Transcriptome and metabolome analysis reveal oral secretions from two lepidoptera species induced defense response in cotton

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Abstract

Cotton is one of the most important economic crops in the world, whereas insect attacks is a prime cause of its losses on yield and quality. Despite it is important, little is known about the mechanism of cotton response to insect. In this study, we simulated insect feeding by applying insect oral secretions (OS) to wounds, and combined transcriptome and metabolome analysis to comprehensively investigate how OS from two major pest species (*Helicoverpa armigera* and *Spodoptera litura*) affect cotton defense responses. Obvious differences were observed that 12,668 and 13,379 genes differently expressed respectively in cotton after being treated with different OS when compared with wounding along. Upon the addition of OS, JA-signaling pathway were rapidly and strongly induced, however SA-biosynthesis related gens were significantly down-regulated. By constructing the co-expression gene network, we identified a hub gene which encoding a leucine-rich repeat receptor kinase may play important role in recognition and signal transduction at early stage. We also found that OS from different insect species altered abundance of flavonoid related compound with different patterns. Interestingly, Gossypol which is a well-known anti-insect compound was kept a relative low content after OS application relative to wounding which implied the existence of the suppression effects of OS to cotton defense response. This study illustrated the transcriptional and metabolic changes of cotton in responding to OS from two chewing insect species, identified potential key gene during the interaction process and finds the evidence for OS's inhibition effects on cotton defense response.

***Key-words*: Oral secretions; *H. armigera; S. litura*; Cotton; Suppression; Host plant defense**

Introduction

The interactions between plants and insects have been exited for 350 million years[1]. To understand the mechanism of plant defense against insect attacks and how insect elude or overcome the barrier could provide us more information to control agricultural pests by using different strategies including classical breeding and transgenic approaches.

Cotton is one of the most important economic crops in the world, not only as the source of natural fiber, but also the third largest field crop in terms of edible oilseed tonnage[2, 3]. However, cotton production is seriously challenged worldwide by diversity arthropod pests, occurring at economically damaging levels every year[4]. The herbivorous insects cotton bollworm (*H. armigera)* and cotton leafworm (*S. litura*) are the two most destructive herbivores feeding on cotton in natural habitats. They direct feeding on cotton leaf, bud and boll, resulting in damages in cotton growth and production. Although the Bt transgenic cotton planted past decades has contribute to resist *H. armigera*, wheres, under the long-term selection pressure of Bt cotton, *H. armigera* has evolveed many types of resistance genes, which makes its resistance efficiency significantly reduced or even completely lost[5-7]. So, it is urgent to develop and utilize new endogenous or exogenous insect-resistant factors to protect cotton production. Although the interactions between insects and host plants have been study intensively[8-11], little is known about the defense response of cotton against chewing insects at transcription and metabolism levels.

Whereas, unlike with study in abiotic stress, it is difficult to control the feeding behavior of herbivory, such as the feeding time and feeding site. In the light of this complexity, it is obvious that a high degree standardization is required for the mechanism investigating. To cope with this dilemma, the application of oral secretions to mechanical wounds has used to mimic herbivory feeding[12, 13]. This method has been demonstrated to simulate real herbivory feeding well[14].

Evidence has accumulated over the past few years to indicated that hormones including jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) signaling pathways paly central roles in regulation of plant defense against pathogen and herbivory[15-18]. Generally, SA activated the plant defense response against biotrophic and hemi-biotrophic pathogens as well as the phloem-feeding insects such as whitefly and acids[19-21]. In contrast, JA and ET are usually associated with defense against necrotrophic pathogens and chewing insects[22, 23]. However, the cross talk

between SA and JA/ET signaling pathways was also provides the plant with powerful regulatory to against complex biotic stress encountered[24].

Transmembrane receptor-like protein kinases (RLKs), which is one of the most important sensory protein groups for plant to perceive environmental cues, such as pathogen-associated molecular patterns (PAMP) or herbivore-associated molecular patterns (HAMP), was widely demonstrated to regulate plant defense response to pathogens or herbivories[25, 26]. Genome-wide sequence analyses reveals a large number of RLKs was contained in higher plants and assigned to 21 subfamilies including the largest family, leucine-rich repeat (LRR)-RLKs[27]. According to previous studies, there are about 60 LRR-RLKs have been functionally defined including BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and FLAGELLIN SENSITIVE 2 (FLS2) which participated in the regulation to plant innate immunity[28, 29].

Plants can produce a large number of structurally and functionally diverse secondary metabolites, many of which have a role in defense against herbivores and pathogen by increase the abundance of compounds with toxic or anti-digestive to make the feeding difficult for attackers [30-33]. Such as benzoxazinoids and phenolamides in *Poaceae* or glucosinolates in *Brassicaceae* [10, 34]. In cotton, there were also some compounds including rutin and gossypol were reported to have anti-insect activity[35].

However, as response, herbivores also have developed various strategies in the continue battle to make them feeding easier. The most straightforward way to cross the defense is to elevate the tolerance to the defensive compounds synthesized by host plants, such as a *CYP6AE* gene cluster in the genome of *Helicoverpa armigera* contributed to detoxification of phytochemicals and insecticides[36]. In addition, they also release specific effector molecules or insect-associated bacteria to suppress the defense response in the host plants [37-41]. Moreover, insect endosymbiosis bacteria also reported to influence the interaction by expanding the food range or changing the phenotype of plants to benefit their host insects (Hosokawa *et al*. 2007; Kaiser *et al*. 2010).

In this study, we investigated the interaction between chewing insects (*H. armigera* and *S. litura*) and cotton plants by mimicking the real insect feeding. The RNA-Seq and untargeted

metabolomics analysis were performed to identify the differential response genes and metabolite features between leaves with wounding and OS treatments.

Materials and methods

Plant treatment and sample preparation

Cotton (*Gossypium. hirsutum*) cultivar Jin668 were sown in plastic pots in nutrient-rich soil with controlled temperature and daylight hours (28-30 ℃, 16h). The second-instar larvae of *H. armigera* and *S. litura* were purchased from Henan Jiyuan Baiyun Industry Co., Ltd (Henan, China). Individual insects were reared in 9 cm petri dishes containing fresh Jin668 leaves.

OS were collected from larvae (third to fifth instar) and kept on the ice during collection and subsequently stored at -70℃ until use. The plants used for the experiment were 30-day old and the third leaf from the top of each plant was selected for treating. Wound+Water (W+W) and Wound+OS (W+OS) treatments were performed by adding10 μL of water or OS to wounds generated by rolling a pattern wheel along the midvein generating two rolls of wounds on each side of two fully expanded leaves per plant. Treatments were all performed at the indicated times prior to harvest. Treated leaves from three individual cotton plants were harvested as one experimental replicate, pooled and flash-frozen in liquid nitrogen, stored at -80 ℃ before conducting the experiment. Three and six replicates were collected respectively for each time point for RNA and metabolite extraction respectively.

RNA isolation and library construction

Total RNA was extracted using polysaccharides & polyphenolics-rich RNApre pure plant kit (Tiangen Biotech, Beijing, China). The RNA concentrations were measured using Qubit 2.0 Flurometer (CA, USA) and RNA integrity was assessed using Agilent Bioanalyzer 2100 system (CA, USA). A total of 1.5 μg of RNA per sample was used for library construction. RNA sequencing was performed on an Illumina Hiseq 4000 platform.

Bioinformatic analysis of RNA-sequencing data

Clean data was obtained by filtering out low quality sequences. TopHat+Cufflinks[42, 43] was utilized to process the RNAseq and obtain the differentially expression genes (DEGs). Genes with *P*-value<0.05 and | log2(fold change) | ≥ 1 were defined as DEGs. The genome of *G. hirsutum* cv. TM-1 was used to be as the reference genome[44]. Gene Ontology was identified in the GO database through WEGO 2.0 (http://wego.genomics.org.cn/). The main metabolic pathways were predicted by KEGG enrichment analysis using KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/).

