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Spodoptera exigua modulates gossypol biosynthesis in cotton *Gossypium hirsutum*

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ABSTRACT

Cotton plants induce high levels of gossypol in response to herbivore damage. However, little is known about the mechanisms by which insect herbivory modulates gossypol biosynthesis in cotton plants. Here, we report the mechanism by which herbivore damage or insect-originated elicitors modulate the biosynthesis of gossypol and jasmonic acid (JA) in plants. *Spodoptera exigua* larval-damaged (HD) cotton plants and mechanically damaged plants treated with *S. exigua* oral secretion (MDOS) showed higher levels of gossypol and JA as well as increased transcript levels of genes involved in the biosynthesis of both secondary plant metabolites, compared to undamaged (UD) or mechanically damaged (MD) plants. In correlation with the observed induction of gossypol and JA, *S. exigua* larvae that fed on HD and MDOS cotton plants showed significantly reduced weight. The findings provide a better insight into the molecular mechanisms mediating herbivore-induced plant defense.

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KEYWORDS
Gossypol biosynthesis; jasmonic acid; *Spodoptera exigua*; induced defense; oral secretion; plant–insect interactions

Introduction

Cotton plants (*Gossypium* spp.) have numerous inducible direct and indirect defense mechanisms that are important for their ability to respond to changing biotic stress, including the induction of gossypol, a phenolic sesquiterpenoid. Gossypol is an important allelochemical produced by the subepidermal glands of some cotton varieties and exhibits antibiosis against some cotton pests (Wu et al. 2010). High gossypol level expressed by some cotton varieties has been shown to affect feeding and development of certain insect pests (Du et al. 2004; Mao et al. 2007; Zhang et al. 2011).

Zhang et al. (2011) demonstrated that exogenous application of jasmonic acid (JA) to cotton plants resulted in increased levels of gossypol, and nymphs of the mealybug *Phenacoccus solenopsis* that fed on JA-treated cotton plants showed reduced growth and development. JA is a key phytohormone involved in plant defense against herbivores (War et al. 2011), which is derived from linolenic acid through octadecanoid pathway and accumulates upon herbivory in plant tissues (Zhang et al. 2008). JA has been repeatedly shown to be the most important mediator of plant-herbivore interactions and is responsible for volatile organic compound (VOC) activation, including the synthesis of terpenes (Baldwin 2010; Degenhardt et al. 2010; Dicke & Baldwin 2010).

Gossypol biosynthesis is well studied in cotton, *Gossypium hirsutum* (Essenberg et al. 1985; Alchanati et al. 1998; Zhou et al. 2013). The first step in the biosynthesis of gossypol and its precursor terpene aldehyde begins with (+)-δ-cadinene synthase (CDNS; referred to as CAD) that catalyzes the cyclization of farnesyl diphosphate (FPP) to (+)-δ-cadinene via a nerolidyl diphosphate intermediate (Essenberg et al. 1985; Alchanati et al. 1998; Zhou et al. 2013). The CAD is encoded by a gene family which is divided into two subfamilies, CAD1-A and CAD1-C (*Cdn1-C1*, *Cdn1-C14*, *Cdn1-A* and *Cdn1-C3*), based on sequence similarities. The diploid genome of *Gossypium* arboreum contains about six members of CAD1-C and a single copy of CAD1-A (Townsend et al. 2005). The second step is hydroxylation of (+)-δ-cadinene to form 8-hydroxy-(+)-δ-cadinene, which is catalyzed by (+)-δ-cadinene-8-hydroxylase, a cytochrome P450 monooxygenase CYP706B1 (Luo et al. 2001; Wang et al. 2003). In plants, cytochrome P450 monooxygenases play essential roles in the biosynthesis of phenylpropanoids, terpenoids, alkaloids and other natural products, and in the catabolism of herbicides and other xenobiotics (Zhou et al. 2011). The expression pattern of CYP706B1 is similar to that of CAD1 in various tissues of cotton plants. The CAD1 transcripts increase dramatically along with the seed maturation in association with an increase in sesquiterpene cyclase activities and subsequently the accumulation of gossypol (Meng et al. 1999). CAD1 enzyme and transcripts are induced in cotton plants infected with the fungal pathogen *Verticillium dahliae*, and in cotton suspension cultures treated with *V. dahliae* elicitors (Chen et al. 1995; Townsend et al. 2005). These studies revealed that CAD1 is the key enzyme, which is developmentally regulated and also induced by pathogen infection in cotton. However, information on the effects of insect herbivory on induction of *CAD1* and *CYP706B1* transcripts remains elusive. Furthermore, little is known about the defense-related function of gossypol in intact cotton leaves against insect herbivores.

