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ORIGINAL PAPER

Transcriptomic and metabolomic reprogramming in cotton after *Apolygus lucorum* feeding implicated in enhancing recruitment of the parasitoid *Peristenus spretus*

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Abstract

Different types of insect feeding can activate distinct plant resistance mechanisms and trigger the generation of specific volatile compounds. Transcriptomic changes and the genomic basis underlying plant defense in response to chewing herbivores or phloem-feeding insects have been relatively well investigated, while better insight into molecular mechanisms underlying the plant defense response, in particular, volatile emissions triggered by sap-feeding insects such as the green plant bug *Apolygus lucorum* is needed. Here, we monitored transcriptomic and volatile metabolomic changes in cotton over time during *A. lucorum* infestation. RNA-seq analysis showed that 1614 transcripts were differentially expressed ($\log_2|\text{Ratio}| \geq 1$; $q \leq 0.05$) in cotton leaves infested by *A. lucorum*. All differentially expressed genes (DEGs) in jasmonic acid (JA; 48 genes) and salicylic acid (SA; 5 genes) pathways were upregulated, highlighting a central role of JA in *A. lucorum*-induced signaling without attenuating the SA pathway. Moreover, all DEGs (30 genes) involved in herbivore-induced volatile biosynthesis were upregulated. Consistently, *A. lucorum*-induced cotton volatile blends and synthetic methyl salicylate significantly attracted the parasitoid *Peristenus spretus*. The present data indicated that cotton plants after *A. lucorum* infestation undergo rapid, extensive transcriptome reprogramming mediated by complex signaling networks in which the JA and SA pathways act synergistically, leading to a specific volatile profile involved in an indirect plant defense.

Keywords *Gossypium hirsutum* · Herbivore-induced plant volatiles · Induced resistance · Plant–insect interactions · Indirect defense · Methyl salicylate

Key message

- We monitored changes in transcriptome and volatile metabolome in cotton infested by *A. lucorum*.
- *A. lucorum* infestation activated JA signaling pathway without attenuating SA pathway.
- Specific volatile blends and synthetic MeSA were attractive to parasitoid wasp *P. spretus*.

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Introduction

Cotton plants are frequently subject to attack by different types of pests, including lepidopteran larvae, whiteflies, aphids and mirids such as the green plant bug, *Apolygus lucorum* (Ayubov and Abdurakhmonov 2018; Llandres et al. 2018). In China, the wide planting of *Bacillus thuringiensis* (Bt) transgenic cotton cultivars to control serious lepidopteran pests has allowed insecticide use to be reduced, which has led to frequent outbreaks of nontarget herbivores such as *A. lucorum* (Lu et al. 2010). *A. lucorum* is polyphagous and has become a key pest of cotton and many other important crops and prefers to feed on fresh leaves, young squares and bolls of cotton (Pan et al. 2019). When plants are damaged by mirid pests such as *A. lucorum*, local and systemic emissions of significant amounts of volatiles are induced as part of the plant defense response (Rodriguez-Saona et al. 2002; Degenhardt et al. 2011; Xiu et al. 2019). For example, the

egg parasitoid *Anaphes iole* was significantly more attracted to cotton volatiles induced by *Lygus hesperus* (Manrique et al. 2005), as well as to individual components in the volatiles such as methyl salicylate (MeSA), (*E*,*E*)-α-farnesene and (*Z*)-3-hexenyl acetate (Williams et al. 2008). Females of the endoparasitoid *Peristenus spretus* are attracted by two active compounds in volatiles from *A. lucorum*-damaged *Ricinus communis* plants in both Y-tube olfactometer trials and field studies (Xiu et al. 2019).

In response to a large variety of insects, plants have evolved an intricate and dynamic defense system to counter the effects of herbivore attack, including structural barriers and toxic chemicals (direct defenses) and attraction of natural enemies of the attacking pests (indirect defense). Both direct and indirect defenses may be present constitutively or only induced after herbivore feeding. Induced defense reprogramming of plants proceeds via the recognition of attackers and the activation of signaling networks, which is followed by transcriptomic changes in the expression of sets of defense-related genes, ultimately resulting in changes in plant phenotype (Stam et al. 2014).

Different insect species feed in a variety of ways. Chewing larvae consume a significant amount of plant tissue and thus extensively wound the host, which then loses water, while phloem-feeding and sap-feeding insects insert stylets between and into plant cells, causing less physical damage. These different types of herbivores trigger distinct signaling pathways, especially between leaf-chewing larvae and phloem-feeding herbivores (Erb and Reymond 2019). Generally, chewing herbivores and necrotrophic pathogens usually elicit jasmonic acid (JA)-mediated plant defenses, while the salicylic acid (SA)-responsive pathway modulates defenses against piercing/sucking insects and biotrophic pathogens (Davila Olivas et al. 2017). These two signaling pathways predominantly act through negative crosstalk (Erb and Reymond 2019), but they may overlap or even have synergistic effects (Zhou et al. 2011; Liu et al. 2016). Crosstalk between different signaling pathways allows plants to orchestrate fast and cost-effective responses to different pests.

Dynamic changes in the transcriptome of cotton plants damaged by chewing larvae of cotton bollworm *Helicoverpa armigera* (Huang et al. 2015; Kumar et al. 2016), cotton boll weevil *Anthonomus grandis* (Artico et al. 2014) and phloem-feeding insects including aphid and whitefly (Dubey et al. 2013; Li et al. 2016) have provided comprehensive insights into the cotton defense mechanisms to these different types of insects. Several potential target genes and key pathways were identified in these studies. In cotton damaged by the chewing *H. armigera* or *A. grandis*, more differentially expressed genes (DEGs) were upregulated than downregulated (Artico et al. 2014; Huang et al. 2015), and almost all the DEGs in the JA and ethylene (ET) pathways were upregulated (Artico et al. 2014; Huang et al. 2015; Kumar et al. 2016). DEGs related to SA biosynthesis and signaling were downregulated in cotton leaves or bolls infested by *H. armigera* (Huang et al. 2015; Kumar et al. 2016). These results suggested that larval feeding activated the JA and ET pathways and that the SA pathway was to some extent suppressed. In contrast, global transcriptome analysis showed that more DEGs were downregulated than upregulated in cotton infested by whiteflies and aphids (Dubey et al. 2013; Li et al. 2016), indicating that these two phloem-feeding insects stimulate transcriptional suppression rather than induction and that they suppress plant defense responses to facilitate their feeding. Additionally, whitefly *Bemisia tabaci* primarily activates the SA-dependent pathway and suppresses JA pathway (Dubey et al. 2013; Li et al. 2016).