Measurement of phytohormones

Cotton leaves (100mg fresh weight) were homogenized with 80% cold methanol (v/v) with internal standard (10ng/ml of 9-, 10-dihydro-JA) and shaken at 4 °C overnight in the dark. The extract was filtered through a 0.22mm nylon membrane and stored at -70 °C prior to measurement. The quantification of the phytohormones was performed on an Applied Biosystems 4000-TRAR high-performance liquid chromatography-mass spectrometry system.

Untargeted Metabolomics data acquisition

In untargeted metabolomics experiment, a Waters Acquity UPLC system (Waters, USA) which was equipped a BEH C18 column (1.7 μm, 2.1 × 100 mm), was used to separate plant extract using a gradient program with a flow rate of 400 μL/min. The gradient program was as the following: 0-1 min, 99% A ,1% B; 1-2 min gradient phase reach 80% A, 20% B; 2-6 min, 60% A, 40% B; 6-7 min, 45% A, 55% B; 7-15 min, 5% A, 95% B; 15-18 min, 5% A, 95% B (solvent A: 99.0% water, 1.0% methanol, 0.1% formic acid; solvent B: 5.0% water, 95% methanol, 0.1% formic acid. All percentages are vol/vol). The temperature of column and sample chamber were held at 45 °C and 4 °C respectively. A Waters Xevo G2xs Q-TOF MS fitted with an ESI source was used to acquire MS and MS^n data. Data was collected in both positive and negative ion mode with MSE method, using 6 V and 15-30 V collision energy for low energy channel and high energy channel respectively. Calibration was performed prior to sample analysis via infusion of a sodium formate solution with mass accuracy within 1 ppm. The capillary voltage was set to 2,500 V for positive ion mode, and the source temperature was held at 100 °C, and the desolvation temperature was set to 400 °C with a nitrogen desolvation gas flow rate of 800 L/h.

Metabolomics Data Processing

Raw data files from UPLC-MSE platform were converted to the CDF format with the Datebridge software. Those converted files were further processed by XCMS [45]and CAMERA [46](all based on R-3.3.0 software), including peak detection, retention time correction, peak filling, annotation of isotopes and adducts. The "centWave" method was selected to perform peak detection, ppm=20, snthresh=10, peakwidth=c (2,10). For retention time correction, the relevant parameters were set as follow: minfrac = 0.7, bw1 = 3, bw2 = 2, mzwid= 0.015. Isotopes annotation and in-source pseudospectra reconstructure were conducted with the R package CAMERA. The loess method[47] based QC samples were used to perform correction for each metabolite feature detected in our experiment and only features with RSD < 30% in QC samples were retained for further analysis. Dataset was submitted to MataboAnalyst 3.0 (http://www.metaboanalyst.ca/) for statistics analysis. Features with >50% missing values were removed, and the remaining missing values were replaced by a small value (half the minimum positive value in the original data). In order to improve the quality of data analysis, the data set was filtered by interquantile range (IQR) which aimed to remove non-informative variables that near-constant values though-out the experiment.

Metabolites identification

The molecular formula of features was predicted by MassLynx software based on accurate m/z and isotopic pattern in positive model (window < 10ppm). Mass fragments of putative molecular were compared with candidate compounds in online mass spectral libraries (METLIN, Massbank, HMDB). Some of them identified by purchased standards.

Results

RNA sequencing of cotton treated with mechanical wounding and oral secretion (OS) from two chewing insects

In order to investigate the responses of cotton plants to these two chewing insects, we mimicked the herbivores' attack by applying the OS of these insects to artificially created wounds. The third fully expanded leaves were wounded with a pattern wheel and immediately applied with 10 μL of OS from *H. armigera* (W+OS_H), or *S. litura* (W+OS_S) or water (W+W) to the puncture wounds

separately (Fig. 1). Samples with water applied to the wounded sites (W+W) were served as control. To assess the global transcriptome profile of cotton plants regulated by W+OS and W+W treatment, we performed deep RNA-Sequencing of the leaf samples treated for 0, 10, 30, 60 and 240 min, untreated plants indicated 0 min.

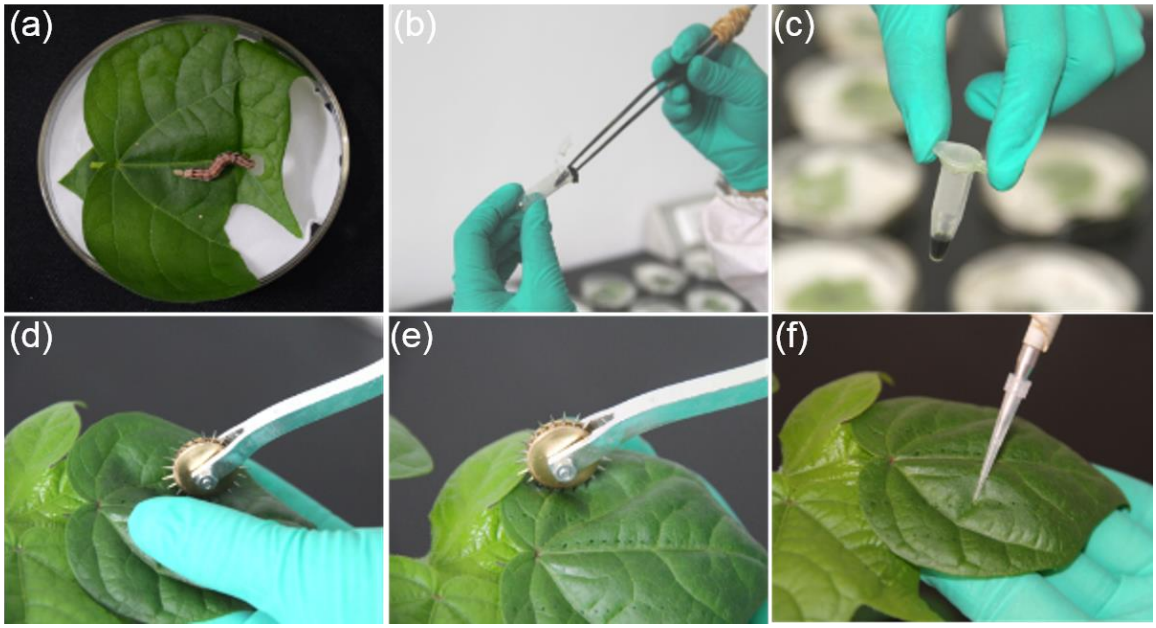

Figure 1. Demonstration of OS collection and application. (a) Raising insects with fresh cotton leaves in petri dish. (b) Collecting oral secretion from insects. (c) Storing oral secretion in plastic tube. (d), (e) Rolling a pattern wheel along the midvein generating two rolls of wounds on each side of two fully expanded leaves. (f) Adding water or oral secretion to wounds.

In total, 39 cDNA libraries were constructed and 288.02G output data were obtained from these libraries with ~25 million reads generated per library in which ~1% of the total paired-end reads were filtered and trimmed (Table 1). All correlation coefficients were more than 0.9 by evaluating the $\log_2$(FPKM) of two replicates in each treatment with Pearson's Correlation Tests, which indicates that the biological replicates are credible (Supplemental Figure. S1).