In this study, we used *Spodoptera exigua*, a key lepidopteran pest of cotton, as a model insect to test the hypothesis that insect herbivore damage or insect-originated elicitors (signal molecules) affect the transcript levels of the genes that are involved in gossypol biosynthesis via the JA signaling pathway in cotton, thereby defending the plant against subsequent herbivore attacks due to increased gossypol levels. First, the levels of gossypol and JA were quantified in undamaged (UD) cotton plants, herbivore (*S. exigua*) damaged (HD), mechanically

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damaged (MD) and mechanically damaged plants treated with *S. exigua* oral secretion (MDOS). Second, the transcript levels of the genes involved in gossypol and JA biosynthesis were quantified in cotton plants from the above treatments. Finally, the development of *S. exigua* larvae which were fed cotton plants from the above treatments was compared.

Materials and methods

Plant growth and insect rearing

Cotton plants (*G. hirsutum,* Var. Max-9, All-Tex Seed, Inc. Levelland, TX) were grown in growth chambers under controlled conditions (23 ± 2°C, 60 ± 5% RH, and a photoperiod of 16: 8 h L: D) using sterilized sunshine potting mixture #8 (SunGro Horticulture, Agawam MA). Plants were watered daily and fertilized twice a week with Scott® peat lite special fertilizer (Scotts-Sierra Horticultural Product Company, Marysville OH), a 20-10-20 water-soluble NPK fertilizer mixture with micronutrients. Six-week-old plants were used for the experiments. *S. exigua* eggs purchased from Benzon Research (Carlisle, PA) were used to start laboratory colonies at Auburn University (Auburn, AL). The larvae were reared on artificial diet prepared from pinto beans, and maintained at 25 ± 1°C, 75 ± 5% RH and a photoperiod of 14:10 h L:D.

Oral secretion collection

Oral secretion (OS) was collected from *S. exigua* larvae as previously described by Zebelo and Maffei (2012). The OS was diluted in 5 mM 2-(*N*-morpholino) ethanesulfonic acid (Mes-NaOH) (pH 6.0) buffer, to stabilize its chemical properties, at the rate 1:5. A 5 µL aliquot of this solution was applied at the site of mechanical damage on cotton leaves with a micro-syringe. The ratios of OS to Mes-NaOH buffer and the amount of OS solution added to mechanically damaged cotton plants were the same as reported in previous studies (Maffei et al. 2004; Maffei et al. 2006; Bricchi et al. 2010; Zebelo & Maffei 2012; Bricchi et al. 2013; Zebelo et al. 2014). Previous studies showed that Mes-NaOH buffer alone has no effect on plant defense signaling (Maffei et al. 2004; Maffei et al. 2006; Bricchi et al. 2010; Zebelo & Maffei 2012; Bricchi et al. 2013; Zebelo et al. 2014).

Treatments

Four cotton plant treatments were tested in this study: (1) Undamaged control plants (UD, neither herbivore nor mechanical damaged), (2) Herbivore (*S. exigua*) damaged plants (HD). To create HD plants, a group of 15 second instar *S. exigua* larvae were introduced into a Petri dish. The larvae were allowed feed on the leaf for 12 h, after which they were removed from the feeding arena, (3) Mechanically damaged plants (MD), made using a pattern wheel (Leonard Adler & Co, Chicago, IL) to create mechanical damage on the leaves and (4) Mechanically damaged plants treated with *S. exigua* oral secretion (MDOS). The OS was applied to the plants using a pipette and clipped with cover slips.