The activation of different signaling networks by the different types of insect feeding is followed by the production of a great diversity of nonvolatile metabolites that are acutely toxic to herbivores and specific volatile compounds that function as ecological signals in direct, indirect and/or priming defenses (Erb and Reymond 2019; Turlings and Erb 2018). This volatile specificity might be closely related to the feeding habit of the herbivore. Whitefly *B. tabaci* alone did not increase volatile emission from cotton (Rodriguez-Saona et al. 2003). Moreover, the volume of volatile emissions from cotton plants simultaneously infested with *B. tabaci* and chewing *Spodoptera exigua* was significantly lower than from plants infested only with *S. exigua.* By contrast, chewing caterpillars (Loughrin et al. 1994; Röse and Tumlinson 2004; Huang et al. 2015) and piercing/sucking *L. hesperus* and *Aphis gossypii* elevated volatile emissions from plants (Rodriguez-Saona et al. 2002; Hegde et al. 2011). The specificity of plant volatiles induced by different herbivores can be sufficiently distinct to be recognized by their natural enemies (Turlings and Erb 2018). For example, *A. iole* females were only attracted by volatiles induced by *L. hesperus*, not by volatiles from plants exposed to a chewing insect (*S. exigua*) or after mechanical damage (Manrique et al. 2005).

Sap-feeding mirids are usually classified with phloem-feeding insects as piercing/sucking herbivores. They use stylets to vigorously puncture plant cells, and their feeding is more intense than that of phloem-feeding insects (Backus et al. 2007). Although an increasing number of studies have revealed changes in the transcriptome and metabolome of plants in response to cell-content feeding insects such as mites and thrips (Martel et al. 2015; Sarde et al. 2019; Zhang et al. 2020), little is known about the molecular mechanisms of cotton plant defense, especially about indirect defense against sap-feeding mirids such as *A. lucorum*. In the current study, transcriptomic changes in cotton plants infested by *A. lucorum* were analyzed at three times (12, 24 and 36 h of exposure) to identify candidate genes and key pathways associated with the activation of cotton plant defense, with

a focus on induced indirect defense against this insect. We also identified volatiles compounds in cotton leaves infested with *A. lucorum* bugs at three times and compared the volatiles after 36 h with those induced in cotton plants by *H. armigera*. Finally, the behavioral orientation of parasitoid *P. spretus* to headspace volatiles from cotton plants after a 36-h exposure to *A. lucorum* or to synthetic MeSA was examined. Our results provide a better understanding of cotton plant response to *A. lucorum* feeding and the mechanisms of induced plant defense against sap-feeding mirids.

Materials and methods

Plant and insect material

Cotton seedlings (*Gossypium hirsutum* cv. CCRI12) were grown singly in plastic pots as described previously (Huang et al. 2018) and used for experiments at 5–6 weeks of age. *A. lucorum* were reared on green beans (*Phaseolus vulgaris* L.) and a 10% sucrose solution soaked in a cotton ball and maintained in a climatic chamber at 29 ± 1 °C, 60 ± 5% relative humidity, and 14 h light: 10 h dark (Lu et al. 2014). Newly enclosed adult bugs were used for the experiments. The parasitoid *P. spretus* was reared on second instar nymphs of *A. lucorum* and maintained with a 10% honey solution in a growth chamber at 25 ± 1 °C, 65 ± 5% relative humidity, and 14 h light: 10 h dark (Luo et al. 2015). Unmated males (1–3 days old) or mated females (2–4 days old), without prior exposure to the prey *A. lucorum* or odors associated with prey–plant combinations, were used for behavioral experiments.

Plant treatments

Fifteen 3-day-old adults of *A. lucorum* were placed on one cotton plant in a nylon mesh cage, starting at 18:00. Cotton plants without *A. lucorum* were treated the same way and used as controls. After 12, 24 or 36 h, the bugs were removed, and plants were used for RNA extraction or volatile collection.

To compare changes in volatiles induced by *A. lucorum* with those induced by *H. armigera*, five plants each of similar size were assigned at random to separate cages to receive the following treatments: (1) infestation with 15 *A. lucorum*; (2) infestation with 6–7 3rd instar larvae of *H. armigera*; (3) no infestation (control). After 36 h, volatiles were collected from the plants as described later.

RNA extraction and sequencing

The first to third developing young leaves of each plant were harvested and pooled at each time and total RNA extracted using a modified hot borate method (Huang et al. 2015). RNA quality was assessed using the Agilent Bioanalyzer 2100 system, and then, 1 μg RNA per sample was used to prepare a cDNA library using the NEBNext Ultra RNA Library Prep Kit according to the manufacturer's instructions. The transcriptome was pair-end sequenced using a PE150 strategy on the Illumina Hiseq2500 platform (Sinobiocore, Beijing, China) as described previously (Wang et al. 2019). Clean reads were mapped to the *G. hirsutum* TM-1 genome downloaded from the CottonGen database (http://www.cottongen.org) using Histat2 software (Li and Durbin 2009). For each time and treatment except for 24 h post-infestation (two samples), transcripts were analyzed using three biological replicates. Transcriptome sequencing data are available at NCBI BioProject under accession number PRJNA688359.

Bioinformatics analysis

Gene function was annotated using the Gene Ontology (GO) database and Blast2GO (http://www.geneontology.org/). Genes were assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using KOBAS 2.0 software (Xie et al. 2011). Transcript abundances of the unigenes were calculated based on the FPKM (expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced). Genes associated with the statistical test $q \leq 0.05$ and the value of $\log_2|\text{Ratio}| \geq 1$ were considered as significantly differentially expressed genes (DEGs). The temporal expression of the DEGs was analyzed using the Short Time-series Expression Miner (STEM) (http://www.cs.cmu.edu/~jernst/stem/; Ernst and Bar-Joseph 2006).

Quantitative real-time PCR analysis

For validating the RNA-seq data, quantitative real-time PCR (qPCR) analysis was performed in biological triplicates using independent plants according to the previously published protocol (Huang et al. 2018). cDNA was synthesized from the total RNA using the FastQuant RT Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. GhACT4 (GenBank: AY305726) was used as a candidate reference gene. All primers used are listed in Table S1. Three biological replicates were analyzed for each time as described above.

Headspace collection and identification of herbivory-induced cotton volatiles

Volatile emissions from at least four insect-exposed or control cotton plants for each treatment and time were collected using a dynamic headspace sampling system (Huang et al. 2018). One pot containing one *A. lucorum*-exposed or

control plant was placed into a glass jar (diameter, 25 cm; height, 60 cm). A stream of activated charcoal filtered air was pumped through the glass jar and a glass tube (8 mm diameter) containing 50 mg Tenax-TA (60–80 mesh; Sigma-Aldrich) at a flow rate of 1.5 L/min for 6 h. The absorbed volatiles were then eluted with 300 µL of hexane, in which 2.595 µg of ethyl caprate (Sigma-Aldrich) was added as an internal standard.

A 1-µL aliquot of the eluted sample was injected into a Shimadzu gas chromatography-mass spectrometry (GCMS-QP2010 SE, Japan) with an Rxi-5Sil MS column (30 m × 0.25 mm × 0.25 µm). The oven temperature was maintained at 40 °C for 1 min and increased to 130 °C at 4 °C min^{-1} (5 min hold), then to 250 °C at 10 °C min^{-1} (5 min hold). MeSA was identified by comparison of its retention time and mass spectrum with those of an authentic standard (Sigma-Aldrich) analyzed under the same conditions. Other compounds were tentatively identified by comparison of mass spectra with those reported in the NIST17 library.

