoid aldehydes, sesquiterpenoids; Heliocides, sesterterpenoids; CDN, -cadinene; G, gossypol; dHG, desoxyhemigossypol; HGQ, hemigossypolone; H_{14}, heliocides 1–4; FDP, farnesyl diphosphate; HMGR, 3-hydroxy-3-methyl glutaryl-CoA reductase; CaMV, cauliflower mosaic virus; T, transformed; R, regenerated, not transformed.

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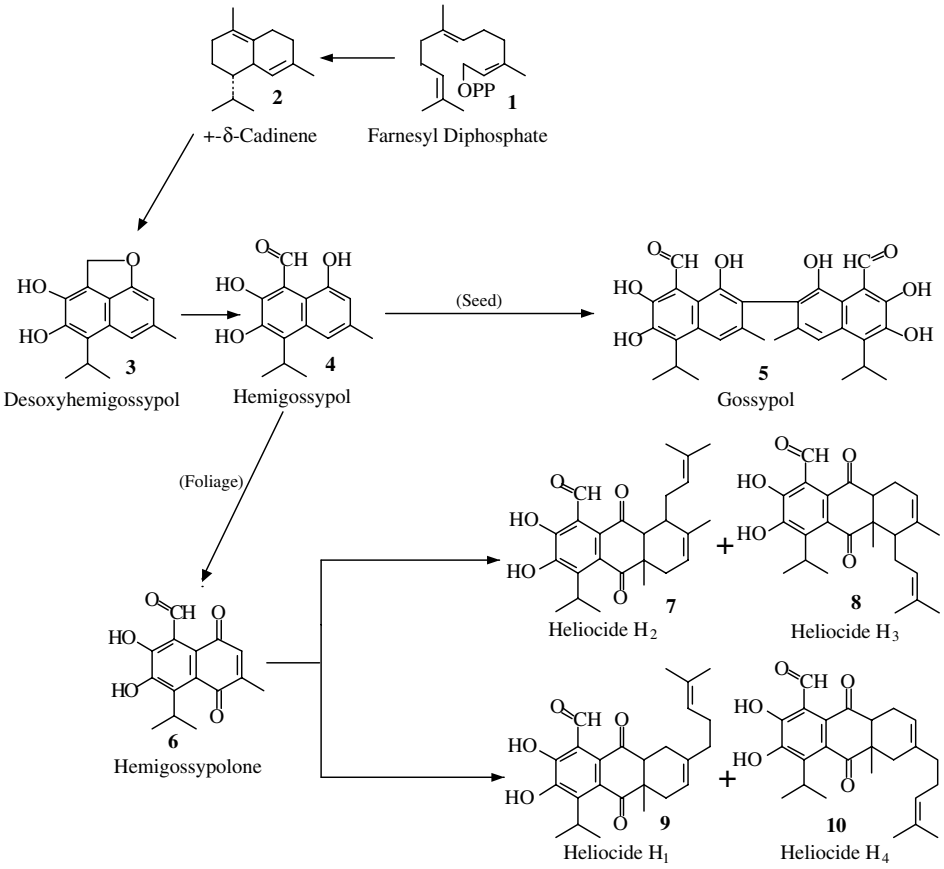

Fig. 1. Stuctures of sesquiterpenoids found in different cotton tissues.

damaged by insects associated with the lack of **5**, **6**, and **7**–**10** in the foliage.

The research on commercializing glandless cotton lasted for about 25 years and stimulated an interest in establishing the relation between lysignous glands and terpeniod deposits. Even though gl_2gl_2, gl_3gl_3 glandless plants normally lack terpenoids, the biosynthesis of desoxyhemigossypol (**3**) and hemigossypol (**4**) can be induced in cotyledons of these plants by infection with *Xanthomonas campestris* pv *malvcearum* (Abraham et al., 1999). Xylem tissue of wild-type cotton does not form lysigenous glands and infection of this tissue with *Verticiullium dahliae* induces the formation of sesquiterpenoids (Bell, 1967). This fungal induction involves an increase in 3-hydroxy-3-methyl glutaryl-CoA reductase (HMGR) and HMGR mRNA (Bianchini et al., 1994) and increases in (+)-δ-cadinene (CDN) synthase (Benedict et al., 1995), CDN synthase mRNA (Alachanti et al., 1998) and *S*-adenosyl-L-methionine: desoxyhemigossypol-6-*O*-methyltransferase (Liu et al., 1999). Australian *Gossypium* subspecies *Hibiscoidea* and *Sturtia* contain immature lysigenous glands but no terpenoid aldehydes (Brubaker, 1996). The authors conclude that the initiation of terpenoid aldehyde formation and gland morphogenesis in these species are uncoupled so that identifying the molecular signals that initiate terpenoid aldehyde formation and not gland formation may be the key to the development of cottonseed free of **5**.