Gossypol extraction and quantification

Gossypol was extracted as previously described by Zhang et al. (2011) with some modifications. Leaves from cotton plants that were either undamaged (UD), herbivore (*S. exigua*) damaged (HD), mechanically damaged (MD) or MD plants treated with *S. exigua* oral secretion (MDOS) were collected at 24 and 48 h after treatment, frozen in liquid nitrogen and kept in a −80°C freezer until use. The samples were ground by using liquid nitrogen in a mortar. Lyophilized leaf samples (100 mg) were weighed into centrifuge tubes and extracted by ultrasonification (3 min) in a solvent (acetonitrile/water/phosphoric acid = 80:20:0.1; 10 mL). The samples were centrifuged (3 min at 2800*g*) and an aliquot of the supernatant was transferred directly into a vial. Synthetic gossypol (95% purity; Sigma-Aldrich, USA) was dissolved in the extraction solvent. Standard curves were obtained for gossypol with concentrations in the range of 5–80 µg mL^{-1} in 5-step increments. Three samples were collected for each treatment. Samples were analyzed on a Waters ESI^--MS (Q-Tof premier, Waters, Vernon Hills IL). Samples were isocratically eluted from a 150 × 3.9 mm I.D Waters (4 µm) C18 Novapak column. The mobile phases were 95% acetonitrile and formic acid. At the flow rate of 0.15 mL min^{-1}, total run time was 10 min. The signal was monitored at 272 nm. Data collection and integration were performed using the waters Empower software (Waters 2489, Milford, MA). The data were analyzed by using one-way ANOVA followed by the Tukey–Kramer HSD multiple comparison test at a significance level of $P < .05$.

Jasmonic acid extraction and quantification

Cotton leaves from the above four treatments (UD, HD, MD and MDOS) were collected 12 h after respective treatments, frozen in liquid nitrogen and kept in a −80°C freezer until use. JA was extracted as previously reported by Occhipinti et al. (2011). The amounts of JA in the samples were determined by comparing retention times and mass spectra of standard solutions with a linear gradient in RP-chromatography (Luna C18, 3.0 × 150 mm, 3.0 µm; Phenomenex, Torrance, CA) and further analysis of samples were analyzed on a Waters ESI^--MS (Q-Tof premier, Waters). Samples were isocratically eluted from a 150 × 3.9 mm ID. Waters (4 µm, particle size) C18 Novapak column. The mobile phases were 95% acetonitrile and formic acid. The flow rate was 0.15 mL min^{-1} and total run time was 10 min. The signal was monitored at 272 nm. Data collection and integration were performed using the Waters Empower software (Waters 2489, Milford, MA). H2-JA (TCI-Europe) was used as internal standard, and precursor ions were detected in negative mode by multiple reaction monitoring (MRM) [M-H] 209 and 211, for JA and H2-JA, respectively. The reported data are the mean values of at least three biological replicates and several technical replicates. The data were analyzed by one-way ANOVA followed by the Tukey–Kramer HSD multiple comparison test at a significance level of $P < .05$.

Gene expression: total RNA isolation and cDNA synthesis

To determine gene expression, leaf samples were collected from the four treatments (UD, HD, MD and MDOS) 24 h after treatment, frozen in liquid nitrogen and kept at −80°C until use. Frozen samples were ground to a fine powder in

Table 1. Primer sequences used for qrtPCR.

Gene	Forward(5′ to 3′)	Reverse(5′ to 3′)	GenBank Acc. No.
Histone(H_3)	GAAGCCTCATCGATACCGT	CTACCACTACCATCATGGC	AF024716
GhACT4	TTGCAGACCGTATGAGCAAG	ATCCTCCGATCCAGACACTG	AY305726
UBP6	GAAAGTGGATTACCCGCTG	CTCTAAGTTTCTGGCGAGGAG	At1g51710
eEF1Balpha2	ACTTGTACCAGTTGGTTATGGG	CTGGATGTACTCGTTGTTAGGC	At5g19510
GhLOX1	*GCCAAGGAGAGCTTCAAGAAT*	*TAGGGGTACTTGGCAGAACCT*	*AY929163*
GhAOS	*ATCATGTAATCCCCGAGTTCC*	*CCAGCTTGATCGTTAGCTGTC*	*KM265129*
Cad1-A	ATAAGGATGAAATGCGTCC	GAAGCTTGGTAAAGTTCCA	Y18484
Cdn1-C1	TTTGCATAGGAAAGAGCTA	CTCTATTGCTGAGCAATCAT	U23206
Cdn1-C14	AACTCAAAAACGCCACCAAC	TAGTCGGAATCGAAGGGATG	U23205
Cdn1-C3	AACTCAAAAACGCCACCAAC	TAGTCGGAATCGAAGGGATG	AF174294
CYP706B1	*GCAAGCCAATTGATTTTGGT*	*GCACCGGGAAAATATCAGAA*	*AF332974*
FPS1	*GGAAACCAGACACTGCCAAT*	*ACACTGCTTGCACTGGTTTG*	*KF871071*