Y-tube olfactometer assays

Behavioral responses of *P. spretus* to *A. lucorum*-induced cotton volatiles (36 h after infestation) were evaluated using a Y-tube olfactometer (Dicke et al. 1990; Xiu et al. 2019). Two streams of charcoal-purified air were each pumped into the olfactometer (1-cm I.D.) at a flow rate of 400 mL/min. An aliquot of 10 µL of the eluted sample from *A. lucorum*-induced or control plant was pipetted on a piece of filter paper (20 × 50 mm). Filter papers and test solutions were replaced every 10 min. An adult wasp was individually released at the stem of the Y-tube olfactometer. If the tested insect failed to choose a side arm during the 5 min, then "no choice" was recorded. To compensate for any position bias, we reversed the side arms after five tests. After testing 10 individuals, the Y-tube was replaced. Moreover, behavioral responses of *P. spretus* to synthetic MeSA at two different concentrations (1 µg/µL and 10 µg/µL) were tested. Here, an aliquot of 10 µL of synthetic MeSA or mineral oil was applied. Thirty males and 30 females were tested in one biological replicate, and three biological replicates were performed in the experiments.

Statistical analyses

Student's *t* tests were used to compare the relative abundance of volatile compounds between herbivore-damaged and corresponding cotton control, and the data were analyzed for normality before performing Student's *t* test. For Y-tube olfactometer assays, *P. spretus* preferences were analyzed using a χ^2 goodness-of-fit tests with the null hypothesis that the parasitoid wasp had no preference for either olfactometer arm (i.e., 50:50 response). Data analysis was performed in SAS 9.4 software (SAS Institute 2012).

Results

Global transcriptome changes in cotton plants in response to *A. lucorum* feeding

Transcriptome sequencing generated 39,986,648–47,672,906 raw reads per sample. After quality control, 38,025,146–44,696,340 high-quality reads were remained. More than 92% (92.86–93.51%) of these reads were mapped to the *G. hirsutum* TM-1 genome. After assembly, 33,862 transcripts were detected in infested and non-infested cotton leaves. Among these transcripts, 1614 were associated with a cutoff of $q \leq 0.05$ and $\log_2|\text{Ratio}| \geq 1$ at one or more times and thus identified as differentially expressed genes (DEGs). Among these DEGs, 1131 were significantly upregulated as result of infestation, 479 were significantly downregulated, and four were mixed in expression (Table S2). For the 36-h continuous exposure, a considerable number of genes were induced after 12 h of infestation (1035 up-DEGs), and the number of upregulated DEGs decreased sharply at the later times (i.e., 24 h and 36 h) (Fig. 1a).

Among the common and exclusive DEGs determined at the three durations of infestation (Fig. 1b; Fig. S1), 11.3% of DEGs were present throughout the 36 h. In addition, 45.3% of DEGs were exclusively modulated within 12 h after *A. lucorum* infestation, suggesting that *A. lucorum* induced a rapid transcriptomic reprogramming and that these DEGs were more likely to play roles in the rapid, specific recognition and early defense signaling events triggered by *A. lucorum* feeding. In the functional classification using Gene Ontology (GO) analysis (Fig. S2), the DEGs were categorized as biological process, cellular component or molecular function. For GO terms of biological process, the top term was metabolic process, followed by cellular process. The data also showed that membrane part, cell part and organelle categories were most abundant in the cellular component ontology. Most of the genes in molecular function were classified into the binding and catalytic subgroup.

To further verify the RNA-seq results, nine DEGs associated with phytohormone biosynthesis and secondary metabolism were randomly selected for qPCR analysis. Expression patterns of the DEGs obtained from qPCR and RNA-seq were highly consistent (Fig. 2), indicating the reliability and repeatability of the RNA-seq results.

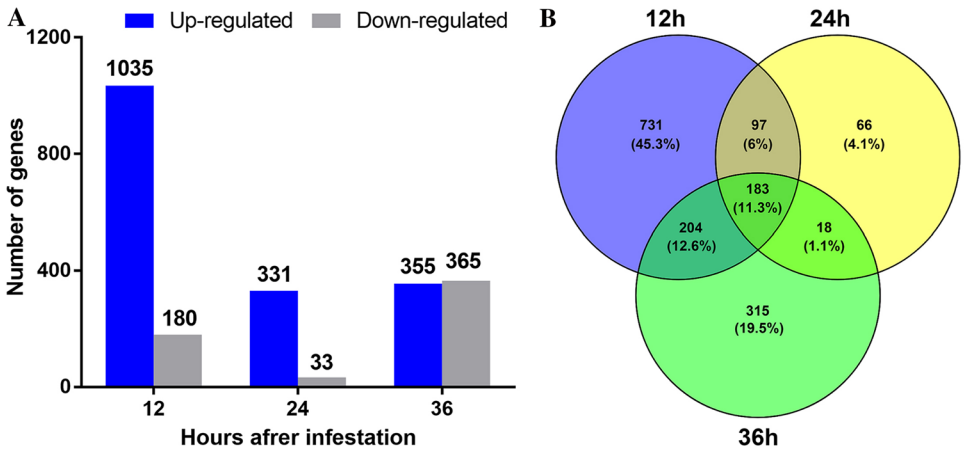

Fig. 1 Overview of DEGs in cotton exposed to *Apolygus lucorum* for 12, 24 or 36 h. The DEGs are those differentially expressed ($\log_2|\text{Ratio}| \geq 1$) with a cutoff of $q \leq 0.05$ for at least one sampling time. **a** Number of up- and downregulated DEGs in cotton at different times of exposure to *A. lucorum*. **b** Venn diagram of common and unique DEGs in cotton at different times of exposure to *A. lucorum*