Table 1. Summary of RNA sequencing data from water, OS treated Jin 668 leaves samples

Sample	Total reads	Clean reads	Filter (%)	GC (%)	T-M[a](%)	U-M[b](%)	M-M[c] (%)
W+W0-1	25,580,881	25,244,634	1.31	44.00	90.20	90.80	9.20
W+W0-2	20,752,818	20,487,486	1.28	44.05	90.50	90.80	9.20
W+W0-3	22,062,260	21,733,630	1.33	44.18	90.60	90.40	9.60
W+W10-1	27,810,211	27,487,832	1.16	44.32	90.60	90.00	10.00
W+W10-2	25,440,159	25,123,857	1.24	44.43	90.70	89.80	10.20
W+W10-3	28,738,714	28,440,200	1.04	44.20	91.00	90.70	9.30
W+W30-1	28,710,201	28,430,016	0.98	44.40	90.80	89.80	10.20
W+W30-2	28,233,357	27,949,612	1.00	44.53	90.70	89.90	10.10
W+W30-3	31,108,247	30,826,636	0.91	44.47	89.70	90.00	10.00
W+W60-1	24,105,389	23,856,117	1.03	44.39	91.00	89.80	10.20
W+W60-2	23,792,105	23,547,415	1.03	44.32	90.90	90.30	9.70
W+W60-3	20,287,474	20,070,968	1.07	44.47	90.80	90.00	10.00
W+W240-1	27,862,784	27,546,840	1.13	43.86	90.60	91.10	8.90
W+W240-2	23,832,048	23,550,933	1.18	44.11	90.00	90.50	9.50
W+W240-3	26,604,487	26,336,600	1.01	44.11	90.80	90.30	9.70
W+OS_S10-1	19,601,696	19,427,502	0.89	44.45	89.10	90.00	10.00
W+OS_S10-2	18,960,130	18,787,823	0.91	44.25	90.50	90.10	9.90
W+OS_S10-3	24,227,022	24,013,801	0.88	44.06	90.20	91.10	8.90
W+OS_S30-1	24,386,653	24,142,463	1.00	44.42	90.20	90.50	9.50
W+OS_S30-2	24,844,820	24,608,507	0.95	44.72	90.00	86.90	13.10
W+OS_S30-3	23,808,343	23,611,557	0.83	44.11	90.00	90.80	9.20
W+OS_S60-1	18,719,087	18,544,496	0.93	45.34	90.20	82.90	17.10
W+OS_S60-2	20,794,084	20,620,502	0.89	44.20	90.20	90.50	9.50
W+OS_S60-3	19,800,816	19,652,314	0.74	43.89	90.40	91.00	9.00
W+OS_S240-1	22,305,783	22,182,898	0.55	44.39	91.10	90.40	9.60
W+OS_S240-2	23,000,828	22,872,108	0.56	44.37	90.90	89.80	10.20
W+OS_S240-3	18,701,351	18,599,608	0.54	44.12	89.30	90.10	9.90
W+OS_H10-1	32,059,094	31,780,293	0.87	44.42	90.50	89.60	10.40
W+OS_H10-2	22,193,429	22,013,797	0.81	44.25	90.40	90.90	9.10
W+OS_H10-3	24,372,606	24,167,068	0.84	44.28	90.40	91.40	8.60
W+OS_H30-1	18,735,462	18,548,498	1.00	44.26	90.40	90.10	9.90
W+OS_H30-2	20,651,459	20,536,606	0.56	44.50	90.30	91.00	9.00
W+OS_H30-3	20,025,114	19,908,250	0.58	44.26	91.70	90.80	9.20
W+OS_H60-1	24,883,408	24,616,552	1.07	44.22	91.30	90.40	9.60
W+OS_H60-2	20,434,408	20,219,097	1.05	43.97	91.00	90.20	9.80
W+OS_H60-3	22,594,514	22,359,036	1.04	44.17	90.60	90.40	9.60
W+OS_H240-	25,671,496	25,397,905	1.07	44.30	90.70	90.40	9.60
W+OS_H240-	29,114,028	28,813,687	1.03	44.23	90.40	90.30	9.70
W+OS_H240-	26,572,257	26,230,454	1.29	44.16	89.00	89.60	10.40

Leaves were harvested 0, 10, 30, 60, 240 min after treating by W+W or W+OS. Every treated-sample contains three biological replicates, [a]Total mapped. [b]Uniquely mapped. [c]Multiple mapped.

Transcriptome profiling of cotton leaves after *H. armigera* OS application

Compared with those in control, genes whose expression levels have changed at least 2-fold with *P*-value (adjusted)<0.05 were considered to show significant differential expression. A total of 12,668 differentially expressed genes (DEGs) were detected in W+W and W+OS_H treated samples at least one time point (Supplemental Table S1). The gene expression levels were used to performed a principal component analysis (PCA) for each of the biology replicates (Fig. 2a). In this analysis, the 10-, 30-, and 60-min time points of W+OS samples clustered farthest from W+W, samples in 240-min almost clustered together, indicating the gene expression level rapidly changed after OS application, and decayed in a short time. It also could be supported by the numbers of DEGs at each time points (Fig. 2b). The cotton plant responded to W+OS_H treatment by differentially expressing 5,494 genes at 10 min, then increased up to 8,153 at 30 min and reached the highest-8,495 at 60 min and reduced to mere 340 at 240 min. There were 1,164 and 1,193 genes with up- or down-regulation were observed in first three time points, whereas only 13 and 58 DEGs occurred at all time points (Fig 2c, d).

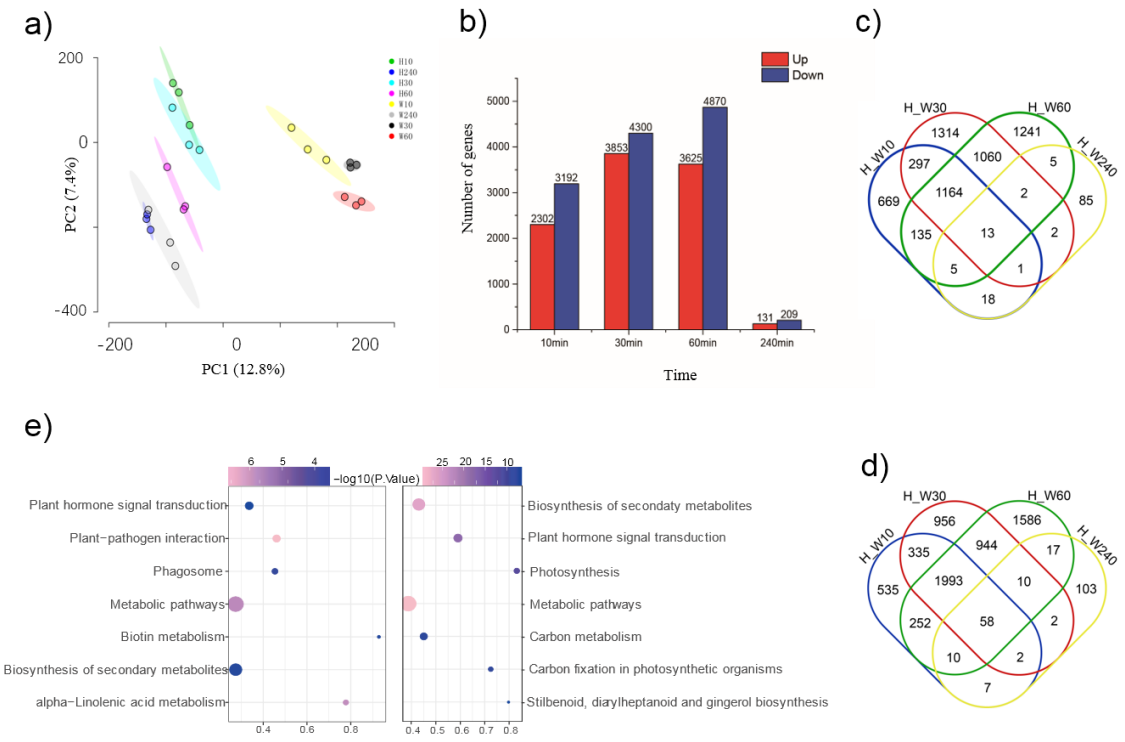

Figure 2.Overview of cotton transcriptome response to oral secretions from *H. armigera*. (a) PCA plot of genes identified by RNA-Seq of inbred line Jin668 treated by W+W and W+OS for the indicated time points. (b) Number of DEGs at each time point. $P<0.05$ (FDR adjusted), and fold change less than 0.5 or greater than 2. (c), (d) Venn diagram showing the distribution and overlap of up-/down-regulated DEGs across all time points. (e), The top 7 pathways of significantly up- (left) or down- (right) regulated genes enriched. ($P<0.05$)

DEGs from all time points were subjected to KEGG pathway analysis to identify the functional categorization. DEGs with up-regulation were assigned to 17 KEGG pathways ($P < 0.05$), including plant-pathogen interaction, alpha-Linolenic acid metabolism, as well as plant hormone signal transduction (Fig. 2e, Supplemental Table S2). For ones with down-regulation were significantly assigned to photosynthesis, Carbon metabolism and other 39 pathways (Fig. 2e, Supplemental Table S2).