liquid nitrogen using a pestle and mortar. Total RNA was extracted from 100 mg of each leaf sample using a Spectrum™ plant total RNA kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. RNA concentration and purity were determined by using a NanoDrop™ Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, DE, USA). RNA integrity was also assessed using 1% agarose gel electrophoresis and ethidium bromide staining. The presence of contaminant DNA in the RNA samples was verified by PCR using specific primers of a known gene and gel electrophoresis analysis. No fragments of genomic DNA were identified in any samples tested in this work. First-strand cDNA was synthesized from 200 ng RNA using a First-Strand cDNA synthesis, which employed a Goscrpit™ reverse transcription system kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Gene expression: real-time PCR

Transcript levels of genes involved in the biosynthesis of gossypol including CAD1 family genes (i.e. *Cad1-A, Cdn1-C1, Cdn1-C14, Cdn1-C3*), (+)-δ-cadinene-8-hydroxylase, a cytochrome P450 monooxygenase *CYP706B1*, JA-related genes (*GhAOS* and *GhLOX1*) and FPP synthase (*FPS1*), (see Table 1) were quantified in the four treatments (UD, HD, MD and MDOS) by quantitative RT-PCR. Real-time PCR was carried out on an ABI 7500 Real Time PCP System (Life Technologies, Carlsbad, CA, USA) with a 96-well rotor. Amplification reactions were performed with 25 µL of a mixture consisting of 12.5 µL of PerfeCTA® SYBR® Green Fastmix®, ROX qPCR Master Mix (Qunita Biosciences, Inc., Gaithersburg, MD, USA), 0.5 µL of cDNA and 100 nM primers (Integrated DNA Technologies, Coralville, IA, USA).

Relative RNA levels were calibrated and normalized with the level of four housekeeping genes: ubiquitin specific protease 6 (*UBP6*), Actin4 (*GhACT4*), the elongation factor 1B alpha-subunit 2 (*eEF1Balpha2*) and Histone 3 (H_3) ribosomal mRNA. The most stable of the four genes (i.e. *GhACT4*) was selected using the NORMFINDER software (Andersen et al. 2004). PCR conditions were determined by comparing threshold values in dilution series of the RT product followed by non-template control for each primer pair. Relative expression levels of genes were calculated by using the Pfaffl method (Pfaffl 2001). A suitable melting curve analysis was always performed. All amplification plots were analyzed with the Expression Suite software v1.0.3 (Life Technologies, Carlsbad, CA, USA) to obtain C_t values. Relative RNA levels were calibrated and normalized with the level of the *GhACT4* mRNA.

Larval feeding assay

To test whether gossypol has an effect on insect herbivory, we carried out a dose-dependent larval feeding test using gossypol-incorporated artificial diets, as described below. Individual pre-weighted second instar larvae were reared on artificial diets with or without gossypol in multiple-well trays (16 wells of 2 cm × 2 cm × 2 cm each). Each tray was sealed with a transparent cover with holes for ventilation and kept under controlled environment (25 ± 1°C, 60 ± 5% RH, and an L:D 14:10 h photoperiod). The larvae were fed with artificial diet incorporated with gossypol at the rate of 0.5–3.5 mg/g of diet in 0.5-step increments. To obtain a test dose, stock solution was prepared by dissolving 50 mg of gossypol (95% purity; Sigma-Aldrich, USA) in 10 ml of ethanol (99.8% purity) and a working solution was provided by diluting in water (0.5–3.5 mg/g). To account for the ethanol in the gossypol preparation, an equal amount of ethanol (0.5%) mixed with water was added to the diet (no gossypol) as a control, in addition to a separate untreated water control. A dose of 3 mg/g of diet was used for the bioassay. The bioassay was arranged in a completely randomized design with 20 replicates. Larval weight and mortality were recorded daily for three days. Insects that failed to move when probed three times with a dissecting needle were recorded as dead. Percent mortality was calculated and body weight was analyzed by using one-way analysis of variance (ANOVA) (SPSS package version 16). Mean separation was performed with Tukey–Kramer Honestly Significant Difference (HSD) at $P = .05$.

