RNAi construct of a cytochrome P450 gene CYP82D109 blocks an early step in the biosynthesis of hemigossypolone and gossypol in transgenic cotton plants

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Naturally occurring terpenoid aldehydes from cotton, such as hemigossypol, gossypol, hemigossypolone, and the heliocides, are important components of disease and herbivory resistance in cotton. These terpenoids are predominantly found in the glands. Differential screening identified a cytochrome P450 cDNA clone (CYP82D109) from a Gossypium hirsutum cultivar that hybridized to mRNA from glanded cotton but not glandless cotton. Both the D genome cotton Gossypium raimondii and A genome cotton Gossypium arboreum possessed three additional paralogs of the gene. G. hirsutum was transformed with a RNAi construct specific to this gene family and eight transgenic plants were generated stemming from at least five independent transformation events. HPLC analysis showed that RNAi plants, when compared to wild-type Coker 312 (WT) plants, had a 90% reduction in hemigossypolone and heliocides levels, and a 70% reduction in gossypol levels in the terminal leaves, respectively. Analysis of volatile terpenes by GC–MS showed that the RNAi plants were reduced in scent activity. HPLC analysis showed that RNAi plants, when compared to wild-type Coker 312 (WT) plants, had a 90% reduction in hemigossypolone and heliocides levels, and a 70% reduction in gossypol levels in the terminal leaves, respectively. Analysis of volatile terpenes by GC–MS showed that the RNAi plants were reduced in scent activity.

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1. Introduction

Cotton plants produce two classes of defense compounds from δ-cadinene (1): those derived from 7-hydroxyxcadalene (6), such as lacinilene C (11a) (LC) (Fig. 1), and those derived from 8-hydroxyxcadalene, such as desoxyhemigossypol (14a) (dHG) and gossypol (16a) (Fig. 2) (Davis and Essenberg, 1995). The 8-hydroxyxcadalene derivatives are present in healthy cotton plants. Gossypol (16a) occurs in the glands of cotton seed, and gossypol (16a), hemigossypolone (18) (HGQ), and heliocides (21–24) (Fig. 2) occur in the glands of the foliage. These compounds protect plants from herbivores (reviewed in Liu et al., 2015). Gossypol (16a), gossypol-6-methyl ether (16b) (MG), gossypol-6,6-dimethyl ether (16c) (DMG), hemigossypol (15a) (HG), and hemigossypol-6-methyl ether (15b) (MHG) are found in the epidermis and cortex of roots (Mace et al., 1974). After infection of xylem vessels with fungal and bacterial pathogens, dHG (14a), desoxyhemigossypol-6-methyl ether (14b) (dMHG) and related compounds (15 and 16) are found in stem stele tissue of infected plants (Mace et al., 1976; Stipanovic et al., 1975a). HG (15a) and dHG (14a) are toxic to fungi and bacteria (Abraham et al., 1999; Mace et al., 1985; Zhang et al., 1993).

The 7-hydroxyxcadalene derivatives [2,7-dihydroxyxcadalene (7a) (DHC), 2-hydroxy-7-methoxycadalelne (7b) (HMC), LC (11a), and lacinilene C 7-methyl ether (11b) (LCME)] are formed in cotton leaves and cotyledons in response to the bacterial blight pathogen (reviewed in Delannoy et al., 2005). These compounds also occur in senescing bracts and gin dust (Greenblatt and Beier, 1983; Stipanovic et al., 1975b). DHC (7a), LC (11a), and LCME (11b) are...
Fig. 1. Proposed intermediates in the biosynthesis of lacinilene C (11a) in Gossypium, and are referred to as 7-hydroxycadalene derivatives (2–11).

Fig. 2. Proposed intermediates in the biosynthesis of gossypol (16a) and heliocides (21–24) in Gossypium, and are referred to as 8-hydroxycadalene derivatives (13–18 and 21–24).
toxic to Xanthomonas campestris pv. malvacearum, the cause of bacterial blight (Essenberg et al., 1990). In field-grown glanded cotton plants, LC (11a) was detected in all plant parts except roots and seeds, and LCME (11b) was found in all cotton parts (Muller et al., 1983).

Only a few enzymes involved in the synthesis of the 7- and 8-hydroxydacadene derivatives (2–11 and 13–24) have been identified. δ-Cadinene synthase was identified and molecularly characterized by Chen et al. (1995). Seed specific RNAi of δ-cadinene synthase was used to generate gossypol-free seed with normal levels of gossypol-related terpenoids in foliage (Sunilkumar et al., 2006). Luo et al. (2001) identified a gland specific cytochrome P450, δ-cadinene 8-hydroxylase (CYP706B1) from Gossypium arboreum. Recombinant CYP706B1 catalyzed the hydroxylation of δ-cadinene (1) to 8-hydroxy-(+)-δ-cadinene (13), a precursor to HG (15a) (Wang et al., 2003). The chemical structures of δ-cadinene (1) and HG (15a) suggests that many additional cytochrome P450 enzymes are necessary for HG (15a) synthesis.

In the present study, a new cytochrome P450 gene involved in the hemis gossypol pathway has been identified from glanded Gossypium hirsutum. RNAi was used to generate transgenic cotton plants that no longer express this gene and its paralogs to confirm that this cytochrome P450 hydroxylase encoded by this gene family is involved in the synthesis of the 8-hydroxydacadene derivatives. The constitutive and induced foliar levels of cotton sesquiterpenes were compared between WT and RNAi plants. A new volatile terpene intermediate was identified in the terminal leaves of the RNAi plants. The results herein indicate that the RNAi construct is blocking the synthesis of dHG (14a), and that there is likely cross-regulation between the 7- and 8-hydroxydacadene derivative (2–11 and 13–24) biosynthetic pathways.

