JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Hemigossypol, a Constituent in Developing Glanded Cottonseed (*Gossypium hirsutum*)

Tanya A. Wagner, Jinggao Liu,* Robert D. Stipanovic, Lorraine S. Puckhaber, and Alois A. Bell

Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2765 F and B Road, College Station, Texas 77845, United States

Supporting Information

ABSTRACT: Gossypol is a dimeric sesquiterpenoid first identified in cottonseed, but found in various tissues in the cotton plant including the seed. From its first discovery, it was assumed that hemigossypol was the biosynthetic precursor of gossypol. Previous studies established that peroxidase (either from horseradish or from cottonseed) converts hemigossypol to gossypol. However, hemigossypol has never been identified in healthy cottonseed. In a temporal study using HPLC and LC-MS, hemigossypol was identified in the developing cotton embryo. It was shown to concomitantly accumulate until 40 days postanthesis (dpa) with gossypol and with transcripts of δ-cadinene synthase and 8-hydroxy-δ-cadinene synthase, genes involved in the biosynthesis of hemigossypol and gossypol. After 40 dpa, hemigossypol and its biosynthetic gene transcript levels declined, whereas the gossypol level remained almost unchanged until the bolls were open. These results provide further evidence to support the previous findings that establish hemigossypol as the biosynthetic precursor of gossypol.

KEYWORDS: *cotton, gossypol, hemigossypol, embryo, Gossypium hirsutum*

INTRODUCTION

Cotton plants (*Gossypium*) produce unique terpenoid aldehydes such as gossypol that protect the plant from herbivory. Gossypol was first identified, and is the primary terpenoid, in cottonseed,[1−3] but it occurs in other plant tissues. It protects the plant from herbivores such as insects, rodents, and birds.[4] Hemigossypol was first identified in infected cotton xylem stem tissue[5] and was identified as part of the resistance response in foliar tissue to *Xanthomonas campestris* pv *Malvacearum*,[6] in the xylem response to infection by *Verticillium dahliae*,[7,8] and in diseased cottonseed.[9] From its first identification,[5] hemigossypol was considered to be the biosynthetic precursor of gossypol, but it had not been identified in healthy cottonseed even though gossypol accumulates to high levels in this tissue (>50 μg/mg of dry embryo in *Gossypium davidsonii*, accession D3D-4[2]). The proposed reaction sequence for the biosynthesis of gossypol is shown in Figure 1. Besides biosynthetic intuition, three investigations support hemigossypol as the biosynthetic precursor of gossypol. Veech et al. in 1976[10] showed that hemigossypol could be converted to gossypol using horseradish peroxidase. More recently, Benedict et al.[11] demonstrated that peroxidase from cotton embryo extracts converts tritium-labeled hemigossypol into tritium-labeled gossypol and that gossypol accumulation during seed development correlated with peroxidase activity. Finally, a dirigent protein was isolated from cotton embryo extracts that controls the enantiomeric ratio of gossypol in the presence of peroxidase and hemigossypol.[12]

We now report a temporal study of terpenoid aldehyde accumulation during cotton embryo development and show that hemigossypol accumulates in this tissue. The accumulation of hemigossypol is compared with that of gossypol and enzymes involved in the biosynthesis of gossypol during embryo development.

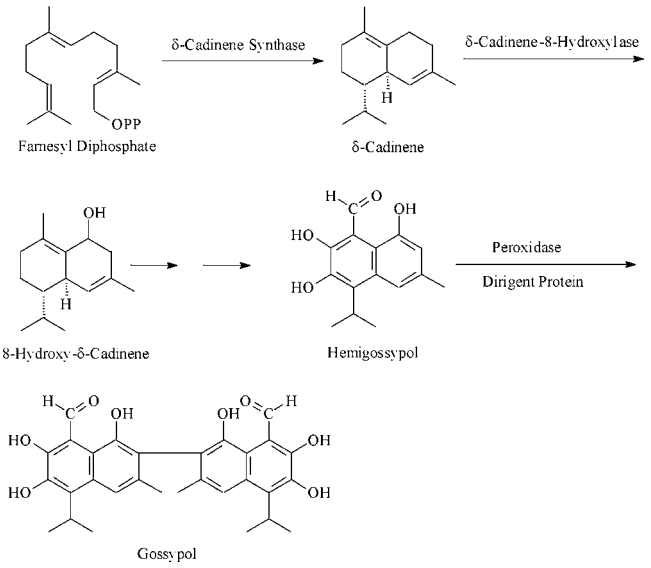

Figure 1. Proposed reaction sequence for the biosynthesis of gossypol.