In this paper, we report the isolation of a cotton variant recovered from transformation–regeneration studies that show a marked suppression of terpenoid aldehydes but a normal production of lysigenous glands. This low terpenoid aldehyde trait is genetically stable through three generations and appears to suppress the initiation of sesquiterpenoids and sesterterpenoids without interfering with gland production.

2. Results and discussion

Plants regenerated from callus grown on kanamycin-containing medium following transformation with a binary vector containing the gene *npt*II were expected also to include an antisense version of CDN synthase. This was verified for the T_0 plants by using Southern hybridization with probes to show that restriction fragments predicted from sites inside the T-DNA cassette were present in genomic digests (Martin et al.,

2003). On the assumption that each regenerated plant would be hemizygous for the insert, the T_0 plants were allowed to self-pollinate and individual progeny were examined for the presence of transgenes as well as levels of sesquiterpenoids present in the seeds produced. Table 1 shows the results for the one specific T_0 regenerated plant and its progeny. The T_0 plant was grown from callus following selection on kanamycin and tested positive for the presence of the antisense construct (Fig. 2). Even though there are multiple copies of CDN synthase genes with similar sequences in the genome, only the antisense copy results in a *Hin*dIII restriction fragment of 1.2 kb when hybridized to the insert from the original *cdn1-C1* clone. Similar tests with *Eco*RI and *Pst*I also indicated the presence of T-DNA components of the expected sizes for the T_0 plant and for 11 of 14 progeny (Martin et al., 2003). Further evidence for transformation of this plant came from Southern hybridization to a fragment from the *npt*II gene that was also present in the T-DNA of the *Agrobacterium tumefaciens* transformation vector to provide resistance to kanamycin. The possibility that the fragments detected could originate from contaminating bacteria containing copies of the T-DNA vector was tested by PCR, using primers that amplify a portion of the non-transferred *vir* genes. No product was detected, even after 45 rounds of amplification in T_0 plant 2C or any of its progeny that were tested. Taken together, these observations demonstrate that at least one copy to the T-DNA was incorporated into the genome of the original T_0 plant. T_0 plant were self-pollinated in order to recover progeny homozygous for the antisense expression of *cdn1-C1* from the CaMV 35S promoter. As is typical for regenerated cotton plants, fertility was low. Assuming that T_0 plant 2C was hemizygous for a single insert, we expected 25% of the progeny would lack the insert and have normal levels of sesquiterpenoids, 50% would be hemizygous and 25% would be homozygous for the antisense insert. Although the detection schemes would not differentiate progeny