2. Results and discussion

2.1. Identification and cloning of a gossypol pathway cytochrome P450 hydroxylase, CYP82D109

Glandless cotton contains no or extremely low levels of gossypol (16a) and related terpenoids (14–24) in its seeds and green tissues, yet it produces gossypol (16a) in the roots. Thus, activation of the gossypol (16a) pathway is not expected in healthy leaf tissue of glandless plants. Additional cytochrome P450 enzymes involved in the gossypol (16a) biosynthetic pathway in glanded cotton were sought. Degenerate primers were used to amplify cytochrome P450 fragments from glanded Paymaster 1218 BG/RR (PM1218) cotton leaf cDNA, and the resulting PCR products were cloned. Gland-associated clones were identified by differential hybridization using probes derived from a glandless Acala cotton leaf cDNA library. The positive clones were further analyzed by RT-PCR; only two clones were found to be expressed in the leaves of glanded cotton, but not in the leaves of glandless cotton. One clone had 99% identity with the G. arboreum δ-cadinene 8-hydroxylase, a cytochrome P450 monooxygenase previously described (Luo et al., 2001). The second was unknown, but had high identity with a soybean cytochrome P450 82A4 (59%); therefore, this unknown clone was further characterized.

A Virtual Northern of leaf mRNA from glanded PM1218 and glandless Acala cotton showed that the transcript corresponding to the unknown cytochrome P450 clone is about 1.9 kb in size, and was expressed in glanded cotton but not in glandless cotton (Fig. S1). The full length cDNA was obtained using the method of Israel (1993). Sequencing gave a cytochrome P450 hydroxylase cDNA of 1935 bp with an ORF that codes for 522 amino acids. The corresponding genomic DNA has a single 96 bp long intron.

The cloned cDNA is predicted to encode a functional cytochrome P450, and the deduced amino acid sequence is 58% identical to CYP82D4 from Vitis vinifera. Therefore, the encoded protein was placed in the CYP82D subfamily and designated as CYP82D109 (David Nelson, Dept. of Microbiology, Immunology and Biochemistry, University of Tennessee, personal communication). CYP82D109 is only 28.3% identical at the amino acid level to cotton CYP706B1 responsible for the 8-hydroxylation of δ-cadinene (1). Sequence analysis and sequence comparison with the sequence of mammalian cytochrome P450 2b4: 1P05 chain A, whose crystal structure is known (MMDb: 1P05_A), resulted in detection of several structural motifs characteristic of eukaryotic cytochrome P450s. The highly conserved heme-binding motif FxxGxxRxCG (Chappell, 1998) was found in CYP82D109 as FGSGRRSCPG and in 1P05 chain A as FSLGKRCGLG whose cysteine side-chain forms a coordinate bond with Fe in the axial position through a sulfur atom on the opposite side of the opening of the active site. The threonine-containing pocket for binding an oxygen molecule with a consensus sequence of (A/G)Gx(D/E)T(T/S) (Durst and Nelson, 1995) was found as AGSDTT in CYP82D109 and as AGTETT in 1P05 chain A, where the pocket is formed by an α helix (AGTETT is part of the helix) running over one side of the flat heme group in the opening of the active side.

Orthologs, their paralogs, and closely related genes of the cloned G. hirsutum CYP82B109 (GhCYP82B109) were identified by BLAST search in the recently available genomes of G. raimondii and G. arboreum (Phytozome8.0 and http://cgp.genomics.org.cn/page/species/index.jsp, respectively). G. hirsutum is an allotetraploid (AD1) derived from a cross between A genome and D genome ancestors resembling the extant G. herbaceum or G. arboreum and G. raimondii (Grover et al., 2008; Paterson et al., 2012). Five genes were identified in each genome and all belong to the CYP82D subfamily. They were designated as G. raimondii or G. arboreum CYP82D109, CYP82D110, CYP82D111, CYP82D112, and CYP82D113 (David Nelson, Dept. of Microbiology, Immunology and Biochemistry, University of Tennessee, personal communication) and their phylogenetic relationship based on nucleotide sequences is given in Fig. 3. The G. raimondii ortholog of GhCYP82D109, Goral.009G331600 (GrCYP82D109) on chromosome 9 is more identical at the DNA level to GhCYP82D109 (99.5%) than is the G. arboreum ortholog, Cotton_A.30463 (GaCYP82D109) on chromosome 10 (97.1%). Therefore, the cloned GhCYP82D109 is a D chromosome homeolog. The GrCYP82D109 and GaCYP82D109 pair shares 97.2% identity. G. raimondii and G. arboreum orthologous pairs GrCYP82D110 and GaCYP82D110, GrCYP82D111 and GaCYP82D111, GrCYP82D112 and GaCYP82D112, GrCYP82D113 and GaCYP82D113 share 98.5%, 97.4%, 97.8%, and 97.9% identities, respectively. GaCYP82D112 and GaCYP82D113 on chromosome 8 were reannotated from Cotton_A.13708 and the combination of Cotton_A.13709 and Cotton_A.13710, respectively, as shown in Fig. S2. GaCYP82D110 was identified on chromosome 7 at positions 115079741–115081619 and the corresponding coding DNA sequence is presented in Fig. S2.