MATERIALS AND METHODS

Plants. *Gossypium hirsutum* plants were grown in the greenhouse as previously described.[13] The first experiment was initiated during the winter season with the cultivar 'Coker 312', and the second experiment was initiated during the summer season with 'Coker 312' and two additional lines: an Acala glandless line and a line of which the seed exhibits a low percentage (ca. 5.5%) of (−)-gossypol. The latter line had the following pedigree: *G. hirsutum* accession 2443 (P.I. 607748)

Received: December 13, 2011
Revised: February 27, 2012
Accepted: February 27, 2012
Published: February 27, 2012

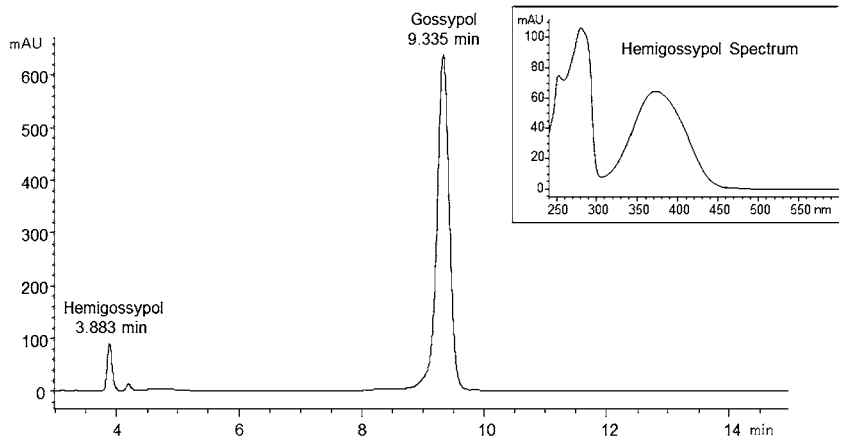

Figure 2. HPLC chromatogram of an extract from 35 dpa 'Coker 312' embryos grown in the winter showing the presence of hemigossypol and gossypol with no other known terpenoids. (Inset) UV spectrum of the hemigossypol peak.

was crossed and backcrossed to cultivar 'Tamcot CAMD-E' to give BC_1F_1 seed.[14] A BC_1F_1 plant selected for low (−)-gossypol based on flower petal analysis was further crossed and backcrossed to 'Suregrow 747' to yield BC_1F_1 seed. The BC_1F_1 plant 45-2 was selected for low (−)-gossypol in petals and selfed to yield the plant 45-2-1. Two additional cycles of selection and selfing yielded the BC_1S_3 plant 45-2-1-2-37, which was further selfed and increased as the low (−)-gossypol line used in this study.

In the first experiment, tagged cotton bolls were harvested at specific days postanthesis (dpa), and the embryos were harvested. With the exception of 25 and 30 dpa embryos, embryos from the same boll were divided into two samples. The first sample was freeze-dried for terpenoid aldehyde analysis, and the second was either immediately frozen at −80 °C or placed into RNAlater solution (Qiagen, Valencia, CA) for RNA extraction. The harvests of embryos from 25 and 30 dpa were too small to divide, so different bolls contributed to the terpenoid aldehyde analysis and the qPCR analysis for these time points. For the second experiment, only terpenoid analysis for the developing embryos was carried out.

Terpenoid Extraction. Terpenoid aldehydes were isolated from the dried embryos following the procedure of Benson et al.[15] About 100 mg of dried embryos was used for most samples. Lesser amounts were used for the youngest embryos for which sample amounts were limited. The smallest amount of tissue used for this analysis was 70 mg. A ratio of 10 mL of extraction buffer per 100 mg of dried embryos was used for the extraction of terpenoid aldehydes.