Transcriptome profiling of cotton leaves after *S. litura* OS application

According to the PCA results, plants treated by W+OS_S exhibited farther distance from W+W at the first three time points which consistent with W+OS_H (Fig .3a). Compared with W+W, a total of 13,379 DEGs were found in W+OS_S, consisting of 5,130, 7,747, 8,894, and 330 genes at 10-, 30-, 60-, and 240-min time points respectively (Fig. 3b, Supplemental Table S3). Similar to the pattern in W+OS_H treatment, W+OS_S-treated samples also had a larger proportion of DEGS

with down-regulation than up-regulation. In addition, more than 6,000 genes significantly response to OS in a short time, Most of the DEGs occurred at first three time points, 3,253 of which were consistently up- or down-regulated in all time points except for 240-min(Fig. 3c, d), which also consistent with the response to W+OS_H . These results indicated that cotton can rapidly response to OS from this two lepidoptera. KEGG pathway analysis assigned all up- or down-regulated DEGs to 23 and 39 pathways respectively(P<0.05). (Supplemental Table S4), up-regulated genes significantly enriched in alpha-linolenic acid metabolism and plant-pathogen interaction, DEGs with down-regulation were enriched specifically in photosynthesis (Fig. 3e).

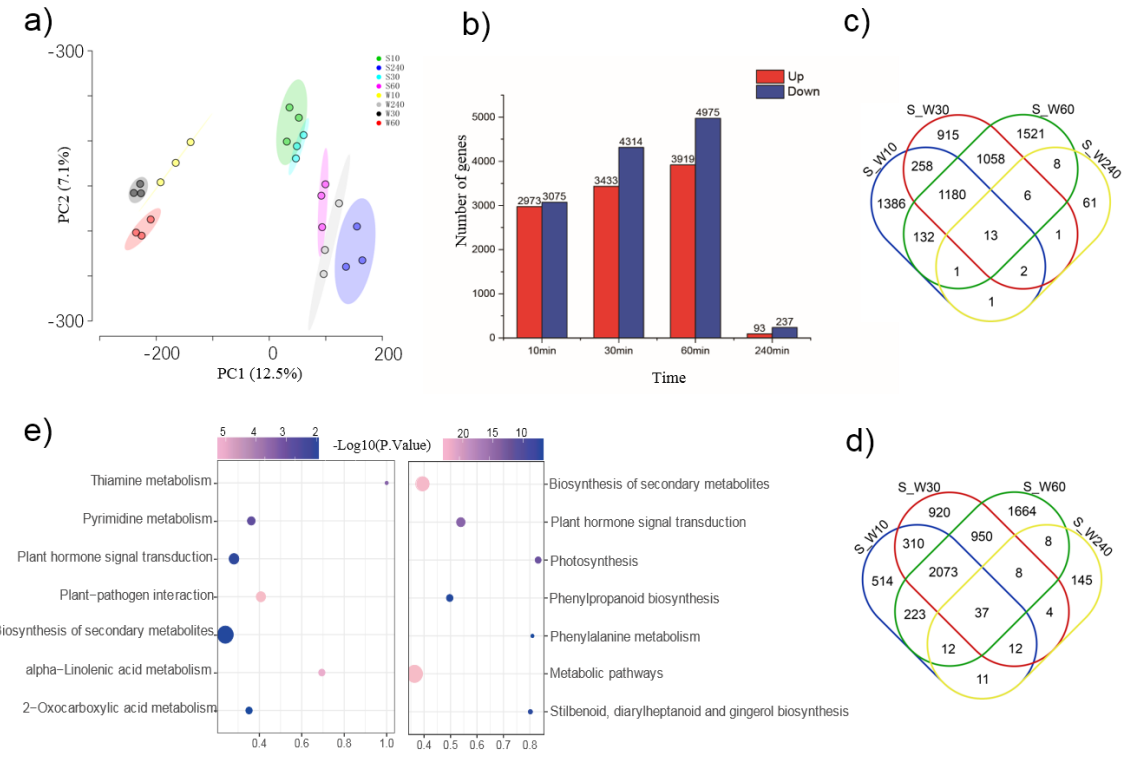

Figure 3. Overview of cotton transcriptome response to oral secretions from *S. litura*. (a) PCA plot of genes identified by RNA-Seq of inbred line Jin668 treated by W+W and W+OS for the indicated time points. (b) Number of DEGs at each time point. P<0.05 (FDR adjusted), and fold change less than 0.5 or greater than 2. (c), (d) Venn diagram showing the distribution and overlap of up-/down-regulated DEGs across all time points. (e), The top 7 pathways of significantly up- (left) or down- (right) regulated genes enriched. (P<0.05)

Furtherly, we conducted comparative analysis of DEGs from two oral secretion treatments and found that 4,471 and 5,433 genes were commonly up-regulated or down-regulate respectively after treating by W+OS_H and W+OS_S (Fig. 4a, b). The amount of common regulated genes was more than unique genes after treating by these two kinds of OS, indicating a high similarity at transcriptional level of cotton response to the OS from these two chewing insect species. The

global description of DEGs were annotated using WEGO analysis, it showed that most of terms which DEGs assigned were observed in both two different OS treatments (Fig. 4c). In details, DEGs significantly enriched in catalytic activity (GO:0003824), binding (GO:0005488), metabolic process (GO:0008152), cellular process (GO:0009987). Terms of genes involved in defense response to fungus, bacterium and fungus were also enriched significantly (GO:0050832, GO:0042742, GO:0009615).