CYP82D111, CYP82D112 and CYP82D113 are three additional paralogs present in both the G. raimondii and G. arboreum genome with nucleotide identity to the cloned GhCYP82D109 ranging from 90.8% to 92.8% (Fig. 3). High sequence homology suggested that all of these genes code for the same functional enzyme to catalyze the same enzymatic reaction step, and therefore constitute a gene family. Syntenic analysis of these paralogs indicates that direct repeat triplication occurred before species divergence of G. raimondii and G. arboreum. Then the upstream copy CYP82D111 underwent inversion in G. raimondii and translocation to chromosome 4 in G. arboreum independently in each respective species (Fig. S3). A remnant 456 bp C-terminal copy containing Cotton_A.13707 was left behind in G. arboreum. Surprisingly, this remnant had highest...
The presence of 1.19 < 3.84; expected 1:1) in the outcross plants 124R, 73R, and 73RC (Fig. S4A), indicating that these plants are likely from calli originated from the same transformation event. In contrast to the progeny lines (Fig. S4), Lines 73R and 85R were further characterized. The probe for Northern analysis was expected to detect the mRNA from CYP82D109 and its paralogs. The full-length mRNAs of CYP82D109 and its paralogs are absent in the leaves of plants from these two independent lines (Fig. S5), indicating that the RNAi construct is very effective in blocking the target gene family.

2.3. Segregation of RNAi phenotype

The reduced terpenoid level phenotype associated with the RNAi construct was observed in subsequent T1 and/or F2 out-crossed progenies derived from 73RA, 73RC, 85RA, and 85RB RNAI plants, representing at least two independent transformation events. 73RA and 73RC were fully fertile. The RNAi transgene segregated as a single dominant locus in the T1 progeny (16 mutant: 4 WT, \( X_{0.05}^2 = 0.27 < 3.84 \); expected 3:1) from each of the two plants. The T1 progeny containing the RNAi transgene all have reduced levels of terpenoids in leaves. Fig. 4 with its large differences in terpenoid aldehyde levels between WT and RNAI plants, and its small variations within the lines clearly shows the reduced levels of terpenoid aldehydes (16a, 18, and 21–24) in the T1 progeny of 73RA compared to the WT and the segregation of the transgene. These results are a confirmation of those shown in Table 1 which came from an earlier experiment for the T0 plants. The presence of the RNAi transgene correlates with reduced levels of the terpenoid aldehydes (16a, 18, and 21–24) present in the leaves in all tests. The strong linkage of the transgene and reduced levels of terpenoid aldehydes (16a, 18, and 21–24) (total) in the leaves of 85RA and 85RB plants or the progeny lines (Fig. S4). Mean ± SE is given for WT (N = 6 leaves). The mean of two biological replicates is given for 85RA and 85RB.

Transgene insertions into the genome in the lines 124R, 73R, and 85R were confirmed by Southern hybridization of the T0 plants or the progeny lines (Fig. S4). Lines 73R and 85R were further characterized. The probe for Northern analysis was expected to detect the mRNA from CYP82D109 and its paralogs. The full-length mRNAs of CYP82D109 and its paralogs are absent in the leaves of plants from these two independent lines (Fig. S5), indicating that the RNAi construct is very effective in blocking the target gene family.

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method based on the coding DNA sequences of the G. hirsutum CYP82D109 orthologs, their paralogs, and closely related genes in the genome of G. raimondii and G. arboreum. Distances were determined by Kimura's two-parameter model. Bootstrap frequencies from 1000 replications are noted next to the branches. The scale is equivalent to 2 nucleotide substitutions per 100 bp. Numbers in parenthesis indicate percent identities with G. hirsutum CYP82D109 DNA sequence.

### Table 1

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2.2. Phenotypes of RNAi plants

To determine the function of the cytochrome P450 hydroxylase encode by CYP82D109 gene family in cotton, a RNAI construct was generated and this construct was used to generate G. hirsutum cotton plants with reduced expression of the gene family. The RNAI 495 bp fragment (positions 457–951 bp in accession number KMS01031) had greater than 89.6% DNA sequence identity with the construct. Analysis of the sesquiterpenoid content of the terminal leaves of eight transgene-containing plants all have reduced levels of terpenoid aldehydes (16a, 18, and 21–24) (4–32% of WT), and heliocides (21–24) (1–15% of WT), and heliocides (21–24) (1–15% of WT) (Fig. 2) present in terminal leaves compared to WT parents. These reductions were greater than that achieved by the antisense suppression of a gossypol (16a) pathway gene (Martin et al., 2003), demonstrating the key involvement of the CYP82D109 family in the gossypol (16a) pathway.

The RNAI transgene was detected in all T0 plants by PCR (not shown), except for 520R which died unexpectedly before a leaf sample was harvested for DNA isolation. The T0 plants 124R, 1315RA, and 1315RB were infertile and died without progeny.

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transgene with the phenotype along with its heritability suggests that the phenotype we have described is caused by the RNAi construct knocking-down the expression of the CYP82D109 gene family, and that this gene family is involved in an early step in the gossypol pathway.