HPLC and LC-MS Analyses. Embryo extracts were analyzed first on a computer-controlled Agilent Technologies (Waldbronn, Germany) 1200 HPLC equipped with a solvent degasser, autosampler, column oven, and diode array detector. The chromatographic procedure was based on the method of Stipanovic et al.[16] A Scientific Glass Engineering (Austin, TX) 250GL4-ODS-H-12/5 (4.6 mm × 250 mm) column maintained at 55 °C was used with an isocratic mobile phase composed of H_2O/ACN/EtOH/IPA/DMF/MeOH/EtOAc/H_3PO_4 (28.0:23.2:19.2:13.9:5.9:5.3:4.4:0.1) and run at 1.25 mL/min. The chromatogram signal was monitored at 272 nm (bandwidth = 20 nm) referenced to 550 nm (bandwidth = 100 nm). Spectra for detected peaks were collected over 240−600 nm. The retention times for gossypol and hemigossypol were 9.5 and 3.8 min, respectively. A Varian 500-MS Ion Trap mass spectrometer equipped with two Varian 212-LC pumps and a Varian 460-LC autosampler (Walnut Creek, CA) was used for the LC-MS analysis. The complex solvent used in the HPLC analysis is not compatible with MS analysis; therefore, the LC-MS chromatography was carried out using a Phenomenex (Torrance, CA) Hypersil C-18, 5 μm (4.6 mm × 250 mm), column with a gradient of ACN and H_2O (both with 0.1% HCOOH) run at 1.00 mL/min with 80% of the flow diverted to waste. The gradient was from 20% ACN to 70% ACN over 7 min, to 80% ACN over 5 min, to 84% ACN over 1 min, and to 100% ACN over 3 min. The MS was operated with a spray chamber temperature of 50 °C using N_2 as the nebulizer gas at a pressure of 35 psi, a drying gas temperature 350 °C, a drying gas pressure 20 psi, a needle voltage 5000 V, and a shield spray 600 V. Electrospray was used to ionize the effluent, and the instrument was run with positive polarity in the MS/MS mode with 70 and 5000 V capillary and needle voltages, respectively. The precursor ion at m/z 261 was detected using a resonant waveform with an excitation storage level of 89.9% and an excitation amplitude of 0.4 V. The LC-MS retention time for hemigossypol was 10.0 min. Major ions were observed at m/z (%) 261 (6.7), 243 (13.6), 219 (100), and 191 (49.5).

RNA Extraction, Reverse Transcription, and Quantitative RT-PCR. Two to three biological samples for each time point were analyzed. Total RNA was isolated from 50−100 mg of tissue for each time point using the RNAeasy kit (Qiagen). The RNA was quantitated using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ). High RNA quality was inferred from 260/280 nm absorbance ratios >1.8. Genomic DNA was removed using the Turbo DNA-free kit (Applied Biosystems, Carlsbad, CA). Total RNA (1 μg) from each sample was reverse transcribed using oligo dT primers and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's directions. The reactions were also done without reverse transcriptase to test for genomic DNA contamination. The cDNA was diluted 10-fold, and 2 μL was used as template for each quantitative PCR reaction.

A 'Coker 312' sequence for δ-cadinene synthase[17] was used for primer design (primer 3) using the criteria specified by Udvardi et al.[18] These primers, forward 5′-ACACCACATCCCTTCGATTC-3′ and reverse 5′-TTGAAATTCCCTTGCTCGTC-3′, amplify a 97 bp fragment. Similarly, the sequence for δ-cadinene-8-hydroxylase[19] was obtained from GenBank and used for primer design. These primers, forward 5′-GGACCTCAAGCAGAAGAACG-3′ and reverse 5′-TGTATCAGTTCCACCGACCA-3′, amplify a 94 bp product. The reference genes *GhMZA* (clathrin adaptor medium subunit), *GhPTB* (polypyrimidine tract-binding protein), and *GhUBQ14* (ubiquitin) were chosen on the basis of the study of Artico et al.,[20] in which these genes were found to be the most stable among 10 reference genes across different staged cotton fruits or across different organs. Primer pairs for *GhMZA*, *GhPTB*, and *GhUBQ14* were the same as previously described.[20] All primer pairs were tested on 'Coker 312' cDNA and genomic DNA in PCR reactions to confirm that there was a single product of the expected size.