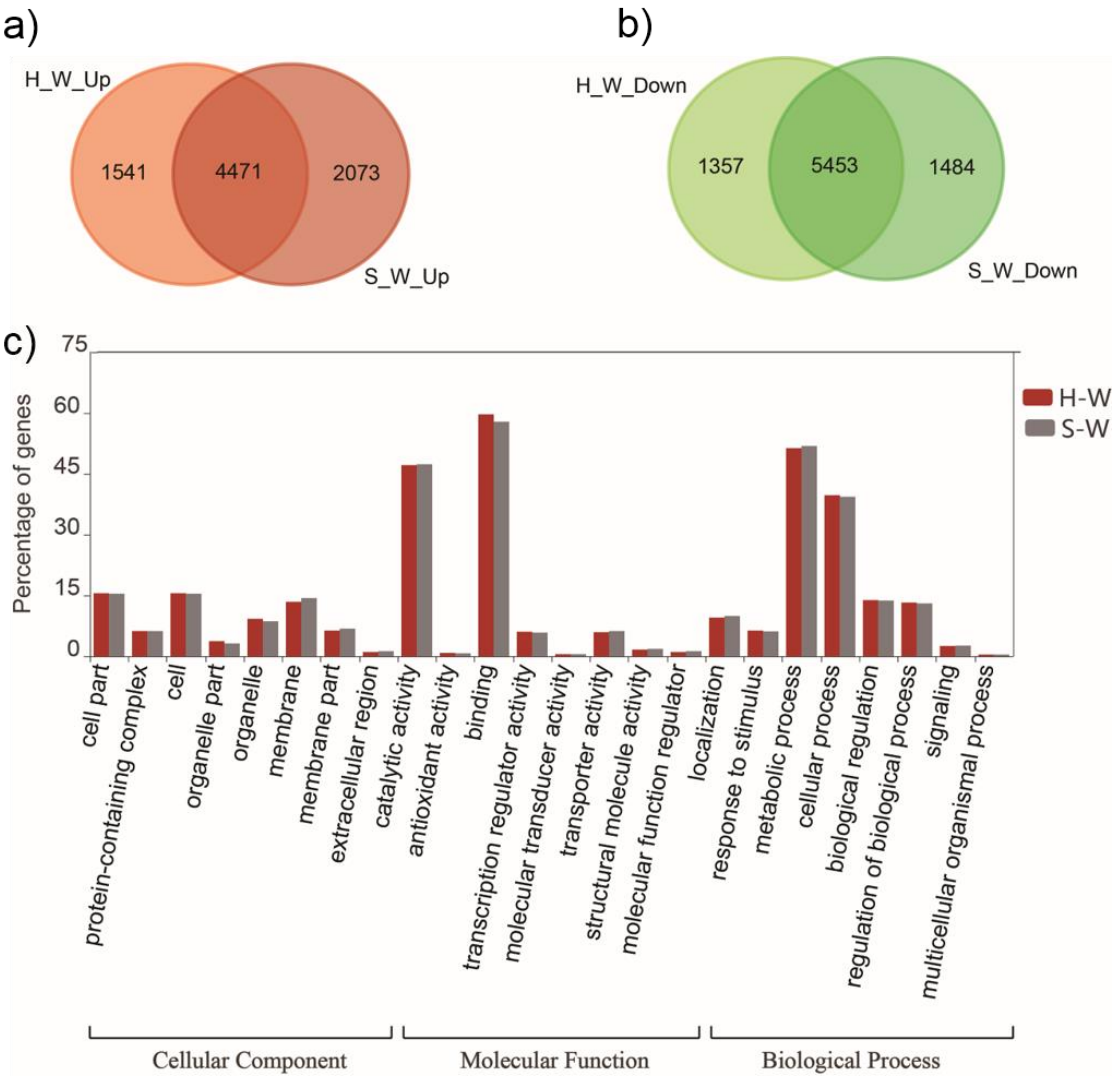

Figure 4. Comparation analysis of the DEGs from W+OS_H and W+OS_S .Venn diagram showing the common and unique up-regulated DEGs (a) and down-regulated DEGs (b) after application by two OS. (c) The GO terms of DEGs from W+OS_H and W+OS_S respectively significantly enriched. P<0.01.

OS induced early signaling pathways

Most major plant responses to herbivores or pathogen attack are primarily regulated via jasmonic acid, salicylic acid and ethylene. KEGG pathway analysis of DEGs (Fig. 2e, 3e) suggested that cotton defense signaling involved pathway are differentially regulated by OS. To understand how is the effects on hormones that elicited by OS , we evaluated the expression profiles of genes participated in JA-, SA-, and ET-responsive genes, such as *LIPOXIGENASE* (*LOX*), *ALLENE OXIDE SYNTHASE* (*AOS*) , *ALLENE OXIDE CYCLASE* (*AOC*), *OXOPHYTODIENOATE-REDUCTASE 3* (*OPR3*), *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 11* (*ACS1*), *1-AMINOCYCLOPROPANE-1-CARBOXYLIC OXIDASE* (*ACO*). In details, *LOX2* was found to be up-regulated after the onset of OS application, *AOC*, *AOS* and *OPR3* were significantly up-regulated after 30 min, and reached highest at 60 min (Fig. 5a), which also could be validated by qRT-PCR (Fig 5b).

Concomitantly, *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) which as the important JA-responsive gene was also observed to significantly increase at 60 min. (Fig. 5a). This result indicated OS from two chewing insect species can induced the expression of JA-related genes. To furtherly validated our result, we examined the content of JA and JA-Ile after treating by W+OS and W+W at different time points. Overall, JA and JA-Ile were rapidly increased in both W+W and W+OS relative to control, and the levels induced by W+OS_H and W+OS_S were more than one-fold greater than induced by W+W (Fig. 5e, f), which consistent with the changes at transcriptional level.

In contrast, genes involve in SA biosynthesis, *PHENYLALANINE AMMONIA-LYASE* (*PAL*) and *ISOCHORISMATE SYNTHASE* (*ICS)*, were found to significantly decrease in W+OS when compared to W+W (Fig .4a). We also investigated the expression of *PATHOGENESIS-RELATED GENE* (*PR1*, *PR5*) which as SA-responsive genes, but no significant alteration was observed. Remarkably, after 10 min of wounding, the transcripts of *PAL* and *ICS* rapidly accumulated and maintained a high level across all time points in W+W relative to control (non-treatment) (Fig. 5a), suggesting they could be induced by wounding and suppressed by OS. Interestingly, although *ACS1* and *ACO* which involved in ET-biosynthesis, were induced by OS, ET-responsive genes, *ETHYLENE-INSENSITIVE 3* and *ETHYLENE-INSENSITIVE3* (*EIN3*, *ERF1*) were found to

significantly decrease at 30 min and 60 min (Fig. 5a), which indicated an underlying mechanism in ethylene signal transduction during plant-insect interaction.

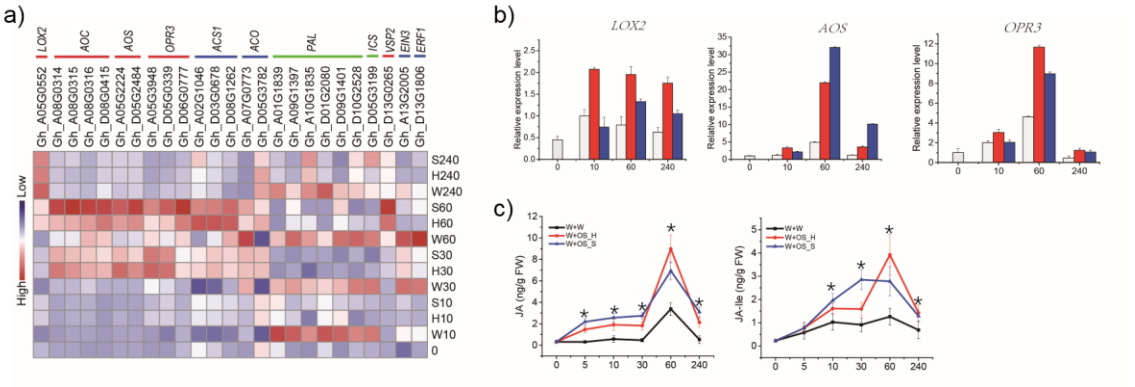

Figure 5. Expression of genes involved in hormones and metabolic changes in JA and JA-Ile. (a), Expression profile JA- (indicated by red line), ET- (blue line) and SA- (green line) related biosynthesis or responsive genes. (b) validation of the gene expression by qRT-RCR. (c) Abundance of JA and JA-Ile after different treatments. Values are mean ± SE (n=5). *, P<0.05 by T-test relative to W+W.

Co-expression network helps to identify defense response related hub gene

To identify the hub genes that response to OS we performed weighted correlation network analysis (WGCNA) on the DEGs (Fig. 6a). The expression matrix of specific transcripts from 39 samples was used to analyze, samples treated with W+W at 10, 30 and 60 min were excluded as the outliers. This analysis eventually identified 10 significant co-expression modules (Fig. 5b). Only 19 genes could not be clustered into any were assigned to grey module. Furthermore, KEGG enrichment analysis for genes in each module was performed respectively (Table 2). According to the results of enrichment analysis, blue module which showed significant association with plant-pathogen interaction was selected for further analysis (Fig. 6c).

Table 2. KEGG enrichment pathway for Co-expression modules.

Module	No. of genes	KEGG enrichment analysis
black	129	Protein processing in endoplasmic reticulum, Protein export, Phagosome,
blue	1525	Plant-pathogen interaction, Plant hormone signal transduction, Metabolic pathways
brown	922	Photosynthesis, Metabolic pathways, Circadian rhythm-plant,
red	189	Flavonoid biosynthesis, Biosynthesis of secondary metabolites, Phenylalanine metabolism
yellow	459	alpha-Linolenic acid metabolism, Plant hormone signal transduction, Zeatin biosynthesis
turquoise	2061	Carbon fixation in photosynthetic organisms, Carbon metabolism, Plant hormone signal transduction
green	421	Pyrimidine metabolism, Glutathione metabolism, Purine metabolism
Pink	57	Butanoate metabolism, Carbon metabolism, Metabolic pathways
magenta	49	RNA degradation, RNA transport, Protein export
purple	48	Ascorbate metabolism, Starch and sucrose metabolism, Aminoacyl-tRNA biosynthesis

The number of genes and annotated top 3 KEGG pathways of 10 modules. ($P < 0.05$).