2.4. Volatile terpene analysis

The volatile terpene profile of the RNAi plants was analyzed to determine alterations in the metabolites in the mutant that might be related to the CYP82D109 gene family coded enzyme. Volatile terpenes were extracted from terminal leaves of the T0 plants and analyzed by GC–MS (Elzen et al., 1985; Fig. 5). An unidentified peak with a retention time (RT) of 18.64 min and a molecular mass of 218 amu was found in the T0 RNAi leaves but not in the WT leaves. This same volatile terpene profile was also seen in the T1 siblings that contain the RNAi transgene, but not in the siblings that do not contain the transgene (data not shown). The compound had a base peak at m/z 175 (100%) and a fragment ion at m/z 147 (52%). The latter ion is consistent with the loss of an isopropyl group from the parent ion, with the subsequent loss of C=O from the base peak. Overall, the mass spectrum was consistent with

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**Fig. 4.** Reductions in levels of hemigossypolone (18) [HGQ], gossypol (16a), and total heliocides (21–24) co-segregate with the RNAi transgene in terminal leaves of the T1 progeny. The segregation of the transgene is shown by PCR in the bottom panel. The top band is the amplicon of RNAi insert, absence of which indicates absence of the transgene. The bottom band is the amplicon of 53EPS gene, serving as a positive control.
Fig. 5. GC–MS chromatograms showing volatile terpenes present in terminal leaves of WT and T2 RNAi plants representing two independent lines. The retention times (min) for the known volatile peaks are as follows: 8.19 – α-pinene, 8.87 – β-pinene, 8.97 – myrcene, 9.66 – trans β-ocimene, 9.86 – cis β-ocimene, 15.56 – β-caryophyllene, and 15.92 – α-humulene. The peak at 16.28 min was a contaminant (i.e. butylated hydroxyl toluene). Peaks at 14.98, 15.10, 15.68, 16.42, and 16.64 min were unknown. The new peak at 18.64 min in RNAi lines is indicated by an arrow.

The compound being a derivative of δ-cadinene (1). Based on this assumption, the compound would have 5 degrees of unsaturation giving a proposed formula of C13H20O. RNAi leaves were extracted and careful evaporation of the extract provided a concentrated of the compound. The concentrate was then subjected to purification using a GC in which the effluent was split to allow collection of the unknown terpene. This sample was then subjected to NMR spectroscopic analysis.

The 1H-NMR spectrum showed a single proton (δ 6.72, C-4; see Fig. 1, structure (4aR,5S)-δ-cadinene-2-one (3) for position numbering) attached to a sp² carbon. A COSY experiment showed this proton was weakly coupled to a methine proton at δ 2.84 (C-4a), and weakly coupled to a methyl group (δ 1.76, C-9). This methyl group showed coupling to one of a pair of methylene protons at δ 2.81 (C-1). This methylene proton also showed weak coupling to a methyl group at δ 1.62 (C-10), and weak coupling to the methylene protons at δ 1.95 (C-7). The methine proton at δ 1.22 (C-5) also showed coupling to the proton at δ 2.84 (C-4a). An isopropyl methine proton showed the expected coupling to two methyl groups (δ 0.84, C-12; δ 1.00, C-13). This supported our assumption that the compound was related to δ-cadinene (1).

The 13C-NMR spectrum indicated the presence of a carbonyl group (ς δ 199.5, C-2) in agreement with the mass spectral fragmentation pattern. HMBC (Fig. S6) and HSQC experiments further clarified the structure. Two sets of carbon–carbon double bonds were evident at δ 125.1 (C-8a) and δ 128.6 (C-8), and δ 134.4 (C-3) and δ 148.0 (C-4), with the latter having the attached proton. The structure shown in Fig. 1 was proposed with the location of the carbonyl carbon and the protons on the methyl group at C-9 and the sp² proton at δ 6.72. This compound, called δ-cadinene-2-one (3), was previously isolated from Heterotheca grandiflora collected in Hawaii (El-Dahmy et al., 1986). There was good agreement between the 1H-NMR spectra reported by El-Dahmy et al. (1986) and that of the cotton compound eluting from the GC at 18.64 min.

A carbonyl group at the 2 position was unexpected given that δ-cadinene-8-hydroxylase has previously been identified as one of the early steps in the gossypol (16a) pathway (Luo et al., 2001). Neither HG (15a) nor gossypol (16a) has a hydroxyl group at C-2. However, enolization of the carbonyl group at C-2 and rearrangement of the C-8–C-8a double bond of δ-cadinene-2-one (3) produces 7-hydroxycalamenene (4), a proposed lacinilene C (11a) pathway intermediate (Davila-Huerta et al., 1995). GC analysis with a longer analysis time of a concentrated leaf extract from healthy RNAi plants indicated the presence of additional compounds, 7-hydroxycalamenene (4) (retention time 20.78 min) and (8S)-(−)-3-hydroxy-α-calacorene (5) (retention time 19.29 min) based on retention times and mass spectra of standards. The latter also is a proposed intermediate of the lacinilene C (11a) pathway (Stipanovic et al., 2006). In addition, a compound with a MS spectrum virtually identical to that of (8S)-(−)-3-hydroxy-α-calacorene (5) was also identified; this may be (85S)-(−)-3-hydroxy-β-calacorene (8) (Fig. 1). These three compounds were present at lower concentrations than δ-cadinene-2-one (3). The carbon-5 (see Fig. 1 for carbon numbering) has the S configuration in δ-cadinene (1) and 3-hydroxycalamene (5) in cotton (Stipanovic et al., 2006). Thus, it is proposed that carbon-5 also has the S configuration in δ-cadinene-2-one (3) in Gossypium.