All reactions were performed in triplicate using Fast-SYBR (Applied Biosystems) and 200 nM gene-specific PCR primers. Reactions (20 μL) were performed using the StepOne real-time PCR system (Applied Biosystems) using a two-step amplification program as follows: 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A standard melting curve protocol began immediately after amplification to determine the quality of the PCR products. Raw

dx.doi.org/10.1021/jf2051366 | J. Agric. Food Chem. 2012, 60, 2594−2598

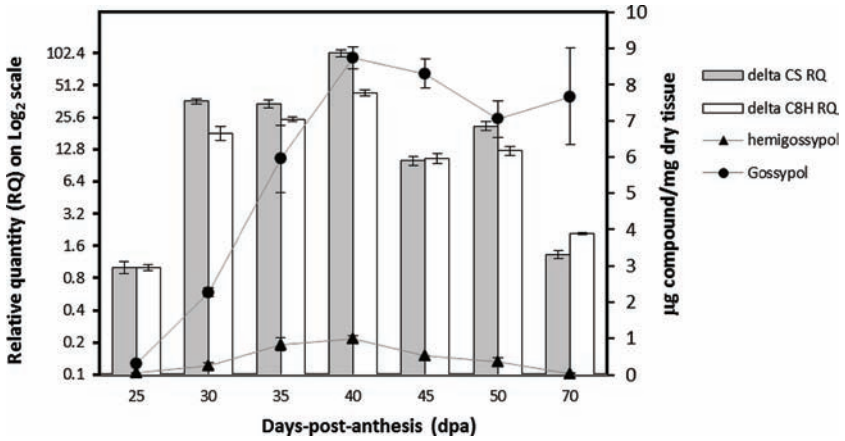

Figure 3. Time course during cotton boll development showing the accumulation of hemigossypol (▲) and gossypol (●) in developing embryos (right *Y* axis) and the relative quantities of (+)-*δ*-cadinene synthase (delta CS; gray bars) and (+)-*δ*-cadinene-8-hydroxylase (delta C8H; white bars) mRNA in developing embryos (left *Y* axis) from the first experiment. For hemigossypol and gossypol accumulation, each point represents the mean (±SE) for embryos harvested from two to four independent bolls. For (+)-*δ*-cadinene synthase and (+)-*δ*-cadinene-8-hydroxylase mRNA accumulation, total RNA was extracted from 'Coker 312' embryos harvested at 25, 30, 35, 40, 45, 50, and 70 dpa from one set of the biological samples used in the terpenoid determination. The levels of (+)-*δ*-cadinene synthase and (+)-*δ*-cadinene-8-hydroxylase were determined by qPCR. *GhMZA* (clathrin adaptor medium subunit) and *GhPTB* (polypyrimidine tract-binding protein) served as internal reference genes. The data are presented as relative quantities (RQ) with the levels at 25 dpa set to 1. The RQ minimum and maximum represent 1 standard deviation of the three technical replicates.

fluorescence values were exported to an Excel file to analyze the PCR data using either real-time PCR miner[21] or LinRegPCR[22] to obtain PCR efficiencies for each reaction and the average efficiencies of genes.

The PCR efficiencies and Ct values were entered into BestKeeper[23] to establish the best reference genes for the experiment. This program confirmed that *GhMZA* and *GhPTB* were the most stable of the three reference genes across embryo development, so they were chosen to normalize *δ*-cadinene synthase and *δ*-cadinene-8-hydroxylase expression. Also, the expression levels of *δ*-cadinene synthase and *δ*-cadinene-8-hydroxylase were highly correlated across the embryo samples. The PCR efficiencies for each gene were entered back into the StepOne 2.1 software program to normalize expression levels and calculate relative quantities. Results are presented as RNA levels (relative fold change ± 1 SD of the three technical replicates) relative to their respective levels at 25 dpa.