Results

Herbivory increases gossypol level in cotton plants

The level of gossypol was significantly higher in HD and MDOS cotton plants compared to UD or MD plants at 24 h after treatment (Figure 1). Similar results were also recorded at 48 h after treatment. However, gossypol levels in MD and UD plants were not significantly different. In general, the amount of gossypol increased with time, from 24 to 48 h after treatment (Figure 2).

Herbivory increases the level of jasmonic acid in cotton plants

The levels of JA in UD, HD, MDOS and MD cotton plants were quantified using HPLC-ESI-MS/MS. The levels of JA in HD and MDOS cotton plants were significantly higher by 3.2- and 2.4-fold than in UD cotton plants, respectively (Figure 2). Similarly, the levels of JA in HD and MDOS cotton

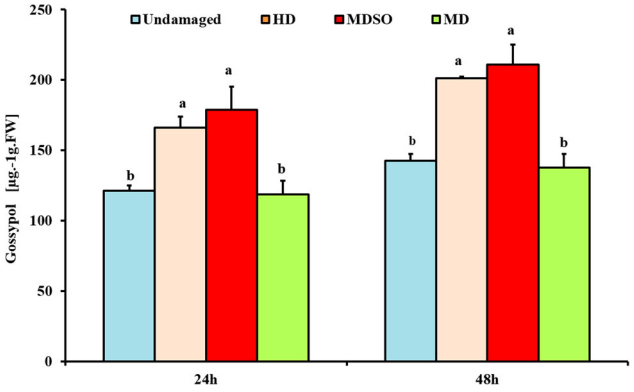

Figure 1. Gossypol level in undamaged (UD), herbivore damaged (HD), mechanically damaged and treated with oral secretion (MDOS) and mechanically damaged (MD) cotton plants 24 and 48 h after their respective treatments. Bars indicate means (±SE) of three replicates; significant differences among different treatments are indicated by different letters on each bar ($P < .05$, ANOVA, Tukey–Kramer HSD multiple comparison test, $n = 3$).

plants were significantly higher by 2.8- and 2.2-fold than the level of JA in MD cotton plants, respectively (Figure 2). There was no significant induction of JA in either UD or MD cotton plants.

Herbivory increases the transcript level of gossypol- and JA-related genes

The transcript levels of genes involved in the biosynthesis of gosspypol (i.e. *FPS1*,*CAD1-A, Cdn1-C1, Cdn1-C14, Cdn1-C3* and *CYP706B)* and JA (i.e. *GhAOS* and *GhLOX)* were higher in HD and MDOS cotton plants than UD and MD plants. The transcript levels of *GhAOS* and *GhLOX1* were increased in HD and MDOS plants by 2.8- and 2.5-fold compared to untreated control, respectively (Figure 3). There was no significant difference on the expression level of *GhAOS* in MD and UD plants. However, the expressions of *GhAOS* and *GhLOX* were higher in HD plants than MDOS, MD and UD plants (Figure 3).

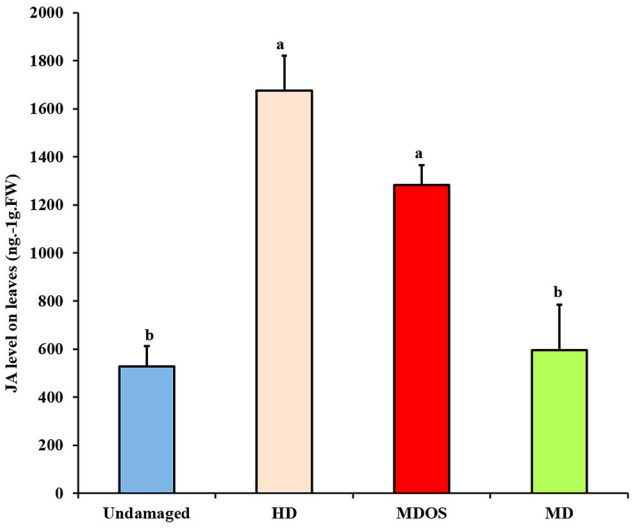

Figure 2. Jasmonic acid (JA) level in undamaged (UD), herbivore damaged (HD), mechanically damaged and treated with oral secretion (MDOS) and mechanically damaged (MD) cotton plants 12 h after their respective treatments. Bars indicate means (±SE) of three replicates; significant differences among different treatments are indicated by different letters on each bar ($P < .05$, ANOVA, Tukey–Kramer HSD multiple comparison test, $n = 3$).