A proposed biosynthetic pathway to lacinilene C (11a) is shown in Fig. 1 with route A from 7-hydroxycalamenene (4), and involves 7-hydroxycedaradene (6) as an intermediate. Based on HPLC retention time and UV spectra, the latter compound has been identified in Thespisia populnea, a small tree that also produces gossypol (16a) (Puckhaber and Stipanovic, 2004), and in cotton stele tissue.
inoculated with the plant pathogen *Verticillium dahliae* (unpublished). *Wang et al.* (2003) proposed an alternative pathway (route B in Fig. 1) for the biosynthesis of lacinilene C (11a) involving 7-hydroxycadalene-2-one (10) as an intermediate. In tritium labeled experiments, *Davis and Eisenberg* (1995) showed that both 7-hydroxycadalene (4) and 7-hydroxy-3-carotenone-2-one (10) were derived from 3-cadinene (1). In routes A and B, 3-hydroxy-

2.5. Leaf terpenoid response to pathogen challenge

The discovery of 3-cadinene-2-one (3) prompted analysis of fully expanded cotton leaves of the RNAi plants for the presence of lacinilenes. Thus, a fully expanded leaf (3rd from apex) was cut from the plant, immediately frozen, and then freeze-dried. The dried tissue was extracted and the extract submitted for HPLC terpenoid analysis (Table 2) using two methods. First, an aliquot of each sample was analyzed for the inducible terpenoid derivatives of 7-hydroxycadalene (6) [i.e., DHC (7a), HMC (7b), LC (11a), LCME (11b) (Fig. 1)] and the inducible terpenoid derivatives of 8-hydroxycadalene [dHG (14a), dMHG (14b), HGAL (17), HG (15a), MHG (15b) (Fig. 2)] using the HPLC ‘stem’ method. Another aliquot was then analyzed by the HPLC ‘leaf’ method which separates gossypol (16a) and the heliocides (21–24) (Fig. 2). HGQ (18), gossypol (16a), and heliocides (21–24) are constitutively expressed in healthy Gosshyposis leaves and their concentrations decrease as leaves expand and mature (Table 1 vs Table 2). A major contributor for the decrease in 8-hydroxy-

**Table 3** Sequofterpenoids present in fully expanded, healthy freeze-dried 3rd leaves from WT and RNAi plants.ª

<table>
<thead>
<tr>
<th>Compound</th>
<th>(µg compound/g dry tissue)</th>
<th>WT (N = 10)</th>
<th>RNAI (N = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lacinilenes (inducible)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacinilene C (LC) (11a)</td>
<td>2.1 ± 0.2</td>
<td>0.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>2,7-Dihydroxycadalene (DHC) (7a)</td>
<td>4.3 ± 2.1</td>
<td>0.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Lacinilene C methyl ether (LCME) (11b)</td>
<td>0.2 ± 0.2</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td>7-Hydroxy-2-methoxycadalene (HMC) (7b)</td>
<td>1.7 ± 1.6</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td><strong>Hemigossypol related compounds (inducible)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemigossypol (HG) (15a)</td>
<td>0.3 ± 0.3</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td>Desoxyhemigossypol (dHG) (14a)</td>
<td>Nd</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td>Hemigossylic acid acetate (HGAL) (17)</td>
<td>Nd</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td>Hemigossypol-6-methyl ether (MHG) (15b)</td>
<td>Nd</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td>Desoxyhemigossypol-6-methyl ether (dMHG) (14b)</td>
<td>Nd</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td><strong>Leaf terpenoid aldehydes (constitutive)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gossypol (16a)</td>
<td>41 ± 9</td>
<td>5 ± 2</td>
<td></td>
</tr>
<tr>
<td>Hemigossypolone (HGQ) (18)</td>
<td>17 ± 5</td>
<td>Nd</td>
<td></td>
</tr>
<tr>
<td>Heliocide H4 (22)</td>
<td>68 ± 27</td>
<td>6 ± 5</td>
<td></td>
</tr>
<tr>
<td>Heliocide H1 (21)</td>
<td>105 ± 40</td>
<td>13 ± 8</td>
<td></td>
</tr>
<tr>
<td>Heliocide H3 (24)</td>
<td>78 ± 19</td>
<td>5 ± 3</td>
<td></td>
</tr>
<tr>
<td>Heliocide H2 (23)</td>
<td>214 ± 50</td>
<td>21 ± 9</td>
<td></td>
</tr>
</tbody>
</table>

ª RNAI-containing plants included in this table were F1 progeny of FM958 X 73RC and DP5415 X 73RC, and T1 progeny of 85RA. All plants were confirmed to contain the transgene by PCR. Mean ± SE is given for each compound. Nd = not detected.
upon inoculation with Fov11 (Table 3). Only a ca. 6% of the increase was devoted to lacinilene (7 and 11) branch. In Fov11 inoculated RNAi leaves, the total concentration of inducible sesquiterpenoid was almost twice the amount observed in inoculated WT leaves, and this induction was channeled solely into the lacinilene pathway (2–11) (7-hydroxycadalene derivatives (7 and 11), Table 3). These results suggest that the 7-hydroxycadalene derivative pathway (Fig. 2) is disabled in the RNAi plants, and the plants respond by diverting its terpenoid biosynthetic potential to compounds in the lacinilene C pathway when activated by the pathogen (Fig. 1).

A possible mechanism for δ-cadinene-2-one (3) buildup in healthy RNAi plants is that δ-cadinene (1) accumulates due to blockage of 8-hydroxycadalene derivative pathway. Leaky suppression of the 7-hydroxycadalene derivative pathway genes allows the synthesis of δ-cadinene-2-one (3) and some downstream compounds (4, 5, and 8) from the increased pool of δ-cadinene (1). In healthy wild type plants, δ-cadinene (1) is efficiently channeled into the constitutive 8-hydroxycadalene derivative (16a, 18, 21–24) pathway.