RESULTS AND DISCUSSION

HPLC and LC-MS Detection of Hemigossypol and Gossypol in the Developing Cottonseed Extracts. Two distinct peaks were detected by HPLC in the developing cottonseed extracts (Figure 2). The major peak corresponded to gossypol as determined by HPLC retention time and UV spectra compared to an authentic sample of gossypol. The smaller peak was not previously detected in cottonseed extracts. This unknown had a retention time of 3.85 min, and its UV spectra had absorbance maxima at 250, 280, and 380 nm (Figure 2). A hemigossypol standard and the unknown had the same retention times and identical absorbance spectra. Both the standard and the unknown could be derivatized with 10% acetic acid and 2% D-alaninol to produce Schiff bases with identical retention times (3.21 min) and UV spectra. Additionally, both the unknown and the hemigossypol standard had identical expected mass fragmentation patterns (Figure S1 in the Supporting Information) and retention times in the LC-MS analysis, thus confirming the identity of the unknown as hemigossypol. The identity of the major peak in the HPLC chromatograms was confirmed to be gossypol by the MS/MS fragmentation obtained from the LC-MS analysis (Figure S2 in the Supporting Information). Other sesquiterpenoids that have been identified in foliar cotton tissues (i.e., hemigossypolone and heliocides)[24] were not detected.

Temporal Results. Bolls from greenhouse-grown plants ('Coker 312') remained closed until about 70 dpa in the winter season in our first experiment. Thus, seed and fruit development extended over a longer time than is commonly observed for cotton in the field. Hemigossypol was sporadically detected at 30 dpa and was at its highest concentration of 1 μg/mg dry embryo tissue at 35 and 40 dpa. Hemigossypol concentration decreased at 45 and 50 dpa and was below 50 ng/mg dry tissue in open bolls (70 dpa) (Figure 3). Gossypol concentration was low (0.25 μg/mg dry embryo tissue) at 25 dpa. It increased rapidly to its peak concentration of 8.9 μg/mg dry tissues at 40 dpa and remained at a high concentration as the boll opened (Figure 3); this is similar to the gossypol profile shown in earlier studies.[24] The time course showed a concomitant increase of hemigossypol and gossypol up to 40 dpa and then a decrease of hemigossypol, whereas gossypol remained elevated until the bolls opened. This pattern is consistent with hemigossypol being an intermediate in gossypol biosynthesis as determined in earlier studies[10,11] (Figure 1).

To confirm that the above results were not a random event, the experiment was repeated with a second set of greenhouse-grown plants in the summer season (second experiment). An Acala glandless line and a low-(−)-gossypol line were analyzed in addition to 'Coker 312' for hemigossypol and gossypol content in developing embryos. Bolls from the second set of plants matured more quickly than those from the first set, with bolls opening 40 dpa. Glandless cotton lacks both lysigenous glands and the associated terpenoid aldehydes.[25] As expected, hemigossypol and gossypol were not detected in the embryos of Acala glandless plants (data not shown). Table 1 shows the amount of hemigossypol and gossypol present in 'Coker 312' and in the line exhibiting the low percentage of (−)-gossypol in seeds. Hemigossypol and gossypol accumulation started earlier and peaked earlier in the second experiment (Table 1) compared to the first (Figure 3). However, the pattern of hemigossypol and gossypol accumulation between Table 1 and

Table 1. Concentration of Hemigossypol (HG) and Gossypol (G) in Developing Cottonseed from 'Coker 312' and a Cotton Line Exhibiting a Low Percentage of (−)-Gossypol (Second Experiment: Bolls Opening at 40 Days Postanthesis (dpa))

	concentration (μg/mg of dry embryos (mean ± SE from three independent bolls))	
sample	HG	G
'Coker 312'		
20 dpa	0.19 ± 0.07	2.08 ± 0.90
25 dpa	0.77 ± 0.04	8.19 ± 0.33
30 dpa	0.59 ± 0.01	10.64 ± 0.82
35 dpa	0.42 ± 0.08	10.44 ± 0.13
40 dpa	0.20 ± 0.03	10.32 ± 0.38
45 dpa	0.06 ± 0.01	9.30 ± 0.27
low (−)-G		
20 dpa	0.35 ± 0.04	3.53 ± 0.34
25 dpa	0.45 ± 0.05	5.53 ± 0.35
30 dpa	0.27 ± 0.02	4.83 ± 0.67
35 dpa	0.18 ± 0.04	6.54 ± 1.29
40 dpa	0.04 ± 0.01	5.00 ± 0.49
45 dpa	0.05 ± 0.01	5.07 ± 0.43