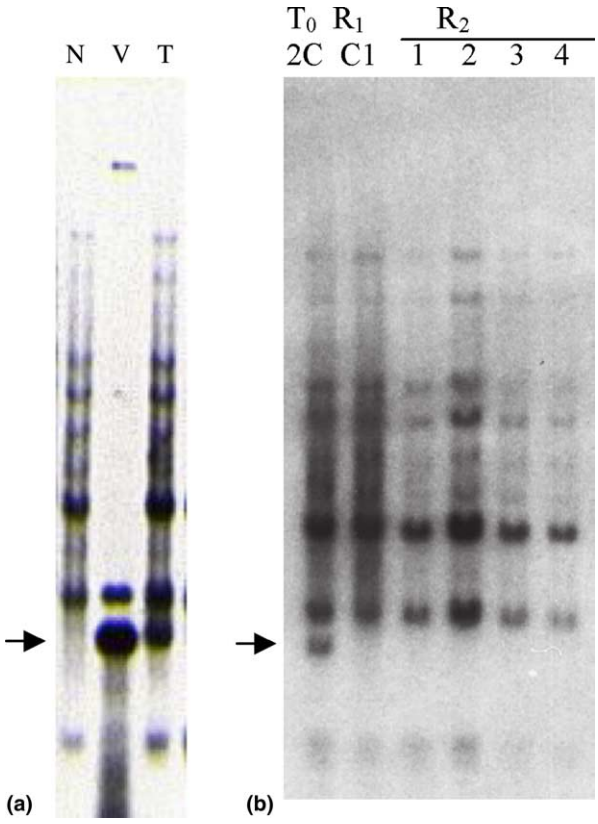

Fig. 2. Southern blot of *Hin*dIII digests of DNA hybridized to probe *cdn1*-C1. Lanes from left to right include DNA from: (A) (N) nontransformed control plant, (V) vector used for transformation, (T) transformed plant. (B) ($T_0$2C) the original T_0 plant, (R_1C_1)R_1 variant progeny C1 and ($R_2$1–$R_2$4), 4 R_2 progeny of the variant. The arrows show the presence of a 1.2kB internal fragment of the antisense *cdn1*-C1 insert, as predicted from the known sequence, from the T-DNA used in transformation.

Table 1
Seed gossypol and T-DNA tests on regenerated transgenic plant 2C and 13 progeny from self-pollination

Plant	Generation	Seed gossypol[b] (μ/mg)	Southern[c] *anti-cdnl-Cl*	Southern[c] *Npt*II	PCR[c] *Vir*D
NTC[a]	R_0	13. 26 ± 2.88 (76 seed)	–	–	–
2C	T_0	(nt, all seed were planted)	+	+	–
2C-C1	T_1	7.19 ± 1.91 (21 seed)	–	–	–
2C-E1	T_1	11.26 ± 0.61	–	–	nt
2C-A3	T_1	9.65 ± 0.59	+	nt	nt
2C-A4	T_1	10.96 ± 2.04	+	nt	nt
2C-B1	T_1	10.84 ± 3.0	+	+	nt
2C-B2	T_1	10.14 ± 2.04	+	nt	nt
2C-D1	T_1	9.54 ± 1.93	+	+	–
2C-G1	T_1	10.04 ± 3.07 (14 seed)	+	nt	nt
2C-G3	T_1	8.96 ± 1.52	+	nt	nt
2C-G4	T_1	8.29 ± 1.27	+	nt	nt
2C-H2	T_1	9.27 ± 2.93 (13 seed)	+	+	–
2C-I2	T_1	11.50 ± 2.94	+	nt	nt
2C-13	T_1	12.06 ± 1.41	+	nt	nt

[a] Data from regenerated but non-transformed control plants.
[b] Average values from 6 seeds (unless noted) selected at random.
[c] +, tested positive; –, tested negative; nt, not tested.

with one or more copies, it seemed likely that plants homozygous for the antisense insert might have lower levels of gossypol-related compounds. In fact, 11 of the 13 fertile T1 plants recovered from the T_0 plant were found to include the antisense *cdn1-C1* gene, and all produced seed with reduced levels of **5** (Table 1). The range of values for different progeny and large standard deviations in levels of **5** measured from seed samples may arise in part from segregation of the antisense construct but also appear to reflect natural variation of **5** in cottensed. (See for example range of **5** in control non-transformed seed in Fig. 3). As shown in Table 1, one plant lacked the antisense construct and produced seed with near control levels of **5**. third plant that lacked the insert did not reproduce so could not be tested for seed **5** levels. Unexpectedly the progeny of the T_0 plant that produced seed with the lowest level of 5, (progeny 2C-C1) lacked any trace of the antisense *cdn1-C1* insert (Fig. 2) or of *npt*II.

Furthermore, a nested PCR procedure for identifying host DNA flanking any T-DNA right border (Spertini et al., 1999) was also negative for this progeny but positive for a plant regenerated following transformation with a similar vector. The procedure is based on amplification using nested primers within the conserved right border region and primer-adapters ligated to *Taq*I restriction fragments. Failure to amplify PCR products infers the absence of the right border and thus of T-DNA in the lowest **5** progeny of the T_0 parent.

Because of the unexpected results for the low-gossypol plant lacking any trace of an antisense construct, seed from the self-pollination of this "variant" plant were grown to test the extent and stability of the low gossypol phenotype. The average levels of **5** (Table 2) from 12 individually extracted seeds produced by each of 13 R_2 progeny ranged from 4.15 to 8.60 μg/mg, well below the range and average (13.26 μg/mg) for control seed. Fig. 3 provides a graphical comparison of the **5** levels in the seed of each population. Despite the fairly broad range of values for each seed source, all of the lowest values came from progeny of the variant plant. The distributions for R_2 and R_3 seed were quite similar, and peaked well below the averages for control or antisense plants (Fig. 3). More than half of the seed from progeny of the low-G variant had less gossypol than the lowest values seen in the controls (Fig. 3). Most importantly, the levels of seed G in the R_2 and R_3 generations were similar and significantly lower than the G content found in either non-transformed controls or from the progency of plants that included antisense *cdn1-C1* construct.