3. Conclusions

A new cytochrome P450 gene (CYP82D109) has been cloned that is present in the leaves of glanded cotton and absent in glandless cotton leaves. The cytochrome P450 hydroxylase encoded by this gene and its family members is involved in the biosynthesis of gossypol-related compounds. Both G. raimondii and G. arboreum contain small gene families that are more than 90% identical to CYP82D109 from G. hirsutum.

RNAi knock-down of the gene family reduced the levels of all the major foliar constitutive terpenoid aldehydes (i.e., heliocides (21–24), HGQ (18), and gossypol (16a)) with overall reduction being greater than 93%. This RNAi phenotype was associated with the buildup of a new volatile compound, δ-cadinene-2-one (3), in the leaves. This compound may be derived from an as yet to be identified δ-cadinene-2-ol (2) which has a hydroxyl group in common with the 7-hydroxycadalene derivatives (i.e., LC (11a), DHC (7a), LCME (11b), and HMC (7b)) and may serve as a precursor to the lacinilenes. 7-Hydroxycadalene derivatives (7 and 11) were not constitutively found in either RNAi or WT healthy leaves. In wild type leaves, pathogen induction induces the formation of both

4. Experimental

4.1. Biological materials

G. hirsutum cotton cultivars used in these experiments were: Coker 312 (WT), an Acala glandless line, Paymaster 1218 BG/RR (PM1218), Deltapine 5415 (DP5415), FiberMax 958 (FM958), and FiberMax 966 (FM966). All cotton plants were grown in the greenhouse as previously described (Robinson et al., 2007). Cotton tissue cultures were grown in a Percival incubator (Percival Scientific, Perry, IA, USA) with a 16 h photoperiod, 28 °C day, and 25 °C night. Agrobacterium tumefaciens LBA4404 (Invitrogen, Life Technologies, NY, USA) was used for cotton transformation. F. oxysporum f. sp. vasinfectum isolate Fov11 (race 1 on cotton; ATCC#46644) was grown as previously described (Liu et al., 2011) for the in vitro fungal challenge.

4.2. Cloning of CYP82D109 cDNA, Virtual Northern, and phylogenetic analysis

The isolation of a cDNA encoding a putative gossypol pathway hydroxylase was performed in multiple steps. First, cDNA from glanded and glandless cotton were synthesized from total RNA extracted from leaves of glanded PM1218 and Acala glandless cotton, respectively, using Super SMART™ PCR cDNA Synthesis Kit (Clontech Laboratories, Palo Alto, CA, USA) following the
manufacturer's recommendations. Both cDNA were labeled with DIG using DIG DNA Labeling and Detection Kit (Roche) following the manufacturer's recommendations. A degenerate primer LP3GSP1 (5’-CGAGARGARTYMGCCIGARMG-3’), corresponding to the conserved peptide sequence of about 20 amino acids upstream of the heme-binding domain of plant cytochrome P450 monooxygenases, paired with AUAP (Clontech) was used to amplify cytochrome P450 cDNA fragments from cDNA of PM1218. The PCR products were cloned into pUC 18 and transformed into *Escherichia coli*. Plasmids were isolated from individual clones.

Individual plasmids were spotted on nylon membranes. After fixing by UV cross-linking, the membranes were probed first with PM1218 DIG labeled cDNA probe. After stripping, the membranes were re-probed with Acala glandless DIG labeled cDNA probe. Plasmids that only hybridized to the ganded cotton probe were sequenced. One of these sequences was highly similar to cytochrome P450 hydroxylase genes and found to be expressed in ganded but not in glandless cotton by RT-PCR analysis using primer pairs GhGosP450f, 5’-TGCCGATGTCTGTCTCATTC-3’ and GhGosP450r 5’-GAGGAAGAGACGAGTAGAGAC-3‘ derived from the determined sequence. The full length cDNA was obtained by screening the PM1218 cDNA using the method of Israel (1993). The presence of the target cDNA was monitored by PCR using the GhGosP450f/r primer pair. After repeated rounds of screening, a single clone with a 1.9 kb insert containing an open reading frame encoding CYP82D109 was obtained and sequenced (accession number: KM501031). The corresponding genomic DNA sequence was also determined (accession number: KM501032).

For expression analysis, the PM1218 and Acala glandless cDNA were subjected to “Virtual Northern blot” according to the manufacturer's recommendation (Clontech). The probe for CYP82D109 transcripts were synthesized and labeled with DIG utilizing PCR DIG Probe Synthesis Kit (Roche) with primer pair primer GhGosP450f/r from PM1218 cDNA. The probe for *G. hirsutum Histone-3* transcripts was also synthesized and labeled likewise using primer pair GhHis3f, 5’-GAAGCTCTCATGATACGTC-3’ and GhHis3r, 5’-CTACACTACCATGTC-3’.

CYP82D109 orthologs, their paralogs, and closely related genes in the *G. raimondii* and *G. arboreum* genome (http://www.cotton-gen.org/ and http://cgp.genomics.org.cn/page/species/index.jsp, respectively) were identified by BLAST search using the CDS of the cloned *G. hirsutum* CYP82D109. A few of these genes were re-annotated as shown in Fig. S1.