Figure 3 is similar, with hemigossypol and gossypol first increasing during embryo development and then hemigossypol dropping to low levels at boll opening, whereas gossypol levels remained elevated to opening. In the second experiment, the gossypol concentration was already >2 μg/mg dry tissue at 20 dpa (Table 1), and hemigossypol concentration peaked at 25 dpa in both 'Coker 312' and the line exhibiting the low percentage of (−)-gossypol in seed. The gossypol levels plateaued at 25 and 30 dpa in corresponding plants. The transient increase of hemigossypol in the midstages of embryo development from these two lines supports the concept[10,11] that hemigossypol is the biosynthetic precursor of gossypol.

In the past, we routinely isolated hemigossypol for experimental studies from *Verticillium dahliae* inoculated cotton stem stele tissues.[5] However, the developing seed embryo may be a more desirable source for hemigossypol. The 1 μg of hemigossypol per milligram of dry 40 dpa embryo tissue is higher than the 0.38 μg of hemigossypol per milligram of dry weight induced by *V. dahliae* in stem stele tissue of 'Seabrook Sea Island 12B2' (*Gossypium barbadense*).[8] The sesquiterpenoids desoxyhemigossypol-6-methyl ether, hemigossypol-6-methyl ether, and desoxyhemigossypol also were induced in the fungal inoculated tissues, making it difficult to purify hemigossypol. Only hemigossypol and gossypol, which are readily separated by chromatography (Figure 2), occur in embryos. Use of embryos also obviates the need for fungal cultures.

δ-Cadinene Synthase and δ-Cadinene-8-hydroxylase mRNA Temporal Expression in Developing Embryos. Quantitative RT-PCR was used to measure the mRNA levels of two enzymes involved in the biosynthesis of hemigossypol over the course of embryo development and gossypol accumulation. δ-Cadinene synthase catalyzes the first committed step in the cyclization of *E,E*-farnesyl diphosphate[26] to the cadinene sesquiterpenoids such as hemigossypol[27] (Figure 1). δ-Cadinene-8-hydroxylase is a P_{450} that catalyzes the hydroxylation of (+)-δ-cadinene at C8[19] (Figure 1). Figure 3 shows the change ($\log_2$ scale) of mRNA expression of δ-cadinene synthase and δ-cadinene-8-hydroxylase during seed development in the first experiment. Expression of these genes was minimal at 25 dpa and set to 1. The rapid increase in expression for δ-cadinene synthase (35-fold) and δ-cadinene-8-hydroxylase (15-fold) at 30 dpa versus 25 dpa occurred when gossypol and hemigossypol were at one-fourth of their highest levels. Expression of δ-cadinene synthase and δ-cadinene-8-hydroxylase genes remained at high levels at 50 dpa (20- and 10-fold versus 25 dpa, respectively) even when hemigossypol levels were decreasing and gossypol levels were stable. When the bolls were open (70 dpa), mRNA levels for δ-cadinene synthase and δ-cadinene-8-hydroxylase returned to levels seen at 25 dpa. Two additional sets of biological samples were analyzed with one of the sets missing many time points (Figures S3 and S4 in the Supporting Information). mRNA levels differed significantly between biological replicates. What was consistent among biological replicates was an initial jump in expression at 30 dpa, a return to baseline levels at 70 dpa, and the coordinated expression of δ-cadinene synthase and δ-cadinene-8-hydroxylase shown in Figure 3.