Table 2
Levels of gossypol in R_3 seeds produced by progeny of variant plant

R_2 plant	R_3 seed gossypol[a] (μ/mg)
2C-C1-A1	6.47 ± 1.76
2C-C1-A2	6.48 ± 1.85
2C-C1-A3	6.18 ± 0.98
2C-C1-B1	6.36 ± 0.72
2C-C1-B2	8.60 ± 0.79
2C-C1-B3	8.54 ± 0.85
2C-C1-B4	4.15 ± 0.44
2C-C1-B5	6.06 ± 0.64
2C-C1-C1	6.83 ± 1.45
2C-C1-C2	7.84 ± 1.05
2C-C1-C3	7.63 ± 1.42
2C-C1-C4	6.08 ± 0.66
2C-C1-C5	6.26 ± 0.51
Overall average	6.73 ± 1.57

[a] Samples of 12 seed from pooled seed from each plant.

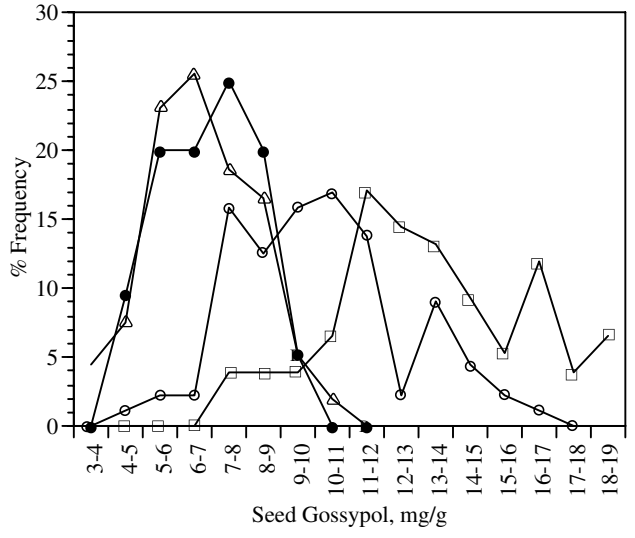

Fig. 3. Relative frequency distribution of gossypol in seed from the low gossypol variant in generation 2 (△) and generation 3 (●). Distributions for a mixture of generation 2 antisense plants (○), and from regenerated Coker 312 controls (□) are also shown.

Foliar concentrations of **5**–**10** (Tables 3 and 4) were also measured for 3rd generation plants. Although there is variation in the measurements of the levels of each compound among individual leaves, the levels found in R_2 progeny of the variant plant are lower than found in control regenerated plants (Table 3). Levels of **5** and **6** are reduced 70% compared to control plants and are similar to levels in the progeny of antisense plants in the same generation (Tables 3 and 4). The reduction of total heliocides (**7**–**10**) by an average of only 30% compared to controls suggests the variant plants would be less vulnerable to insects than glandless varieties.

Glandless cotton was developed by combining at least two recessive genes (gl_2gl_2, gl_3gl_3) and plants with this genotype lack seed G and in green tissues, lack **5**–**10** (Lusas and Jividen, 1987). Analysis of crosses to Gl_2Gl_2, Gl_3Gl_3 varieties suggested that each dominant allele acts in an additive fashion to increase gland number and **5** (Yuan et al., 2001), leading to the concept that the size and number of glands is directly

Table 3
Gossypol (**5**), hemigossypolone (**6**) and heloicides$_{1-4}$ (**7**–**10**) in leaves of progeny of the variant plant and controls