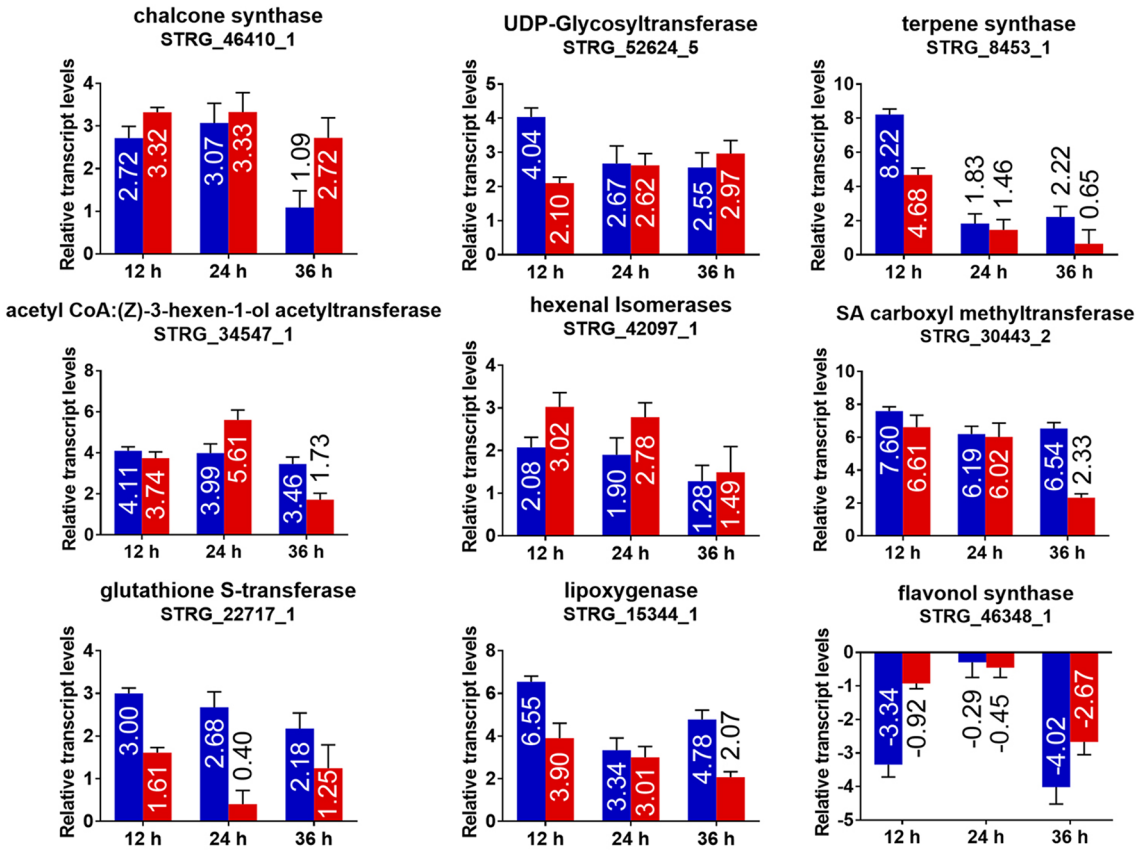

Fig. 2 Comparison of expression levels of selected genes in RNA-seq (blue bar) and qRT-PCR (red bar) analysis. Relative transcript levels (mean ± SE) of the selected genes in cotton plants infested by *A. lucorum* at three times (12, 24 and 36 h of exposure) were compared with the corresponding control

Temporal changes of cotton transcriptome in response to *A. lucorum* infestation

In the STEM analysis of temporal patterns of the transcriptome, 1201 of 1614 DEGs clustered into eight expression profiles (Fig. S3; Table S3). Type I DEGs consisted of profiles A-D, and most were upregulated as result of infestation. Among Type I DEGs, 93% of the genes (645 genes) that had $\log_2|\text{Ratio}| \geq 1$ at 12 h or more times were rapidly and strongly upregulated after 12 h of infestation. Although expression levels of 544 genes in profiles A (127 genes) and B (417 genes) decreased at later times, 101 genes were still highly expressed at 36 h (profiles C and D). In contrast, most Type II DEGs were downregulated and had distinct

expression profiles (profiles E–H). Among these Type II DEGs, 314 genes (62%) were downregulated at 12 h or more times, 33 genes increased in expression at later times (profiles E and F), and the 282 genes in profiles G and H had lower expression levels throughout the 36 h.

DEGs associated with phytohormones and transcription factors elicited by *A. lucorum* infestation

Twenty-seven JA biosynthesis genes and 21 JA signaling genes were differentially upregulated. Among these, 35 genes were upregulated for at least two sampling times, and most of these genes were rapidly and strongly upregulated after 12 h of infestation (profiles A, B and H). Additionally, most of the DEGs associated with ET (17 of 18), SA (5 of 5), auxin (10 of 14), cytokinin (4 of 7), abscisic acid (3 of 4) and gibberellic acid (2 of 2) were upregulated, whereas all genes associated with brassinosteroids (BRs) (5 of 5) were downregulated (Fig. 3).

Also, the expression profiles of genes known or predicted to be involved with transcription factors were investigated. A total of 149 DEGs, including 118 upregulated and 31 downregulated genes, were involved in possible signaling processes elicited by *A. lucorum* infestation. Among the 118 upregulated genes, the largest family was AP2-EREBP (37 of 41) (Table S4).

A. lucorum-induced reconfiguration of primary metabolism

Although most DEGs related to amino acid metabolism (63 out of 71 DEGs), carbohydrate metabolism (38 out of 51) and nucleotide metabolism (18 out of 26) were induced, all of the 16 DEGs associated with photosynthesis were downregulated (Table S5). Notably, all but one DEG involved in photosynthesis were significantly downregulated only at 36 h.

DEGs associated with defense and secondary metabolism induced by *A. lucorum*

The cluster of defense-related DEGs included 53 genes, which may be involved in plant defense against various abiotic and biotic stresses, and 40 were upregulated (Fig. S4). It is noteworthy that some ABC transporter G family members were differentially expressed in response to *A. lucorum* feeding and are known to be involved in defense and to transport benzenoid volatiles across the plasma membrane in *Petunia hybrida* (Adebesin et al. 2017).

For volatile secondary metabolite pathways, numerous genes were differentially expressed after *A. lucorum* infestation (Fig. 4). Terpenoid biosynthesis (21 upregulated genes) comprised the largest subgroup, with all of the eight differentially expressed terpene synthase genes upregulated (two ocimene synthases [unpublished data] and two linalool synthases, one cadinene synthases and three bifunctional (*E*)-nerolidol/(*E*,*E*)-geranyllinalool synthases involved in the synthesis of the herbivore-induced volatile homoterpenes 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) in cotton (Huang et al. 2018)). All of the five DEGs for phenylpropanoid/benzenoid biosynthesis were upregulated. For green leaf volatiles pathway, all of the four DEGs were upregulated including three acetyl CoA:(*Z*)-3-hexen-1-ol acetyltransferase, which catalyzes the formation of (*Z*)-3-hexen-1-yl acetate (D'Auria et al. 2007), and one hexenal isomerase that converts *Z*-3-hexenal to *E*-2-hexenal (Spyropoulou et al. 2017).

Moreover, many genes for some key proteins/enzymes responsible for the biosynthesis of nonvolatile metabolites that contribute to pest and disease resistance such as cytochrome P450 (CYP), glutathione *S*-transferases (GSTs) and UDP-dependent glucosyltransferases (UGTs) (Baek et al. 2019) were also upregulated (Fig. 4). Eight GSTs and 10 UGTs were differentially expressed, and all were upregulated. Most (21 of 29) CYP genes were upregulated. Besides their roles in nonvolatile secondary metabolites, CYPs also play important roles in the biosynthesis of many volatiles. Among these upregulated CYP genes, two were annotated as phenylalanine *N*-monooxygenase, involved in the synthesis of the floral scent 2-phenylethanol in *Plumeria rubra* (Dhandapani et al. 2019), and three are involved in the synthesis of herbivore-induced volatiles DMNT and TMTT in cotton (Liu et al. 2018).

Increased emission of volatile blends induced by *A. lucorum* infestation

In this study, relatively small amounts of α-pinene, (*Z*)-3-hexenyl acetate, β-caryophyllene and α-humulene were emitted from undamaged control plants. However, plants after 12, 24 and 36 h of infestation released significantly greater amounts of volatiles (Table 1; Fig. S5), which consisted of terpenes, hydroperoxide lyase (HPL)-derived green leaf volatiles and shikimic-acid-derived phenylpropanoids/benzenoids. Sixteen compounds were detected after 12 h, with seven significantly higher than emitted from the control plants. Of 23 compounds detected after 24 h, 13 were significantly higher than in the control emissions. After 36 h of infestation, 28 compounds were detected, and 13 were significantly elevated compared with the controls (Table 1; Fig. S5).