To understand how genes in this module to response OS, we examined the expression profile of genes involved in plant-pathogen interaction (Fig. 6d), and found these genes exhibited similar expression pattern in W+OS and W+W, most of them were up-regulated after treating by OS when compared with W+W at same stage, such as *KINASE HEAT SHOCK PROTEIN* (*HST*) and *RESPIRATORY BURST OXIDASE HOMOLOG A* (*RBOHA*) and *WRKY22* which had been widely reported to involved in defense response. Notably, abscisic acid (ABA) signaling components, *CALCIUM-DEPENDENT PROTEIN 4* (*CPK4*, *Gh_A03G1505*, *Gh_A13G0566*, *Gh_D02G1973)* and *CALCIUM-DEPENDENT PROTEIN 32* (*CPK32*, *Gh_D09G1163*) which regulated ABA-responsive genes expression via phosphorylating *ABF4* were significantly induced at 10, 30, and 60 min after OS application (Fig. 6d).

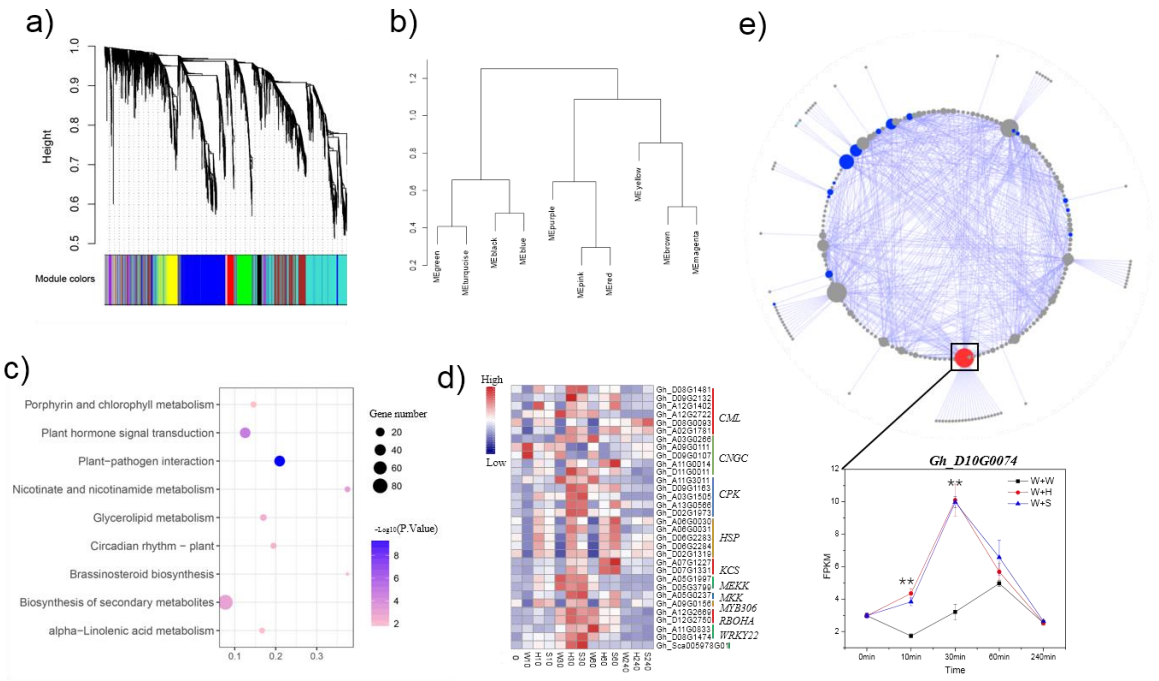

Figure 6. Co-expression network analysis identify hub gene in the process of plant response to OS. (a), Overview of the WGCNA based on DEGs. (b), The modules clustered and its relationships. (c), Pathways of genes in blue module enriched. *P*<0.05. (d), The expression profiles of genes in blue module also involved in plant-pathogen interaction pathway. (e), Co-expression network of genes in blue module and the expression pattern of hub gene which represented by red node, genes involved in plant-pathogen interaction were indicated by blue nodes. Values are mean ± SE (n=3). *, *P*<0.05 by T-test relative to W+W.

Hub gene in this module was screened based on high connectivity with other genes (Fig. 6e) Subsequently, *Gh_D10G0074*, which has a high amino acid sequence identity (55%) with *PEP1 RECEPTOR 1* encoding a leucine-rich repeat receptor kinase in *Arabidopsis thaliana* was identified as the hub. In our study, the transcript abundance of *Gh_D10G0074* was induced rapidly at 10 min after OS application and at least 3-fold up-regulated relative to W+W at 30 min (Fig. 6e).

Overview of metabolomic profiling of cotton response to oral secretions

Secondary metabolites are generally regarded to play important roles in plant adaption to environment stress. To find metabolites related to cotton defense response induced by herbivory, an untargeted metabolomics experiment was carried out (Supplemental Fig. S2a). We analyzed the content of metabolites extracted from cotton leaves which were induced by W+W and W+OS treatment at different time points (1 h, 4 h, 10 h), and manually annotated some of them (Supplemental Fig. S2b). After data pre-process and filtering, a total of 11,155 mass signals were

picked from metabolomics dataset, and 9,809 with RSD < 0.3 in QCs were selected to further analysis (Supplement Fig. S2c). To confirm the reliability of our experiment, we used all samples (including QCs and subject samples) for principal component analysis it showed that all of QC samples in our experiment were clustered well (Supplemental Fig. S2d), suggesting that our experiment system had a stable performance during metabolites detection.

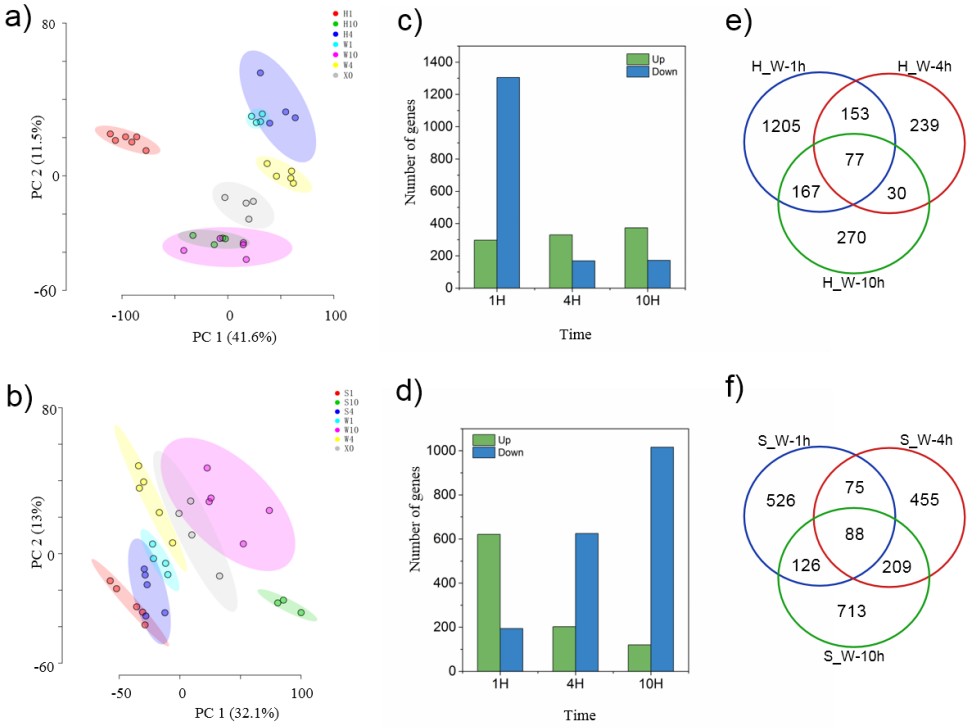

Figure 7. Overview of cotton metabolic response to W+W and W+OS. PCA plot of metabolite features of inbred line Jin668 treated by W+W, W+OS_H (a) or W+OS_S (b) for the indicated time points. Number of significantly regulated metabolite features in W+OS_H (c) and W+OS_S (d) respectively at each time point. Venn diagram showing the distribution and overlap of significantly changed metabolite features in W+OS_H (e) and W+OS_S (f). P<0.05 (FDR adjusted), and fold change less than 0.67 or greater than 1.5.