Plant,	Hemigossypolone (**6**) (μg/mg leaf)	Gossypol (**5**) (μg/mg leaf)	Total heliocides$_{1-4}$ (**7**–**10**) (μg/mg leaf)
Control[a]	0.40 ± 0.013[b]	0.14 ± 0.07	1.72 ± 1.33
Variants[c]			
2C-C1-A2	0.043 ± 0.013 (89.3%)	0.049 ± 0.037 (65.0%)	1.30 ± 0.42 (24.4%)
2C-C1-A3	0.074 ± 0.058 (81.5%)	0.041 ± 0.037 (70.7%)	1.68 ± 1.24 (2.4%)
2C-C1-B2	0.066 ± 0.050 (83.5%)	0.050 ± 0/015 (64.3%)	0.099 ± 0.070 (94.2%)
2C-C1-B4	0.345 ± 0.238 (13.8%)	0.024 ± 0.015 (82.9%)	1.03 ± 0.47 (40.1%)
2C-C1-B5	0.084 ± 0.0005 (79.0%)	0 (100%)	0.55 ± 0.023 (68.0%)
2C-C1-C1	0.0065+0.024 (83.8%)	0.066 ± 0.028 (52.9%)	1.68 ± 0.50 (2.3%)
2C-C1-C3	0.135 ± 0.036 (66.3%)	0.110 ± 0.025 (21.4%)	2.42 ± 0.58 41% increase
2C-C1-C4	0.160 ± 0.090 (60.0%)	0 (100%)	0.67 ± 0.24 (61.0%)
Average % reduction	70%	70%	31%

The values in parentheses are the percent reduction compared to the concentration in control plant leaves.
[a] Data from 15 individual control plants.
[b] Data from 3 replicates of each plant.
[c] Standard deviations.

Table 4
Gossypol (**5**) hemigossypolone (**6**) and heloicides$_{1-4}$ (**7**–**10**) in leaves of second generation (T_2) antisense-containing progeny of plant 2C and controls

Plant	Hemigossypolone (**6**) (μg/mg leaf)	Gossypol (**5**) (μg/mg leaf)	Total heliocides$_{1-4}$ (**7**–**10**) (μg/mg leaf)
Control[a]	0.40 ± 0.013[b]	0.14± 0.07	I.72 ± 1.33
Variants[c]			
2C-G1-B1	0.09 ± 0.10 (77.5%)	0.02 ± 0.01 (85.7)	0.47 ± 0.38 (82.7%)
2C-G3-A1	0 (100%)	0.03 ± 0.01 (78.6%)	0.34 ± 0.18 (80.0%)
2C-G4-A2	0.07 ± 0.03 (82.5%)	0.05 ± 0.02 (64.3%)	1.25 ± 0.32 (27.2%)
2C-H2-A1	0 (100%)	0.03 ± 0.01 (78.6%)	1.27 ± 0.72 (26.2%)
2C-H2-A5	0.41 ± 0.20 (2% increase)	0.08 ± 0.01 (62.9%)	1.34 ± 0.30 (22.1%)
Average % reduction	52%	74%	48%

The values in parentheses are the percent reduction compared to the concentration in control plant leaves.
[a] Data from 15 individual control plants.
[b] Data from 3 replicates of each plant.
[c] Standard deviations.

correlated to **5** content. For the low **5** variant described here, that assumption cannot be valid. Glands visible in leaves show a typical pattern of distribution (Fig. 4(b)). The average number of glands visible in lengthwise cross sections of seed from progeny of the variant was 121 ± 27.5 versus 125.7 ± 22.5 for seed from the non-transformed control (Fig. 4(a)). Similarly, although there was wide variation in the size of glands in both sets of seed, the relative number of dark pixels (representing gland tissue) per seed surface (as determined using image analysis software) was almost identical. Thus the difference in seed gossypol is not simply a consequence of a reduction in number of glands or gland size.

The molecular basis for low levels of gossypol (**5**) in the antisense-free line is not known. Since it segregated from a transgenic plant that did include an inverted copy *cdn1-C1* one possibility is that an interaction initiated from pairing between hetero-alleles led to a rearrangement or loss of a cadinene synthase gene, mimicking the creation of gene "knock-outs". However, such rearrangements would be expected to lead to missing bands or shifts in the sizes of *Hin*dIII fragments that hybridize to the *cdn1-C1* probe, neither of which were detected among the low-gossypol progeny (Fig. 2). Another possibility is retention of a short undetected fragment of the gene that somehow induces post transcriptional gene silencing or RNA inhibition (RNAi)