After the 36-h exposure to insects, the number and quantity of volatiles emitted from cotton infested only with *H. armigera* alone or with both pests were higher than emitted from cotton plants after *A. lucorum* infestation. Of 35

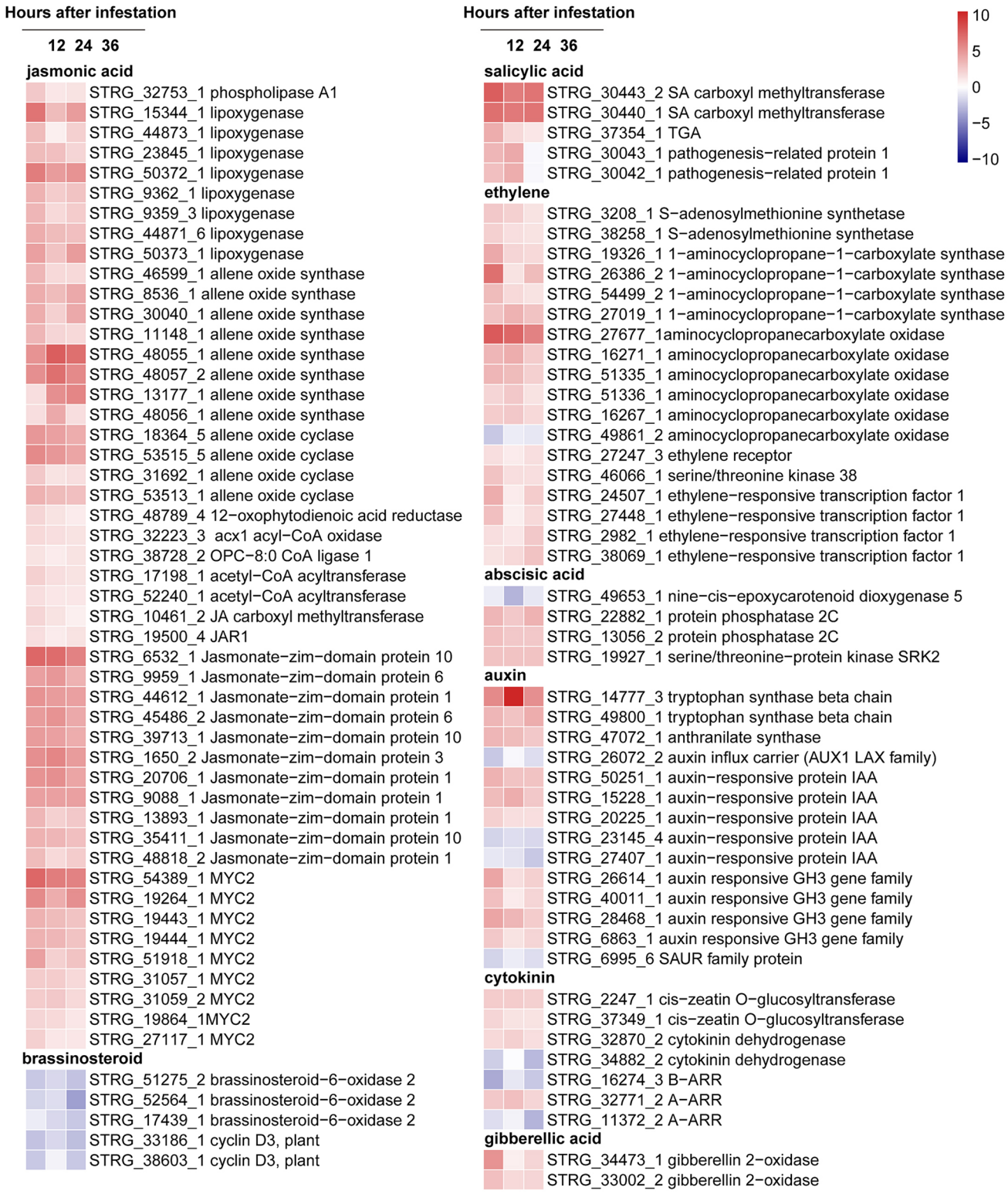

Fig. 3 *Apolygus lucorum*-responsive DEGs involved in phytohormone biosynthesis and signaling

volatiles emitted from *H. armigera*-infested cotton plants, 25 compounds were significantly higher than from control plants (Table S6). Of 37 volatiles detected after the dual infestation, 15 were significantly higher than determined in our recent study (Huang et al. 2018). Interestingly, one induced volatile (retention time = 18.78 min in Table 1; Fig.