'Coker 312' and the line exhibiting the low percentage of (−)-gossypol are of diverse heritage. The two experiments were conducted during summer and winter when the times between anthesis and boll opening are quite different. The finding that hemigossypol accumulates in these diverse lines and at different times of the year indicates that this is a general phenomenon in cotton and not unique to 'Coker 312'. Furthermore, the hemigossypol concentration profile followed a bell-shaped curve that corresponded to high levels of mRNA transcription of δ-cadinene synthase and δ-cadinene-8-hydroxylase (genes involved in the biosynthesis of hemigossypol) between 30 and 50 dpa during embryo development in the first experiment. In contrast, gossypol levels reached a plateau at a time point when the hemigossypol level reached a maximum. These results provide further evidence to support the previous findings[10,11] that hemigossypol is the biosynthetic precursor of gossypol.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Mass spectra (MS/MS) of hemigossypol (Figure S1) and gossypol (Figure S2) and time courses during cotton boll development showing the relative mRNA levels of (+)-δ-cadinene synthase and (+)-δ-cadinene-8-hydroxylase from two additional sets of biological samples (Figures S3 and S4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (979) 260-9233. Fax: (979) 260-9319. E-mail: jinggao.liu@ars.usda.gov.

Funding

This research was supported in part by Cotton Incorporated. Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

delta CS, (+)-δ-cadinene synthase; delta C8H, (+)-δ-cadinene-8-hydroxylase; dpa, days postanthesis; GhMZA, *Gossypium*

hirsutum clathrin adaptor medium subunit; GhPTB, *Gossypium hirsutum* polypyrimidine tract-binding protein; GhUBQ14, *Gossypium hirsutum* ubiquitin 14; RQ, relative quantity.

REFERENCES

(1) Marchlewski, L. Gossypol, a component of cottonseed. *J. Prakt. Chem.* **1899**, *60*, 84−90.

(2) Stipanovic, R. D.; Puckhaber, L. S.; Bell, A. A.; Percival, A. E.; Jacobs, J. Occurrence of (+)- and (−)-gossypol in wild species of cotton and in *Gossypium hirsutum* var. marie-galante (Watt) Hutchinson. *J. Agric. Food Chem.* **2005**, *53*, 6266−6271.

(3) Stipanovic, R. D.; Puckhaber, L. S.; Liu, J.; Bell, A. A. Total and percent atropisomers of gossypol and gossypol-6-methyl ether in seeds from pima cottons and accessions of *Gossypium barbadense* L. *J. Agric. Food Chem.* **2009**, *57*, 566−571.

(4) Bottger, G. T.; Sheehan, E. T.; Lukefahr, M. J. Relation of gossypol content of cotton plants to insect resistance. *J. Econ. Entomol.* **1964**, *57*, 283−285.

(5) Bell, A. A.; Stipanovic, R. D.; Howell, C. R.; Fryxell, P. A. Antimicrobial terpenoids of *Gossypium*: hemigossypol, 6-methoxyhemigossypol and 6-deoxyhemigossypol. *Phytochemistry* **1975**, *14*, 225−231.

(6) Abraham, K. J.; Pierce, M. L.; Essenberg, M. L. The phytoalexins desoxyhemigossypol and hemigossypol are elicited by *Xanthomonas* in *Gossypium* cotyledons. *Phytochemistry* **1999**, *52*, 829−836.

(7) Garas, N. A.; Waiss, A. C. Jr. Differential accumulation and distribution of antifungal sesquiterpenoids in cotton stems inoculated with *Verticillium dahliae*. *Phytopathology* **1986**, *76*, 1011−1017.

(8) Bianchini, G. M.; Stipanovic, R. D.; Bell, A. A. Induction of δ-cadinene synthase and sesquiterpenoid phytoalexins in cotton by *Verticillium dahliae*. *J. Agric. Food Chem.* **1999**, *47*, 4403−4406.

(9) Halloin, J. M.; Bell, A. A. Production of nonglandular terpenoid aldehydes within diseased seeds and cotyledons of *Gossypium hirsutum* L. *J. Agric. Food Chem.* **1979**, *27*, 1407−1409.

(10) Veech, J. A.; Stipanovic, R. D.; Bell, A. A. Peroxidative conversion of hemigossypol to gossypol. A revised structure for isohemigossypol. *J. Chem Soc., Chem. Commun.* **1976**, *1976*, 144−145.