S. exigua damage (HD) and mechanically damaged and treated with *S. exigua* oral secretion (MDOS) were found to increase the transcript levels of most of the genes involved in the biosynthesis of gossypol, such as *FPS1*,*CAD1-A, Cdn1-C1, Cdn1-C14, Cdn1-C3* and *CYP706B1* in cotton plants. The transcript levels of the *FPS1* gene were higher in HD and MDOS cotton plants by 2.1- and 1.8-fold, respectively, than in control and MD cotton plants. The transcript levels of CAD1 family genes such as *CAD1-A, Cdn1-C1, Cdn1-C14, Cdn1-C3* and *CYP706B1* were increased 24 h after herbivore damage (HD) by 2.2-, 5.9-, 2.0-, 3.7- and 4.2-fold compared to undamaged (UD) control, respectively (Figure 3). Most genes were consistently up-regulated by herbivory but not by mechanical damage. Interestingly, the transcript levels of *Cad1-A, Cdn1-C1, Cdn1-C14, Cdn1-C3* and *CYP706B1* were higher 24 h after mechanical damage and oral secretion treatment (MDOS) by 3.0-, 2.7-, 3.1-, 3.4- and 2.0-fold, respectively (Figure 3). There was no significant difference on the expression level of *Cdn1-C3 and FPS1*genes in HD and MDOS plants.

Gossypol impacts survival and development of S. exigua larvae

Larval mortality was significantly higher in the larvae that fed on gossypol-treated diet compared to untreated control (Figure 4). For instance, larval mortality in the cohort fed on gossypol-treated diet was twice (65%) as high as untreated control (0%) (Figure 4). Moreover, larval weight of *S. exigua* that fed on gossypol-treated diet was significantly reduced, when compared with those larvae fed on untreated diet and ethanol-treated diet (Figure 4).

Discussion

The transcript levels of jasmonic acid (JA) and gossypol-related genes were increased in *S. exigua*-damaged plants and mechanically damaged plants subsequently treated with OS. The increased transcript level of these key genes might be linked with increased level of JA and gossypol observed in these cotton plants. Interestingly, defense responses (defense-related gene expression, JA and gossypol content) of herbivore-damaged cotton plants were distinctly different from mechanically damaged (MD) or undamaged (UD) plants but showed similar response in MDOS plants. These results demonstrate that elicitors in herbivore OS are responsible for activating defense-related genes. Furthermore, higher level of gossypol showed negative consequences on subsequent feeding by *S. exigua* larvae and eventually increased larval mortality. The results of this study are in agreement with previous findings by McAuslane et al. (1997) and Alborn et al. (1996) which showed that *S. exigua* and *Spodoptera littoralis* larvae-damaged cotton plants contained much greater quantities of gossypol than control plants and were less preferred in feeding bioassays.

In plant defense signaling cascade, JA plays a central role in regulating defense responses to herbivores that inflict various types of tissue damage (Howe & Jander 2008). DNA microarray studies show that the JA pathway has a dominant role in regulating changes in gene expression in response to herbivory (Arimura et al. 2000; Reymond et al. 2000; Reymond et al. 2004; Howe & Jander 2008; Bricchi et al. 2013). Our result showed that HD and MDOS plants induced