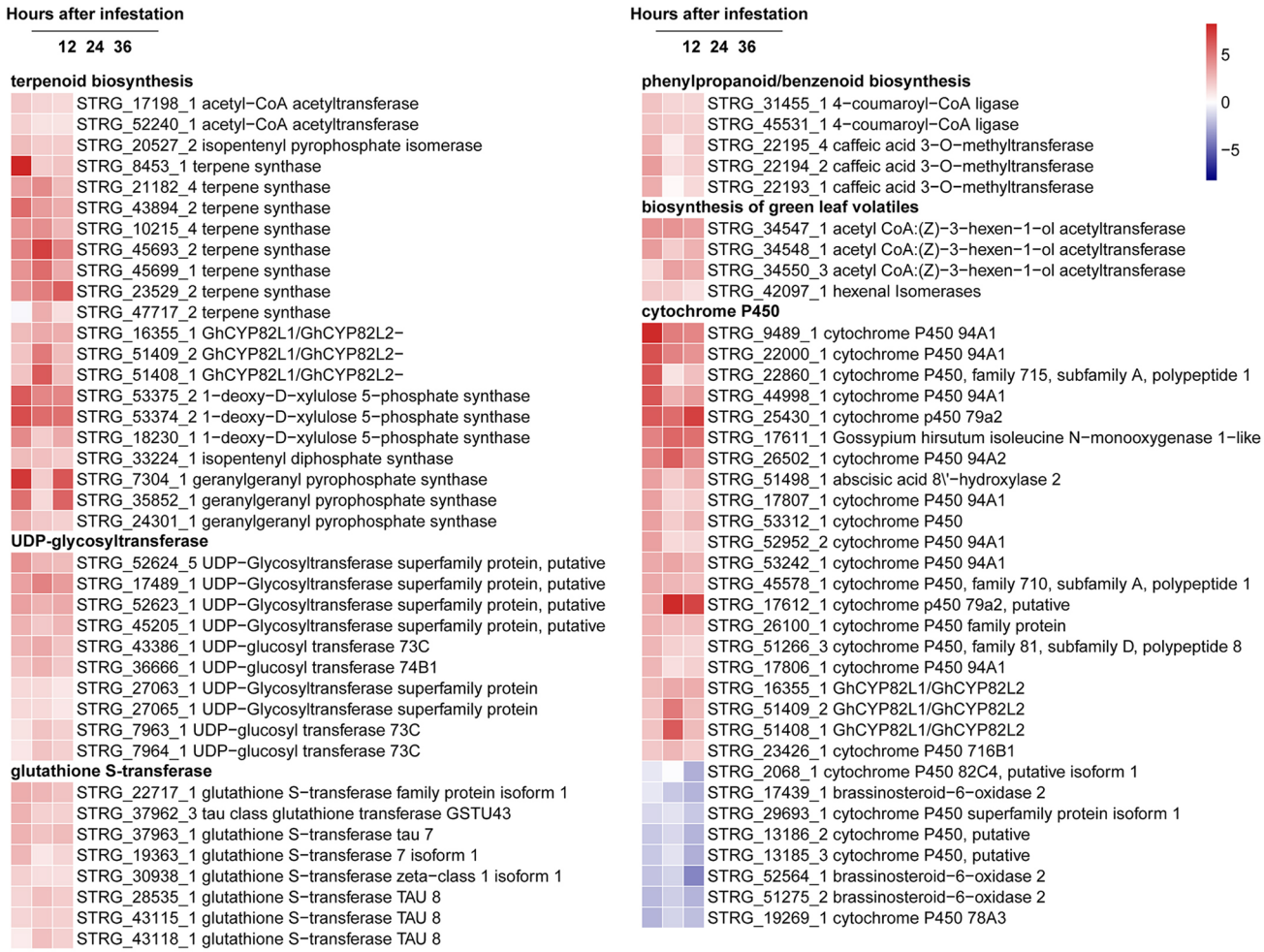

Fig. 4 *Apolygus lucorum*-responsive DEGs involved in secondary metabolism

S5) was present only in plants exposed to *A. lucorum* alone or with *H. armigera*, not after attack by *H. armigera*. This compound, identified as MeSA (Fig. S6), thus seems to be induced specifically by *A. lucorum*.

Attractiveness of *A. lucorum*-exposed cotton plants and synthetic MeSA to parasitoid *P. spretus*

In the Y-tube olfactometer assay, *P. spretus* showed a significant preference for the cotton volatiles after 36 h of *A. lucorum* feeding compared with the control volatiles (Fig. 5a; mated females, $\chi^2 = 20.056$, $P < 0.01$; unmated males, $\chi^2 = 6.531$, $P < 0.05$). Furthermore, the behavioral responses of *P. spretus* to synthetic MeSA at two concentrations (1 µg/µL and 10 µg/µL) were investigated. The results confirmed that *P. spretus* was significantly attracted by synthetic MeSA at the tested concentrations (Fig. 5b; 1 µg/µL: mated females, $\chi^2 = 6.870$, $P < 0.01$; unmated males, $\chi^2 = 11.864$, $P < 0.01$; 10 µg/µL: females, $\chi^2 = 8.667$, $P < 0.01$; males, $\chi^2 = 16.615$, $P < 0.01$). Males and females did not differ significantly in response to *A. lucorum*-induced volatiles or synthetic MeSA.

Discussion

Similar to phloem-feeding aphids and whiteflies, mirid bugs generally pierce plant tissue with their stylets and secrete saliva to solubilize cellular matter (Showmaker et al. 2016). We previously reported that simultaneous infestations of *A. lucorum* bugs and *H. armigera* caterpillars elevated volatile emissions from cotton (Huang et al. 2018). However, other studies have shown that the whitefly *B. tabaci* suppressed the emission of cotton volatiles induced by *S. exigua* caterpillars (Rodriguez-Saona et al. 2003). Thus, different types of herbivore damage can induce different volatile phenotypes, mediated by different defense signaling pathways (Clavijo McCormick 2016). Chewing *H. armigera* caterpillars triggered JA signaling pathway (Huang et al. 2015). Phloem-feeding insects such as whiteflies and aphids usually induce

Table 1 Proportions (% of internal standard ethyl caprate) of headspace volatiles from cotton plants infested by *Apolygus lucorum* for 12, 24 or 36 h and from corresponding control plants

Compound	Calculated RI/published RI	RT	12 h		24 h		36 h	
			Control	Infested	Control	Infested	Control	Infested
1. (*E*)-2-Hexenal	851/851	6.39	nd	nd	nd	0.94 ± 0.30*	nd	0.91 ± 0.55
2. (*Z*)-3-Hexenol	854/849	6.46	nd	nd	nd	0.46 ± 0.28	nd	nd
3. 2-Methylbutyl acetate	878/882	7.18	nd	nd	nd	0.81 ± 0.24*	nd	1.17 ± 0.64
4. α-Pinene	935/938	9.02	nd	nd	1.23 ± 0.34	3.16 ± 0.30**	1.89 ± 0.25	3.06 ± 0.79
5. β-Pinene	977/973	10.60	nd	nd	nd	0.32 ± 0.32	nd	0.72 ± 0.46
6. β-Myrcene	988/990	11.06	nd	nd	nd	5.19 ± 4.02	nd	5.27 ± 1.85*
7. (*Z*)-3-Hexenyl acetate	1002/1008	11.61	nd	20.08 ± 8.48*	nd	12.63 ± 4.36*	1.12 ± 0.49	22.14 ± 10.45
8. 1-Decyne	1021/1024	12.30	nd	0.81 ± 0.47	nd	2.22 ± 1.72	nd	6.33 ± 1.30**
9. (*Z*)-β-Ocimene	1035/1039	12.82	nd	nd	nd	nd	nd	2.65 ± 1.04*
10. (*E*)-β-Ocimene	1047/1050	13.24	nd	64.89 ± 20.16**	nd	34.80 ± 9.25**	nd	130.98 ± 57.77*
11. Linalool oxide	1085/1075	14.74	nd	nd	nd	nd	nd	3.57 ± 0.30**
12. Methyl benzoate	1091/1095	15.01	nd	2.61 ± 1.09*	nd	nd	nd	3.78 ± 2.87
13. Linalool	1098/1107	15.28	nd	10.09 ± 6.58	nd	9.12 ± 2.31**	nd	37.98 ± 14.01*
14. (*E*)-2-Hexenyl propionate	1106/1111	15.56	nd	nd	nd	nd	nd	1.51 ± 0.88
15. DMNT	1113/1114	15.82	nd	22.79 ± 9.65*	nd	11.11 ± 1.91**	nd	46.75 ± 17.89*
16. (*Z*)-3-hexenyl isobutyrate	1142/1144	16.88	nd	0.69 ± 0.59	nd	0.03 ± 0.03	nd	0.78 ± 0.70
17. (*E*)-2-hexenyl isobutyrate	1150/1152	17.15	nd	1.11 ± 0.64	nd	0.39 ± 0.24	nd	3.16 ± 1.85
18. Methyl salicylate	1191/1192	18.78	nd	1.36 ± 1.10	nd	2.17 ± 1.15	nd	5.36 ± 3.10
19. (*E*)-2-Hexenyl butyrate	1194/1195	18.89	nd	3.48 ± 1.57*	nd	11.29 ± 4.49*	nd	7.59 ± 2.36**
20. (*Z*)-3-Hexenyl 2-methylbutanoate	1232/1231	20.24	nd	7.72 ± 3.24*	nd	3.01 ± 0.83**	nd	8.04 ± 3.85*
21. (*Z*)-1-Ethoxy-4-methyl-2-pentene	1239/1238	20.49	nd	10.45 ± 6.24	nd	4.40 ± 1.36*	nd	27.40 ± 15.96
22. Indole	1292/1297	22.47	nd	4.10 ± 2.37	nd	5.28 ± 1.94*	nd	21.45 ± 10.22*
23. (*E*)-2-Hexenyl tiglate	1334/1338	24.07	nd	nd	nd	nd	nd	1.59 ± 1.10
24. Methyl anthranilate	1337/1343	24.19	nd	nd	nd	nd	nd	1.03 ± 0.61
25. β-Caryophyllene	1426/1422	27.84	8.95 ± 1.32	13.75 ± 3.75	33.55 ± 9.04	22.16 ± 3.92	25.90 ± 9.45	25.79 ± 5.94
26. α-Humulene	1467/1461	29.59	4.24 ± 1.59	6.78 ± 1.71	10.48 ± 2.88	7.62 ± 1.17	7.93 ± 3.08	9.09 ± 1.77
27. α- Farnesene	1516/1511	31.35	nd	nd	nd	2.42 ± 0.78*	nd	6.83 ± 1.96**
28. δ-Cadinene	1527/1520	31.76	nd	0.85 ± 0.30*	nd	1.31 ± 0.46*	nd	4.64 ± 1.56*
29. TMTT	1581/1575	33.14	nd	nd	nd	2.29 ± 1.78	nd	11.97 ± 3.11**