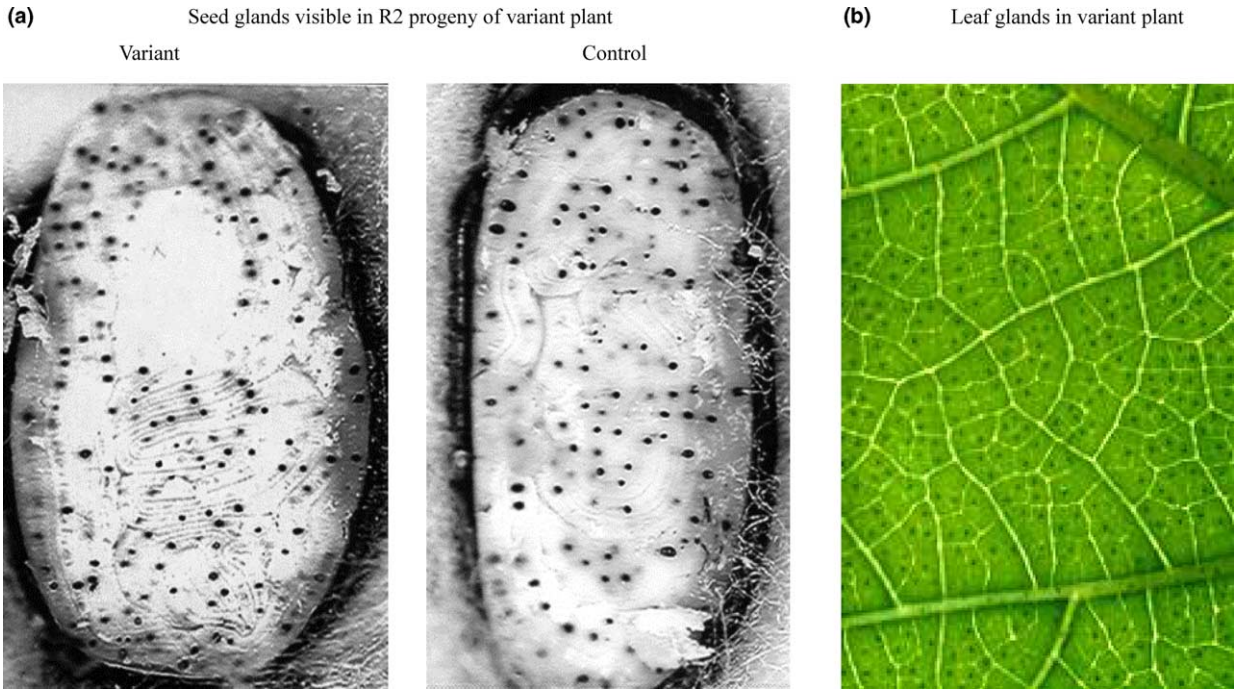

Fig. 4. Pigment glands in cotton tissues. (a) Seeds of a low gossypol variant and a regenerated Coker 312 control. (b) Leaf section from progeny B5 of the low gossypol variant 2C-C1 showing presence of a typical array of glands.

(Vaucheret et al., 2001), but this would also imply retention of an active promoter. Silencing of endogenous genes by homologous transgenes has been reported in maize (Sidorenko and Peterson, 2001) and tobacco (Matzke et al., 2001). In both reports, the changes were shown to be epigenetic, involving paramutation and transvection, respectively. However, in both cases, promoter elements included in the transgene constructs matched those in the host and were implicated in silencing. In this study a CaMV 35S promoter was included in the construct tor antisense expression, so interactions with a CDN synthase gene promoter would not be expected. At this point our best explanation for the origin of the low gossypol plant that includes no trace of transforming DNA remains simply "somaclonal variation". Somaclonal variation is a general term describing recovery of aberrant plants or progeny following regeneration from callus tissue and may involve genetic or epigenetic causes.

3. Conclusions

A plant recovered following self-pollination of a plant transformed to include an antisense version of CDN synthase was found to have significantly lower levels of gossypol (**5**) than the parental cultivar and all other progeny. Because CDN synthase catalyzes the first unique step in gossypol (**5**) biosynthesis, it was assumed the plant was homozygous for one or more copies of the antisense *cdn1-C1*. However, as opposed to the original transformed parent or other low-gossypol progeny, no trace of any segment of the transforming DNA was found in the low gossypol plant. Progeny from self-pollination of the plant were consistently low in gossypol (**5**) in both leaves and seeds. Seed gossypol (**5**) level in these plants is very near the level considered safe for human consumption. Whether the original transformation event led to disruption and loss of expression of one or more members of the CDN synthase gene family, or if low gossypol (**5**) levels resulted from an event completely independent of the transformation, the recovered plant provides a potentially useful source of low gossypol cotton.