Coding DNA sequences of the identified genes were aligned using the Clustal W program (Thompson et al., 1994) and adjusted manually. Phylogenetic analyses were performed using the neighbor-joining method (Saitou and Nei, 1987) implemented in the phylogenetic analysis program of MEGA 6.0 (Tamura et al., 2007). Kimura's two-parameter distance option and pair-wise deletion of the gap option were used. Statistical support for the derived tree was calculated using 1000 bootstrap replicates.

4.3. Construction and molecular analysis of RNAi plants

4.3.1. Cloning and cotton transformation

A 495 bp fragment from the first exon of CYP82D109 was amplified from WT genomic DNA using the following primers: F XbaAscRNAI 5’-CGTCGACAGATCTCCGAGGAGTCTATCCTCACTACA-3’ and R BamSwaRNAI 5’-CAAGATCTCATTCATATGCTATCCTCCGGAGAC-3’. Using restriction enzymes present in the primers, the PCR fragment was cloned into a binary vector pPGC9411 [Arabidopsis Biological Resource Center (ABRC) accession No. CD3-447] on each side of the chalcone synthase intron, such that one side was in the sense orientation and the other was in the antisense orientation. A EcoRI/HindIII fragment, containing the RNAi hairpin construct and the 5’ and 3’ regulatory elements, was then inserted into the binary vector pORE-04 (ABRC accession No. CD3-923) to form p60RNAI-P04. This construct, carrying NptII as the plant-selectable marker gene, was introduced into *Agrobacterium* strain LBA4404, which was then used to transform WT as described (Rathore et al., 2006).

4.3.2. Confirmation of plants containing the RNAi construct

Terminal leaves from WT and RNAI containing plants were harvested and stored at –80 °C. DNA was isolated from cotton following the procedure of Li et al. (2001). Total RNA was isolated from cotton tissue using the RNeasy Plant Kit (Qiagen, USA). Nucleic acid was quantitated using a NanoDrop 2000 (ThermoScientific, USA). To detect the RNAI transgene by PCR, genomic DNA was diluted (most often 1:10 with H2O) and used as a template in two PCR reactions, and later in a multiplex reaction. The primers: F RNAI 5’-CGTCGACAGATCTCCGAGGAGTCTATCCTCACTACA-3’ and R 599CHS 5’-TCCGACCACTATAAGAAAG-3’ were used to amplify a 1.1 kb fragment of the RNAI construct. The second set of primers amplified a control gene (5-enolpyruvylshikimate-3-phosphate synthase, 5ESP3): F 781 5’-CGTCGACAGATCTCCGAGGAGTCTATCCTCACTACA-3’ and 5ESP3 R1283 5’-CTTCGAACCTCGCCGAAT-3’ to give a genomic product of 700 bp. Both fragments were amplified from the DNA of RNAI transgene containing plants. Only the 5ESP3 fragment was amplified from WT and null segregants. For Southern and Northern hybridization, nucleic acids were separated on an agarose gel (Sambrook and Russell, 2001), transferred to a positively charged nylon membrane (Roche), and UV-cross-linked (Spectrolinker XL-1000, Spectronics Corporation). The membrane was hybridized to DIG-labeled probes, washed, and visualized with CSPD (Roche) following the manufacturer’s protocols. The probe used in the Southern hybridization was amplified from p60RNAI-P04 using the PCR DIG Probe Synthesis Kit (Roche) with the same primers used to amplify the transgene above. The probe used in Northern hybridization was amplified from the CYP82D109 cDNA using the PCR DIG Probe Synthesis Kit (Roche) and primers: F CYP82D109-631 5’-CTTCGAACCTCGCCGAAT-3’ and R CYP82D109-1130 5’-TCCGACCACTATAAGAAAG-3’.

4.4. Analysis of terpenoid aldehydes, lacinilenes, and volatile terpenoids from leaves

4.4.1. Determination of gossypol (16a) and related sesquiterpenoids (7, 11, 14–18, and 21–24) via HPLC analysis

Freeze-dried leaf tissue was extracted using the procedure of Benson et al. (2001) with a 20 mg tissue to 1 ml solvent ratio. The extracts which contained gossypol (16a) and related sesquiterpenoids (7, 11, 14–18, and 21–24) were then analyzed on a computer-controlled Agilent Technologies (Waldbrohn, Germany) 1200 LC equipped with a solvent degasser, autosampler, column oven, and diode array detector. Two HPLC methods, ‘leaf’ and ‘stem’, were employed. Because of stability issues with the lacinilenes (7 and 11), all extracts were analyzed using the ‘stem’ method within 6 h of preparation. The extracts then were stored at –20 °C for 48–72 h until analysis using the ‘leaf’ method. The ‘leaf’ method (Stipanovic et al., 1988) was used to separate and quantitate HGQ (18), gossypol (16a), and H heliocides (21–24). The ‘stem’ method is detailed in Bianchini et al. (1999). It was originally developed to separate the HG related compounds (HGQ (18), HG (15a), dHg (14a), HGAL (17), MHG (15b) and dMHG (14b)) and the gossypol related compounds (gossypol (16a), MG (16b), DMG (16c)). In the present work, the individual lacinilenes (i.e., LC (11a), DHC (7a), LCME (11b) and HMC (7b)) were resolved. When all of the HG (15a), gossypol (16a), and lacinilene (7 and 11) related compounds were present, they were all well resolved for quantitation except dMHG (14b) and HMCG.
(7b) which co-eluted. To deal with this overlap, LC spectra and standard µg vs area curves were obtained for pure HMC (7b) and pure dMHG (14b) solutions, plus a series of mixtures of these compounds (specifically, mixtures with dMHG (14b):HMC (7b) ratios of 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, and 1:8). To distinguish whether one or both dMHG (14b) and HMC (7b) were present in an extract, the extract’s dMHG + HMC peak spectrum was compared with the spectra from the series of standard mixtures via the overlay feature of the Agilent ChemStation software and the best ratio match selected. Quantitation of the dMHG (14b) and HMC (7b) in the extract was then obtained using the µg vs area standard curve slope for the ‘best ratio match’ mixture and the extract’s dMHG + HMC peak area.