Numbers for compounds correspond to those in Fig. S5 and Supplementary Table S6. Data (mean ± SE) were analyzed for significant differences in *A. lucorum*-induced cotton compared to the corresponding control using Student's *t* test (*$P < 0.05$, **$P < 0.01$)

DMNT = 4,8-dimethylnona-1,3,7-triene; TMTT = 4,8,12-trimethyltrideca-1,3,7,11-tetraene; RI = retention index; RT = retention time; nd = not detected

the SA signaling pathway and suppress the JA signaling pathway (Stam et al. 2014). Because mirid bugs employ "lacerate and flush" feeding strategies to cut plant cells (Showmaker et al. 2016), they cause more physical damage to cells than do phloem-feeding aphids and whiteflies. So, we propose that the sap-feeding attack by *A. lucorum* activates a signaling network(s) (mainly JA signaling pathway) that is clearly distinct from that induced by phloem feeders. As expected, our results demonstrated that *A. lucorum* infestation activated JA-mediated signaling without attenuating the SA pathway. Specific volatile blends that were consequently emitted were attractive to the parasitoid wasp *P. spretus* (Fig. 5a). Moreover, MeSA, that was induced specifically by *A. lucorum* but not by *H. armigera* (Table S6; Fig. S6), was found to attract *P. spretus* when tested alone at two concentrations (Fig. 5b).

Transcriptomic changes in plants in response to insect attack constitute a dynamic and complex reprogramming

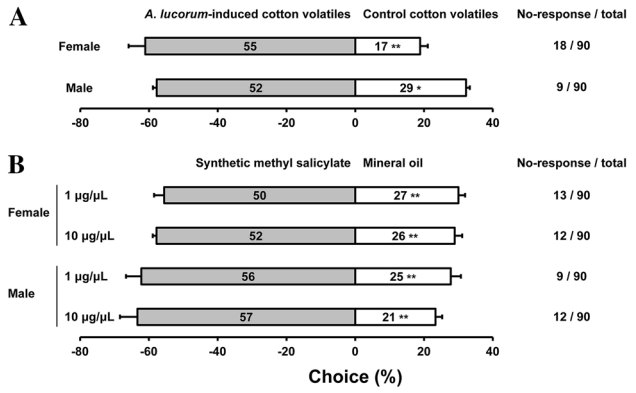

Fig. 5 Y-tube olfactometer assays of female adults of *Peristenus spretus* in response to headspace volatiles from cotton plants infested for 36 h by *Apolygus lucorum*. Data (mean ± SE) were tested for significant differences using Student's *t* test (* $P < 0.05$)

of signaling and biosynthetic pathways and can vary with the duration of attack (Guo et al. 2018a). In this study, the transcriptomic analysis showed that *A. lucorum* infestation induced rapid and strong changes in signaling and other regulatory processes in addition to primary and secondary metabolic processes. A considerable number of DEGs were rapidly and strongly upregulated at 12 h of the onset of herbivory, while the number of upregulated DEGs decreased sharply at the later times (Fig. 1a). However, the most significant changes in the emission profile of *A. lucorum*-induced volatiles were observed at 36 h. Such poor correlation between the transcriptomic and volatile metabolomic data could be attributed to post-transcriptional and post-translational regulation of these DEGs.

Upregulated DEGs were more common than downregulated DEGs after *A. lucorum* infestation, indicating that *A. lucorum* feeding stimulated more gene expression. This result is similar to transcriptomic changes in cotton plants responding to chewing *H. armigera* (Huang et al. 2015) and *A. grandis* (Artico et al. 2014), rice responding to *Chilo suppressalis* (Liu et al. 2016), maize responding to *Ostrinia furnacalis* (Guo et al. 2018a) and *Mythimna separata* (Qi et al. 2016), but contrary to that of cotton responding to aphid and whitefly (Dubey et al. 2013). The difference in global transcriptome changes between sap-feeding and phloem-feeding insects likely reflects the different amounts of damaged caused by the two types of stylet feeders (Voelckel and Baldwin 2004).

In Clusters A-C of the DEGs, 81 transcription factors were identified, predominantly from three gene families: AP2-EREBP (31 genes), MYB (12 genes) and WRKY (9 genes). These genes might be involved in different pathways of cotton plants to elicit a rapid, effective defense against *A. lucorum*. A comparative transcriptomic analysis of cotton cultivars resistant and sensitive to the whitefly *B. tabaci* showed that WRKY40 expression was elevated in the resistant cultivar compared to the susceptible cotton (Li et al. 2016). Recently, transcription factors that regulate the biosynthesis of plant volatile secondary metabolites have received increasing attention. For example, MYB transcription factor EMA1 from *Medicago truncatula* regulates the emission of the volatile methyl anthranilate (Pollier et al. 2019). In strawberry, the FaERF#9-FaMYB98 complex regulates synthesis of an important fruit aroma compound, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (Zhang et al. 2018). In *Citrus sinensis*, two ERF-type transcription factors, CitAP2.10 and CitERF71, activate *CitTPS* genes that are involved in the synthesis of important volatile mono- and sesquiterpenes in fruit (Shen et al. 2016; Li et al. 2017). In banana, MabZIP4 and MabZIP5 are involved in regulating aroma formation during fruit ripening (Guo et al. 2018b). Other transcription factors such as AtMYC2 in *Arabidopsis thaliana* (Hong et al. 2012), GaWRKY1 in cotton *G. arboretum* (Xu et al. 2004), AaERF1 and AaERF2 in *Artemisia annua* (Yu et al. 2012) and ZmEREB58 in *Zea mays* (Li et al. 2015) are also involved in the accumulation of volatile sesquiterpenes. The function of these differentially expressed TFs in the biosynthesis of *A. lucorum*-induced volatiles should be further investigated in the future to understand the regulatory mechanisms underlying the production of these important volatiles.