In glandless cotton the combination of recessive genes *gl2gl2*and *gl3gl3*results in the suppression of both lysigenous glands and the initiation of the formation of terpenoid aldehydes. Data from the cotton variant described here clearly indicates an uncoupling of gland and terpenoid aldehyde formation similar to Brubaker's work (Brubaker, 1996). The cotton variant differs from the Australian *Gossypium* subspecies *Hibiscoidea* and *Sturtia* in that mature glands are present in the cottonseed. It is concluded that since the terpenoid aldehydes are markedly suppressed in the cotton variant, the low terpenoid aldehyde trait reflects the initiation of their biosynthesis and may be related to the molecular signal affecting biosynthesis independently of gland morphogenesis. The reduction is independent of gland number and appears to show stable inheritance, so offers an

alternative, potentially additive, source for the long sought goal of low gossypol seed.

4. Experimental

4.1. Vector sources and construction

The cloning plasmid, pRTL2 (obtained from James Carrington, Biology Dept., TAMU) which has a dual 35S Cauliflower Mosaic Virus (CaMV) promoter was used to create an antisense cassette of *cdn1*-C1 (obtained in pBluescript SK from Peter Heinstein, Purdue University). Digestion of both plasmids with restriction endonucleases *Xho*I and *Bam*HI permitted religation of the *cdn1*-C1 fragment into pRTL2 in an inverted configuration while removing a TEV translation leader from pRTL2. The resulting 35S-anti-*cdn1*-C1 cassette was then excised for direct transfer into the *Pst*I site of binary vector pCGN1578 (obtained from Calgene). The result was a vector with a T-DNA segment that included the CaMV36S-antisense *cdn1*-C1-CaMV35S terminator complex near the right border together with a similar cassette for expression of *npt*II to permit selection of transformed callus on kanamycin. The T-DNA vector was introduced into *A. tumefaciens* strain EHA101 for plant transformation.

4.2. Recovery of transformed antisense and control plants

Following protocols developed by Firoozabody et al. (1987) and Bayley et al. (1992), hypocotyl sections of *Gossypium hirsutum*L. cv. Cqker 312 were incubated for 3 days with the modified *A. tumefaciens*before being transferred to medium containing carbenicillin (400 μg/ml) and kanamycin sulfate (50 μg/ml) and placed in a 28 °C incubator with illumination for 16 h per day. Calli that arose after 3 weeks were transferred to fresh medium with the same antibiotics and thereafter, subcultures were started every 4–6 weeks.

Friable, embryogenic sectors that developed were transferred to regeneration medium without hormones. Developing embryos that displayed both cotyledons and radicles were transferred to peat pots and eventually to a greenhouse designated for transgenic plant containment. Regenerated control plants were initiated under identical conditions following treatment with *Agrobacterium* lacking the antisense *cdn1*-C1 cassette.

4.3. Verification of transformed plants by Southern hybridization and PCR

DNA was extracted from young leaves after grinding in liquid N_2. The buffers and protocols described by Paterson et al. (1993) were followed with minor exceptions, in that centrifugation was at 3000*g* rather than 2700*g* and the centrifugation following chloroform:isoamyl alcohol extraction of proteins was for 15 rather than 5 min. Dissolved DNA was treated with RNAse (40 μg/ml) and reprecipitated before use.

Following digestion with *Hin*dIII, *Pst*I or *Kpn*I, 10 μg samples of DNA were loaded into wells for electrophoresis in 1% agarose gels. Blotting onto Zeta-Probe GT membrane was as recommended by the supplier (Bio-Rad). Labeling of both the *cdn1*-C1 cDNA and *npt*II probes with [α-^{32}P] dCTP took advantage of a random priming kit purchased from GibcoBRL. Hybridization, washing and detection on Kodak XR-5 film followed standard procedures (Sambrook et al., 1989).