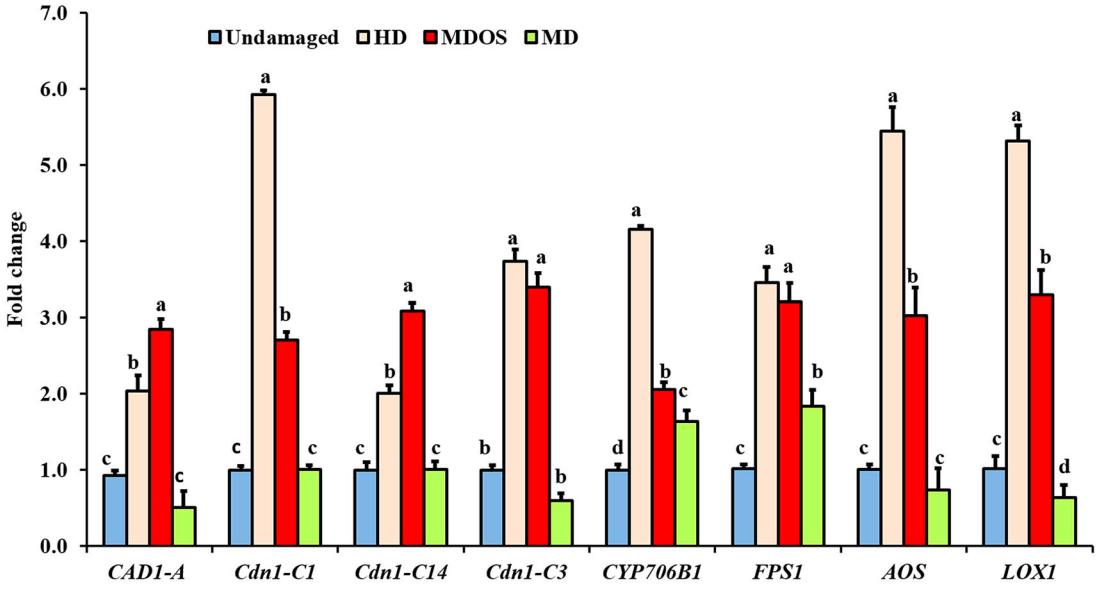

Figure 3. Differential expression of genes involved in biosynthesis of jasmonic acid (JA) in undamaged (UD), herbivore damaged (HD), mechanically damaged and treated with oral secretion (MDOS) and mechanically damaged (MD) cotton plants. Lipoxygenase (*GhLOX1*), *Allene oxide synthase (GhAOS)*, (+)-δ-cadinene synthase (CDNS; referred to as CAD), cytochrome P450 monooxygenase (*CYP706B1*) and farnesyl diphosphate synthase (FPS1). QRT-PCR analyses are shown as fold change in expression. Bars indicate means (±SE) of three replicates; significant differences among different treatments are indicated by different letters on each bar ($P < .05$). $n = 3$, Tukey–Kramer HSD multiple comparison test.

increased level of JA and the transcript levels of genes involved in JA biosynthesis (*GhAOS* and *GhLOX1*). Moreover, the expression of these JA-responsive genes was previously shown to be induced in cotton plants exogenously treated with JA or methyl jasmoniate (MeJA) or both (Miyazaki et al. 2014). Opitz et al. (2008) reported that exogenous application of JA induces increased level of gossypol and other terpenoid production in cotton plants.

The biosynthesis of sesquiterpene compounds, in general, takes place in the cytosol, with FPP as a common substrate. FPP synthase (*FPS1*) is a 1–4′ prenyltransferase that catalyzes FPP, which is a precursor for a structurally diverse class of terpenoides including gossypol (Gershenzon & Croteau 1993; Bohlmann et al. 1998). The transcript levels of *FPS1* were higher in plants that were herbivore damaged (HD) or mechanically damaged and treated with insect oral secretion (MDOS) than in undamaged (UD) or mechanically damaged (MD) plants. The first step in gossypol biosynthesis is regulated by *CAD1 family genes* (Cad1-C1, Cad1-A, Cdn1-C3, Cdn1) (Townsend et al. 2005; Zhou et al. 2013). *S. exigua* larval damage increases the transcript levels of *CAD1 family genes* (Cad1-C1, Cad1-A, Cdn1-C3, Cdn1), which regulate the catalysis of the cyclization of 2E,6E-FPP to (+)-δ-cadinene via a nerolidyl diphosphate. Interestingly, the transcript levels of *CAD1 family genes also increased in MDOS*. These results demonstrate that elicitors in herbivore OS were responsible for activating defense-related genes.