Consistent with the transcriptome, metabolomics profiles of cotton leaves treated by W+OS showed a strong difference compared with W+W. PCA with all filtered metabolite features showed a clear separation between W+OS and W+W treatment at 1 h, 4 h and 10 h respectively (Fig. 7a, b), which suggested that there were great metabolic changes between plant response to mechanical wounding and OS. In addition, the samples at 1 h with W+OS_H treatment clustered farthest from W+W at the same stage, 10-h W+OS_H and W+W sample kept a relatively close distance and closer to control (untreated plants) when compared to other stages (Fig. 7a), suggesting that metabolic differences disappeared with time increased. However, different pattern was observed in W+OS_H, which exhibited obvious differences even at the last time points (Fig. 7b).

In details, there were 297, 330 and 373 features were significantly up-, and 1305, 169, 171 were down-regulated after 1h, 4h, and 10h (P<0.05, Fold change >1.5 or < 0.67) respectively in W+OS_H relative to W+W (Fig. 7c). At the initiation of W+OS_S treating, there were much more features with up- than down- regulation, whereas, it reverted at 4- h, the great number of down-regulated features was observed at 10 h after W+OS_S treatment (Fig. 7d). To integrated the significantly altered features, we found that there was a limited overlap across all three time points (Fig. 7e, f), indicating the dynamic changes in cotton plants response to OS.

OS-induced changes on flavonoids related metabolism in cotton plants

Flavonoids in plants have been widely reported to serve a multitude of functions including being pigments and antioxidant, some of them participated in plant defense against biotic and abiotic stresses[48, 49]. To investigate the effects on metabolomic level in cotton response to OS, we focused on several compounds that annotated as flavonoids or related (Fig. 8a).

For these compounds previously reported to have anti-insect activity, such as chlorogenic acids, syringin and procyanidin B5, we found that it could be induced after treating by W+OS_H for at least one time point (Fig. 8b-d). In contrast, the content of syringin and procyanidin B5 in W+OS were down-regulated at 10 h when compared with W+W (Fig. 8b-d), which implied the inhibition effect of OS on plant defense response. In addition, isoquercitrin and rutin which had been shown to be important in protecting cotton from herbivory feeding[35] did not exhibit significant difference between W+W and W+OS in our study. For other compounds, quercetin 3-O-malonylglucoside and quercetin 3-xylosyl-(1->2)-alpha-L-arabinofuranoside that derived from isoquercitrin and quercetin respectively, exhibited significant increases at 10h after W+OS_H, whereas no significant difference was observed in W+OS_S (Fig. 8g, i). The content of quercetin 3-sambubioside and quercetin 3-(2G-xylosylrutinoside) were also significantly induced at 10 h, and kaempferol 3-O-glucoside was observed to increase at last two stage in W+OS_H (Fig. 8f, h), induction effects to these three compounds can be found in W+OS_S at 10 h, however it was not significant in statistics except for kaempferol 3-O-glucoside. These results indicated that OS from two caterpillar species could affect the accumulation of flavonoids with different patterns, and it seemed like that cotton plants responded more strongly to OS from *H. armigera*.

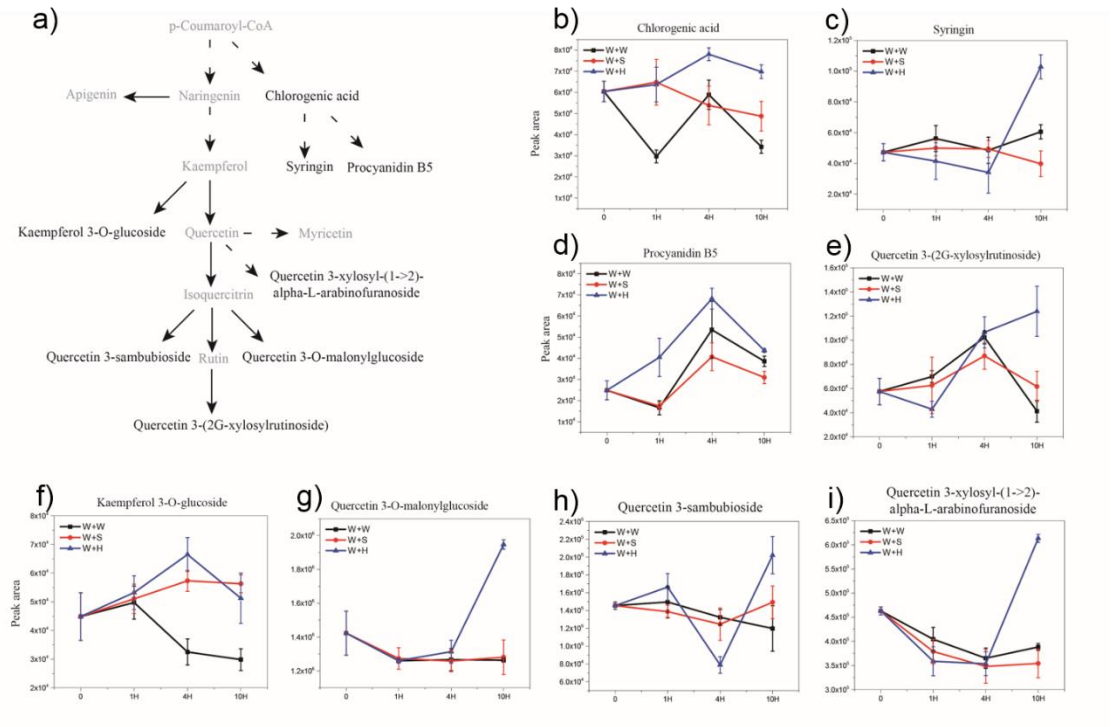

Figure 8. Effects of OS and wounding on flavonoid related metabolites. (a), Flavonoids related biosynthesis pathway. (b-i), Metabolites that were significantly altered by OS. Values are mean ± SE (*n*=3).

Gossypol biosynthesis pathway was influenced by OS

As a representative sesquiterpene derivative compounds in cotton, gossypol is considered to have important role in cotton against insect infestation (Parrott, 1990; Liu *et al*. 1999). In order to investigate the changes of gossypol, we analyzed the expression of genes involved in gossypol biosynthesis pathway, including (+)-Δ-*CADINENE SYNTHASE* (*CDN*), *CYTOCHROME P450 MONOOXYGENASE*, (*CYP706B1*, *CYP82D113*, *CYP71BE79*), *ALCOHOL DEHYDROGENASE* (*DH1*), and *2-OXOGLUTARATE/FE*(*II*)*-DEPENDENT DIOXYGENASE* (*2-ODD-1*) (Fig. 9).