Polymerase chain reaction (PCR) amplification to test for the presence of contaminating Agrobacterium used primers specific for the *VirD4* region which is not included in the T-DNA (Porter et al., 1987). Primer sequences and reaction conditions were described previously (Martin et al., 2003). Tests for the presence of cotton DNA flanking the right border of T-DNA took advantage of a modified adaptor-ligation PCR technique and a pair of nested primers in the T-DNA right border (Spertini et al., 1999). DNA samples (1 μg) extracted from plant 2C-C1 and a control transformed plant (LT3) were digested at 65 °C overnight with *Taq*I. Double stranded adaptors with *Taq*I overhangs were prepared and ligated to the *Taq*I fragments with T4 DNA ligase as directed (Spertini et al., 1999). The resulting adaptor-tailed *Taq*I fragments were then subjected to nested amplification with two pairs of primers where one primer targets RB sequences and the other the adaptor (Spertini et al., 1999). Hot start conditions and Accutaq LA polymerase (Sigma) were used to permit long extensions. Cycling parameters for both primer sets were as described, including the recommended increase to 37 rather than 32 cycles in the first round due to the relatively large genome of cotton. Following completion of the second-round of amplification with nested adaptor and RB primers, the products were subjected to agarose gel electrophoresis and examined under UV illumination using ethidium bromide for band detection.

4.4. Analysis of cottonseed gossypol (**5**) content

The amount of **5** in individual seeds was determined via HPLC(Stipanovic et al., 1988). The powder from individual seeds ground in liquidN_2 was transferred to a 125 ml Erlenmyer flask and extracted by adding 20 ml of EtOH:H_2O:ether:HOAC (59:24:17:02), capping and shaking on a gyratory shaker at 350 rpm for 30 min at room temp. Each extract was filtered through a Whatman #1 filter into a 50 ml flask using a Buchner funnel. One ml of each sample was removed and filtered through a 0.45 μm filter into a crimp-top vial for HPLC analysis. HPLC was performed on a Scientific Glass

Engineering 250 GL4-ODS-H12/5 analytical column using a Hewlett–Packard 1090 Liquid Chromatograph equipped with a diode array detector and multichannel integrator. Conditions and solvents were as described by Martin et al. (2003). Quantities of **5** in each sample were calculated based on a calibration curve made using 25–6000 ng of authentic **5**, which eluted at 10.37 min. The curve fits a linear model ($Y = 1238X - 8.11$) where Y is the area of the peak at 10.37 min in mAU and X is the amount of **5**, in μg.

4.5. Analysis of hemigossypolone (6) and heliocides in leaves

Fully expanded leaves from the 3rd to 5th node down from the apex of the main stem were collected, frozen in liquid N_2 and lyophilized. Individual dried leaves were ground in a mortar and 100 mg of the resulting powder was transferred to a 125 ml Erlenmeyer flask. For extraction, hexane:EtOAc (3:1 v/v, 10 ml), 10% HCl (20 ml) and glass beads (15 ml) were added before capping the flask and shaking for 30 min at room temp on a gyratory shaker. Extracts were filtered through fritted glass into a 50 ml pear-shaped flask. The residue from grinding was washed with hexane:EtOAc (3:1 v/v, 3×5 ml) and the washings were also filtered into the pear-shaped flask. The residue remaining after removing the solvent with a rotary evaporator was dissolved in four consecutive washes with hexane:EtOAc (3:1 v/v, 150 ml). The washes were combined and loaded onto a silica Sep-Pak. The Sep-Pak was dried with N_2 and the sample was eluted with isopropyl alcohol:acetonitrile:H2O :EtOAc (37:22:41:6, 5 ml). The eluant (1 ml) was taken for HPLC analysis as described for **5**.

The amounts of **5**, **6** and **7**–**10** were determined by comparison to standard curves made using the authentic compounds **5**, **6**, **7** and **8**. Amounts (in μg) of **6** (retention time 3.09 min) were calculated using the equation $Y = 1810X - 33.54$ where Y is the area of the HGQ peak in mAUs and X is the amount. The equation for **7**–**10** (retention times 11.27, 11.68, 12.84 and 13.59, respectively) is $Y = 1652X - 89.70$ in all cases.

4.6. Image analysis

Surfaces of split seeds were examined under an Olympus dissecting microscope with a Panasonic KR22 digital camera attached to a desktop computer running Image Pro Plus (V4.5). Images were captured and the interior part of the seed defined using the wand tool. After adjusting contrast to eliminate below-surface glands, the number of pixels in dark area (glands) and light areas were calculated and recorded. Glands visible at the surface were manually counted from prints.

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