The second step in gossypol biosynthesis is hydroxylation of (+)-δ-cadinene to form 8-hydroxy-(+)-δ-cadinene, which is catalyzed by (+)-δ-cadinene-8-hydroxylase, a cytochrome P450 monooxygenase CYP706B1 (Luo et al. 2001; Wang et al. 2003). In plants, cytochrome P450 monooxygenases play essential roles in the biosynthesis of phenylpropanoids, terpenoids, alkaloids and other natural products and in the catabolism of herbicides and other xenobiotics (Zhou et al. 2011). The transcript levels of the *CYP706B1* gene were also significantly higher in HD and MDOS cotton plants than in UD or MD plants. Together, these results show a coordinated up-regulation of the genes that regulate enzymes which catalyze reactions in the gossypol biosynthesis pathway upon

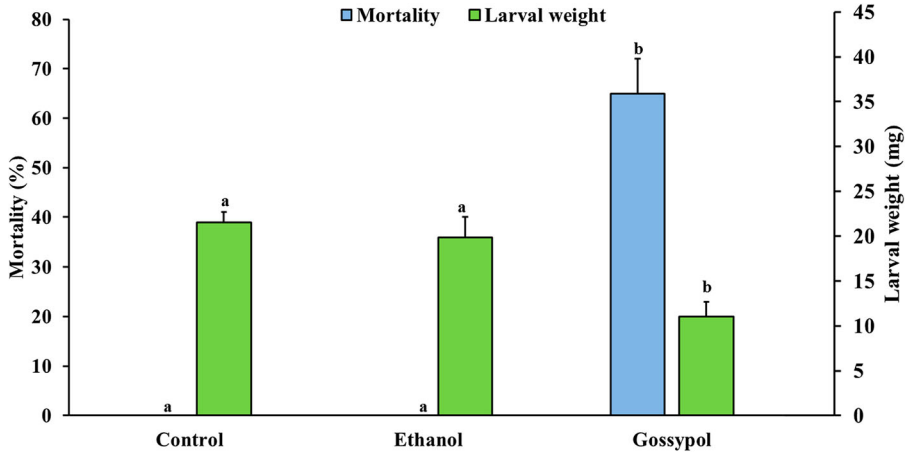

Figure 4. Larval weight and mortality rate of developing *S. exigua* larvae fed on artificial diet incorporated with gossypol, ethanol and untreated (control). Bars indicate means (±SE) of eight replicates; significant differences among different treatments are indicated by different letters on each bar ($n = 20$, $P < .05$, Tukey–Kramer HSD multiple comparison test).

herbivore damage in cotton plants. This coordinated induction leads to a rapid accumulation of gossypol in insect herbivore-damaged cotton plants.

Gossypol has been demonstrated to have an antibiosis effect against many chewing and sucking insect pests. For example, Stipanovic et al. (2008) demonstrated that gossypol reduces survival and pupal weights and increases days-to-pupation of *Heliothis virescens* larvae. Du et al. (2004) found that the high gossypol level in cotton has an antibiotic effect on *Aphis gossypii* in terms of aphid longevity and fecundity. Our data show that the quantity of gossypol was higher in HD and MDOS cotton plants than in UD or MD cotton plants. The data also show that *S. exigua* larvae that fed on gossypol-incorporated artificial diet showed significantly higher mortality rate than those fed only the artificial diet. Our results are consistent with the report by Mao et al. (2007) that lepidopteran larvae which fed on gossypol-rich artificial diet had higher mortality and reduced growth rates.

Previous studies revealed that the key enzymes that regulate gossypol biosynthesis are developmentally regulated and can be induced by pathogen infection in cotton plants (Meng et al. 1999; Townsend et al. 2005). Our results suggest that *S. exigua* larval damage and its OS increase the transcript level of JA-responsive genes and genes that regulate the key enzymes in the biosynthesis of gossypol and subsequently led to increased quantities of JA and gossypol in cotton plants. The increased level of gossypol in HD and MDOS plants might correlate with the increased level of JA observed in HD and MDOS plants. Although no isogenic mutant line for the JA pathway is available in cotton, our data clearly showed that the level of JA was significantly high in HD and MDOS plants. *Spodoptera* spp.-damaged cotton plants release VOCs in greater quantity to deter insect pests and attract natural enemies (Morawo & Fadamiro 2014; Sobhy et al. 2014). This phenomenon might have widespread agro-ecological significance including not only in plant–insect interactions but also in insect pest management.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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