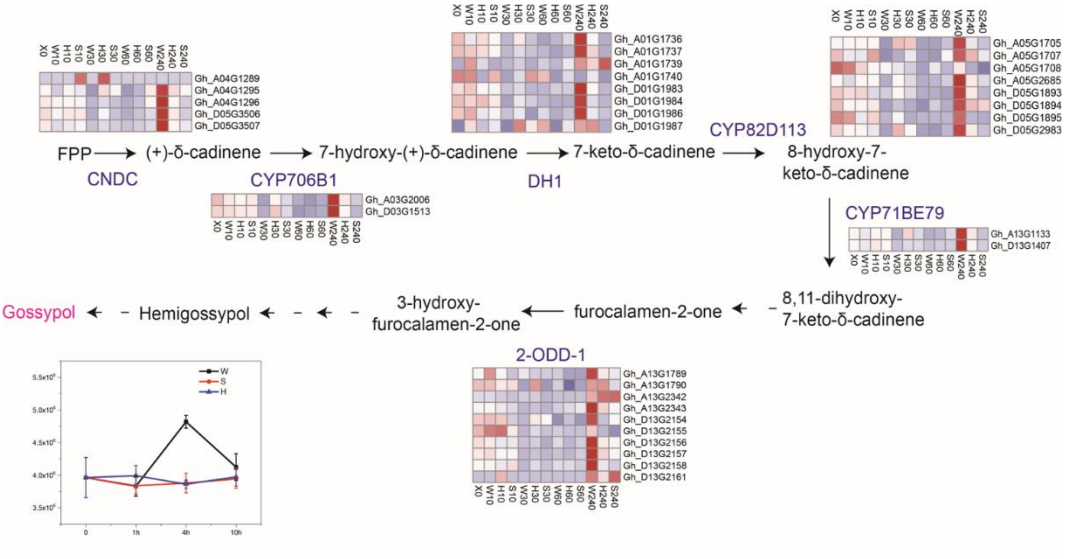

Figure 9. Expression of genes in gossypol biosynthesis pathway and the abundance of gossypol after treating by W+W and W+OS. Values are mean ± SE (n=5). *, $P<0.05$ by T-test relative to W+W. (+)-δ-cadinene synthase (CNDC), cytochrome P450 monooxygenase (CYP706B1, CYP82D113, CYP71BE79), alcohol dehydrogenase 1(DH1), 2-oxoglutarate/Fe(II)-dependent dioxygenase (2-ODD-1).

Interestingly, almost all the homologous genes involved in this pathway we presented exhibited higher expression level at 4 h after W+W treating, once the OS was applied, the abundance of transcripts was significantly reduced (Fig. 9), which may imply that the OS from these two caterpillar species could suppressed the gossypol biosynthesis via down-regulated the expression level of genes in this pathway. To validated the hypothesis, we examined the content of gossypol after treating by W+W and W+OS at different time points. It showed that the content of gossypol reached highest level at 4 h after wounding, and remained lower abundance after W+OS treatments throughout the experiment, which consistent with the pattern at transcriptional level (Fig. 9). These results suggested that some specific components in OS play important roles in plant-insect interaction by manipulating plant defense responses.

Discussion

Previous study has demonstrated oral secretion from herbivory is involved in plant defense response[50, 51]. In this study, we combined transcriptomic and metabolomic analysis to investigate the effects of oral secretions from two classic lepidopteran insect species on cotton defense response. Considering the intention of focusing on getting more complete information during the process, samples for transcriptome experiment were harvested at 0, 10, 30, 60, 240 min, and for metabolism analysis at 1, 4, 10 h after treatments. Despite only very small amount of OS

(10 μL) was applied to artificial wounds, the significant difference of transcriptional and metabolic responses of cotton to W+OS could be observed in a very short time. However, the transcriptional difference was almost vanished at 240 min (Fig. 2b, 3b), which different from the similar study in maize that more than 2,000 genes were still observed with significant regulation after treating by *Mythimna separata* OS at 6 h[12],which may due to the the species specificity of host plants and insects.

Comparing the genes that significantly changed at transcriptional level, more than 55% of DEGs with up-regulation were simultaneously observed at W+OS_H and W+OS_S, and even more than 66% of ones with down-regulation were overlapped, it also could be supported by the comparative GO enrichment analysis (Fig. 4c), whereas the pattern of changes at metabolism exhibited more distinct. KEGG pathway enrichment analysis showed that genes involved in photosynthetic pathways were significant down-regulated after treating by OS (Fig. 2e, 3e). It consistent with the concept that pathogen infection can inhibit photosynthetic, including photosystem II (PSII)activity , CO_2 fixation, and globally down-regulated photosynthetic genes[52, 53]. Recent study indicated that ETI (effector-triggered immunity) inhibited the photosynthesis by activating *MITOGEN-ACTIVATEDPROTEINKINASES 3/6* (*MPK3/6*)[54]. In our study, *MAPK3* was induced rapidly after OS treatment (Supplemental Fig. S3). These results implied that OS may also induced cotton plant ETI in our study like pathogens.

As a major hormone in plant signaling pathway, JA plays an important role in plant defense against chewing insects and insect OS.[55, 56]. Mutant plants with deficiency in JA signaling are more susceptible to insects than is the wild-type plant [57, 58]. In our study, the contents of JA or JA-ILE were increased after cotton plants were treated with W+OS, when compared those in plants treated with either W+W at all time points (Fig. 5c). As for another important signaling pathway, SA-biosynthesis related genes were significantly down-regulated (Fig .5a). The interaction between ABA and ET signaling is mutually antagonistic in vegetative tissues[59], which cloud explain that ET-biosynthesis genes were up-regulated but ET regulated genes, such as *EIN3*, *ERF1*, were significantly down-regulated , as *CDPK4* and *CDPK32* which positively regulate ABA signaling were strongly induced in our study (Fig. 6d). These results indicated JA and ABA signaling pathways were dominant a in cotton response to OS.

By using co-expression network analysis, we identified a hub gene (*GhD10G0074*) in the module specifically enriched in plant-pathogen interaction pathway. It has a high amino acid sequence identity (55%) with *PEP1 RECEPTOR 1* (*PEPR 1*) encoding a leucine-rich repeat receptor kinase in *Arabidopsis thaliana*. In previous studies, *PEPR 1* has been reported as a receptor for endogenous defense signal, Pep1, a 23-aa peptide which activates transcription of the defensive gene defensin (*PDF1.2*) and activates the synthesis of H_2O_2 to amplify innate immunity response to pathogen attacks, [60-63]. In our study, the expression of *PEPR 1* was and significantly induced at the initiate of OS treating (Fig. 6e), indicating the rapid recognition of cotton to signals, which as the base of triggering next defense response. These results also demonstrated that the early signals recognition and transduction were conserved in higher plants.

Plants produce a large number of functionally diverse secondary metabolites, some of which play important roles in plant response to biotic stress. (Kondo, *et al*. 1992). In our study, OS from two caterpillar species can affect the accumulation of flavonoids with different patterns, but OS from *S. litura* was weaker than *H. armigera*. It is noteworthy that OS from *H. armigera* can induce the accumulation of some anti-insect substances such as syringin and procyanidin B5, however, OS from *S. litura* did not, even slightly inhibited. As one of the most important toxic compounds to herbivores, gossypol exhibited a down-regulation after treating by W+OS at 4 h when compared with W+W (Fig. 9). These results remained us that as a counter strategy specific effector or microbe in OS may play critical roles in plant-insect interaction by manipulating plant defense responses.

Supplementary Materials

Additional file 1: Figure S1. Pearson's correlation coefficient testing the correlations of gene expression level between two biological repeats randomly selected form the three replicates.

Additional file 1: Figure S2. Overview of metabolic profile of cotton responses to different treatments.

Additional file 3: Figure S3. The expression level of *MPK3*. Relative expression of *Gh_D03G1283* validated by qRT-PCR.

Additional file 4: Table S1. Differentially expressed genes after treating by W+OS_H. (XLSX 1,862 KB)

Additional file 5: Table S2. Pathways analysis of differentially expressed genes after treating by W+OS_H. (XLSX 15 KB)

Additional file 6: Table S3. Differentially expressed genes after treating by W+OS_S. (XLSX 1,282 KB)

Additional file 7: Table S4. Pathways analysis of differentially expressed genes after treating by W+OS_S. (XLSX 15 KB)

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Authors' contributions

SX designed and coordinated the study. HS, HL, YW, ZX, SL, BL, JL, LS, QW carried out all the experiments. HS performed statistical and bioinformatics analysis of transcriptomics and metabolomics data and wrote the draft of the manuscript. All authors approved the final manuscript.

Conflicts interest

All the authors have declared no conflict of interest.

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