(11) Benedict, C. R.; Liu, J.; Stipanovic, R. D. The peroxidative coupling of hemigossypol to (+)- and (−)-gossypol in cottonseed extracts. *Phytochemistry* **2006**, *67*, 356−361.

(12) Liu, J.; Stipanovic, R. D.; Bell, A. A.; Puckhaber, L. S.; Magill, C. W. Stereoselective coupling of hemigossypol to form (+)-gossypol in moco cotton is mediated by a dirigent protein. *Phytochemistry* **2008**, *69*, 3038−3042.

(13) Robinson, A. F.; Bell, A. A.; Dighe, N. D.; Menz, M. A.; Nichols, R. L.; Stelly, D. M. Introgression of resistance to nematode *Rotylenchulus reniformis* into upland cotton (*Gossypium hirsutum*) from *Gossypium longicalyx*. *Crop Sci.* **2007**, *47*, 1865−1877.

(14) Bell, A.; Puckhaber, L.; Kim, H.; Stipanovic, R.; Percival, E. Genetic approaches for increasing percentages of (+)-gossypol levels in cotton. In *Genetic Control of Fiber and Seed Quality*; Benedict, C. R., Jividen, G., Eds.; Cotton Inc.: Cary, NC, 2000; pp 218−230.

(15) Benson, C. G.; Wyllie, S. G.; Leach, D. N.; Mares, C. L.; Fitt, G. P. Improved method for the rapid determination of terpenoid aldehydes in cotton. *J. Agric. Food Chem.* **2001**, *49*, 2181−2184.

(16) Stipanovic, R. D.; Altman, D. W.; Begin, D. L.; Greenblatt, G. A.; Benedict, J. H. Terpenoid aldehydes in Upland cottons − analysis by aniline and HPLC methods. *J. Agric. Food Chem.* **1988**, *36*, 509−515.

(17) Sunilkumar, G.; Campbell, L. M.; Puckhaber, L.; Stipanovic, R. D.; Rathore, K. S. Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 18054−18059.

(18) Udvardi, M. K.; Czechowski, T.; Scheible, W.-R. Eleven golden rules of quantitative RT-PCR. *Plant Cell* **2008**, *20*, 1736−1737.

(19) Luo, P.; Wang, Y. H.; Wang, G. D.; Essenberg, M.; Chen, X. Y. Molecular cloning and functional identification of (+)-δ-cadinene-8-hydroxylase, a cytochrome P450 mono-oxygenase (CYP706B1) of cotton sesquiterpene biosynthesis. *Plant J.* **2001**, *28*, 95−104.

(20) Artico, S.; Nardeli, S. M.; Brilhante, O.; Grossi-de-Sa, M. F.; Alves-Ferreira, M. Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. *BMC Plant Biol.* **2010**, 10, article 49.

(21) Zhao, S.; Fernald, R. D. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J. Comput. Biol.* **2005**, *12*, 1045−1062.

(22) Ruijter, J. M.; Ramakers, C.; Hoogaars, W. M. H.; Karlen, Y.; Bakker, O.; van den Hoff, M. J. B.; Moorman, A. F. M. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* **2009**, *37*, e45.

(23) Pfaffl, M. W.; Tichopad, A.; Prgomet, C.; Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper − Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **2004**, *26*, 509−515.

(24) Martin, G. S.; Liu, J.; Benedict, C. R.; Stipanovic, R. D.; Magill, C. Reduced level of cadinane sesquiterpenoids in cotton plants expressing antisense (+)-δ-cadinene synthase. *Phytochemistry* **2003**, *62*, 31−38.

(25) McMichael, S. C. Combined effects of glandless genes gl2 and gl3 on pigment glands in the cotton plant. *Agron. J.* **1960**, *52*, 385−386.

(26) Benedict, C. R.; Alchanati, I.; Harvey, P. S.; Liu, J.-G.; Stipanovic, R. D.; Bell, A. A. The enzymatic formation of δ-cadinene from farnesyl diphosphate in extracts of cotton. *Phytochemistry* **1995**, *39*, 326−31.

(27) Davis, G. D.; Essenberg, M. (+)-δ-Cadinene is a product of sesquiterpene cyclase activity in cotton. *Phytochemistry* **1995**, *39*, 553−567.