4.4.2. Volatile terpenoid analysis

The procedure of Elzen et al. (1985) was modified for smaller amounts of tissue. Thus, fresh terminal leaf tissue (1 g) was extracted for 1 h with Et2O (20 ml) in a beaker. The solution was decanted into a 50 ml pear flask and evaporated in vacuo in an ice bath to near dryness. The extract was resuspended in CH3CO2H/n-pentane (1 ml, 1:9) and evaporated to near-dryness under a slow N2 stream in the fume hood at room-temperature. The sample was subjected to GC/MS analysis and purification by a preparative GC.

4.4.3. GC/MS analysis and preparative GC

GC analyses were run on a Finnigan Trace Ultra GC coupled to a Thermo Electron DSQ mass spectrometer (Austin, TX, USA). Separation was achieved using a Scientific Glass Engineering BPT fused-silica column (25 m × 0.22 mm, 0.25 µm film thickness) with an initial column temperature of 60 °C which was increased to 180 °C at 10 °C/min, and then to 240 °C at 15 °C/min with a hold time of 10 min. The injection was in splitless mode with a flow of 50 ml/min, splitless time of 1 min, surge pressure of 0.44 psi at a constant flow rate of 1.0 ml/min of He with vacuum compensation. The inlet temperature and the transfer line were set at 200 °C. The compound appeared at 18.64 min. EIMS 70 eV, m/z (rel. int.): 218 [M]+ (53.7), 176 (22.2), 175 [M – H]+ (100), 174 (13.6), 148 (52.8), 147 [M – iPr – CO]+ (52.2), 133 (43.3), 131 (15.4), 120 (37.9), 119 (30.6), 117 (13.36), 114 (13.4), 106 (12.4), 105 (94.2), 91 (45.3), 79 (19.9), 77 (24.1), 69 (11.8), 55 (14.8), 41 (25.8).

The preparative GC was performed on a Varian 3700 using a 1.83 m × 4 mm I.D. glass column packed with 5% BP-1 on Chromosorb G–HP, with an inlet temperature of 200 °C. The oven was programmed from 60 to 250 °C at 10 °C/min and held for 10 min. The sample was collected using a Brownlee–Silverstein thermal gradient collection system (Brownlee and Silverstein, 1968) in which the effluent was split using an all glass variable ratio splitter, with part going to the flame ionization detector and the second diverted to either waste or a collector. This allowed the compound, eluted from the preparative column at 17.98 min, to be collected and then submitted to NMR spectroscopic analyses.

4.4.4. 1H and 13C NMR spectroscopic analyses of 3-Cadinene-2-one (3)

1H and 13C NMR spectra were acquired on a Bruker Avance III 500 instrument equipped with a HCN cryoprobe (Billerica, MA, USA) in CDCl3. 1H, 13C, DEPT, HSQC, and HMBC spectra were used to assign all proton and carbon chemical shifts. 1H NMR (500 MHz, CDCl3): δ 0.84 (3H, d, C12), 1.00 (3H, d, C13), 1.23 (1H, m, C6), 1.22 (1H, m, C5), 1.76 (3H, s, C9), 1.66 (1H, m, C8a), 1.62 (3H, bs, C14), 1.95 (2H, bs, C2), 2.06 (1H, sept, C1), 2.81 (1H, bd, C1), 2.84 (1H, bs, C4a), 3.50 (1H, d, C4), 6.72 (1H, s, C6). These chemical shifts are in good agreement with those reported by El-Dahhiny et al. (1986). 13C NMR (125 MHz, CDCl3): δ 15.8 (q, CH2, C14), 13.8 (q, CH3), 18.5 (q, CH3, C12), 21.0 (t, CH2, C6), 21.7 (q, CH3, C13), 27.1 (d, CH, C11), 31.8 (t, CH2, C7), 41.1 (d, CH, C15a), 43.2 (t, CH2, C7b), 44.2 (d, CH, C16), 125.1 (s, C=, C27), 128.6 (s, C=, C14), 134.4 (s, C=, C13), 148.0 (d, CH=, C14), 199.5 (s, C=O, C2). Important HMBC proton-carbon couplings are shown in Fig. S6.

4.5. Fov11 in vitro leaf assay

The third leaf from the plant apex was excised and cut in half. One half was placed in a petri dish containing filter paper and MS salts (3 ml); the other half was placed in a dish that contained filter paper and MS salts +1% sucrose (3 ml). The latter leaves were inoculated with a suspension of 2 × 105 Fov11 conidia/ml by placing 100 µl drops at each of four spots on the leaf and puncturing through the drops with a 23 ga needle. The leaves were infected with more than Fov11, because the sucrose encouraged the growth of other resident fungal spores and bacteria on the leaves. After inoculation, plates were closed and placed in the growth chamber (Percival Scientific) for 3 days as described in Section 4.1. Tissue was then collected, frozen (liq. N2), freeze-dried, and analyzed for terpenoids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.02.016.

References

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