Phytohormones such as JA, ET and SA have essential roles in a complex regulatory network that mediates direct and indirect plant defenses against insect herbivore attack. Distinct transcriptional responses and signal-transduction pathways are often activated in plants to response herbivores depending on the attacker and its feeding guild (Stam et al. 2014; Erb and Reymond 2019). Chewing herbivores (such as beetles and caterpillars) and cell-content feeding insects (such as mites and thrips) predominantly trigger the JA pathway, while phloem feeders (such as whiteflies and aphids) frequently activate the SA pathway (Zhang et al. 2009; Clavijo McCormick 2016; Arena et al. 2018). Components of SA pathways often antagonize JA signaling and therefore suppress JA-dependent defenses, whereas evidence is accumulating that the two signaling pathways can act synergistically (Stam et al. 2014; Erb and Reymond 2019). Our data showed that *A. lucorum* elicited the expression of genes associated with JA and SA (Fig. 3), which is consistent with overlapping or synergistic effects of JA and SA in rice attacked by *C. suppressalis* (Zhou et al. 2011; Liu et al. 2016) and in *A. thaliana* attacked by *Brevipalpus yothersi* (Arena et al. 2018), but different from the antagonistic effects in cotton during an infestation of *H. armigera* (Huang et al. 2015) or whitefly (Dubey et al. 2013; Li et al. 2016). In addition, almost all DEGs genes involved in ET biosynthesis and signaling were upregulated after *A. lucorum* infestation (Fig. 3), suggesting that the ET-mediated signaling was

also activated and involved in cotton-induced responses to *A. lucorum* and other herbivores (Artico et al. 2014; Huang et al. 2015; Kumar et al. 2016). An integrated signaling network activated by *A. lucorum* infestation, especially positive crosstalk between JA, SA and ET signaling pathways, might contribute to plant adaptation to herbivores of different feeding guilds, which allow cotton plants to fine-tune their defense induced by *A. lucorum* and consequently lead to the production of volatiles induced specifically by this pest. Interestingly, all of the five DEGs associated with BRs were downregulated (Fig. 3), which provided clues about negative crosstalk between BR and JA, and suggested that *A. lucorum* infestation negatively affected BR-aided growth.

Gossypol and its derivatives are the most important insecticidal compounds in cotton. Several cadinene synthase genes and five other genes (*CYP706B1*, *CYP82D113*, *CYP71BE79*, *DH1* and *2-ODD-1*) responsible for intermediates in the gossypol biosynthetic pathway were recently characterized (Tian et al. 2018). Some toxic secondary compounds are synthesized de novo only after the rupture of multiple cells by chewing, not by piercing or sucking actions (Stam et al. 2014). Here, we found that almost all characterized gossypol genes, except for 2-ODD-1, were present in the transcriptome induced by *A. lucorum*, whereas only one upstream gene (cadinene synthase gene) was differentially expressed (Table S2). In this context, *A. lucorum* infestation activated a specific and somewhat mitigated cotton defense strategy instead of a gossypol-related defense, which might be explained by less extensive damage caused by stylets of mirids compared to chewing herbivores.

Thirty genes related to the biosynthetic pathways of volatile terpenoids, shikimic-acid-derived phenylpropanoids/benzenoids and HPL-derived green leaf volatiles were upregulated (Fig. 4). Consistently, *A. lucorum*-exposed cotton emitted 13 terpenes, 4 phenylpropanoids/benzenoids and 12 green leaf volatiles (Table 1; Fig. S5). It is noteworthy that (*E*)-β-ocimene, DMNT and linalool were the three most abundant of these compounds. Volatile terpenoids mainly including monoterpenes, sesquiterpenes and homoterpenes are catalyzed by terpene synthases and cytochrome P450 proteins (Huang et al. 2013, 2018; Liu et al. 2018). As expected, eight genes (*GhTPS4*, *GhTPS10*, *GhTPS12*, *GhTPS14*, *GhTPS15*, *GhTPS16*, *GhCYP82L1* and *GhCYP82L*) that contribute to the production of (*E*)-β-ocimene, (3*R*)-linalool, (3*S*)-linalool, DMNT, TMTT and δ-cadinene (Huang et al. 2018; Liu et al. 2018) were strongly upregulated in response to *A. lucorum* feeding (Fig. 4).

Intriguingly, many key upstream genes involved in phenylalanine ammonia lyase (PAL) for the phenylpropanoid pathway, HPL for green leaf volatiles pathway and HMG-CoA reductase (HMGR) for sesquiterpene pathway were not differentially expressed. However, 12 genes required for catalyzing the last step in the formation of cotton volatiles, including eight terpene synthase genes, three acetyl CoA:(*Z*)-3-hexen-1-ol acetyltransferase genes and one hexenal isomerase gene, were upregulated (Fig. 4). These findings further suggest that cotton plants fine-tune their induced defense response to *A. lucorum.*

Additionally, genes for many other key proteins/enzymes involved in biosynthetic pathways of natural antibiotics, including *CYP*, *GST* and *UGT* genes, were upregulated (Fig. 4), suggesting their possible roles in cotton plants induced resistance to *A. lucorum*. It is important to note that there is a trade-off between the synthesis of defensive metabolites and growth-related metabolites, as exemplified here by the upregulation of defense-related genes concomitantly with the downregulation of photosynthetic genes and partial suppression of amino acid metabolism, carbohydrate metabolism and nucleotide metabolism (Table S5), which are general plant responses to insect feeding (Zhou et al. 2011; Huang et al. 2015; Liu et al. 2016). Thus, cotton plants redirect limited resources by altering their primary metabolism to activate defense-related processes.

Herbivore-induced plant volatiles can function in an indirect plant defense through the recruitment of beneficial natural enemies (Turlings and Erb 2018), as supported here by the attraction of parasitoid *P. spretus* to *A. lucorum*-induced volatile blends and synthetic MeSA (Fig. 5). MeSA is a common component in insect-induced volatile blends of many plant species reported to function as a signal to attract diverse natural enemies. In a meta-analysis of natural enemy responses to MeSA, Rodriguez-Saona et al. (2011) found that 41 of 91 observed insect species were significantly attracted to synthetic MeSA in the field. In cotton fields, MeSA may also attract various carnivorous predators (Yu et al. 2008). Besides its role as an active signal in indirect defense, MeSA can also directly repel some predatory insects (Lin et al. 2016) and herbivores (Groux et al. 2014). Moreover, MeSA is also involved in plant–plant communication and can activate defense responses and improve herbivore resistance in undamaged neighboring plants (Filgueiras et al 2019). MeSA is synthesized from SA by the action of SA carboxyl methyltransferase (SAMT), translocated through the phloem and then converted back to the active signal SA in systemic tissues (Zhao et al. 2009). In transgenic hairy roots of soybean that overexpressed *GmSAMT1*, the SA pathway appeared to be modulated, and plants had enhanced resistance against nematodes compared with the wild type (Lin et al. 2013). Notably, transcriptomic data obtained in our study revealed that two DEGs annotated as SAMT were significantly upregulated at three times (12, 24 and 36 h of exposure) in *A. lucorum*-infested cotton plants (Fig. 3), which is consistent with the detection of MeSA in the emission profile of *A. lucorum*-induced volatiles (Table 1).

In this study, we provide a comprehensive view of sap-feeding *A. lucorum*-induced responses, mainly indirect defense responses, in upland cotton by monitoring dynamic transcriptomic and volatile metabolomic changes. Integrative analysis of transcriptome and volatile metabolome revealed that the *A. lucorum*-induced plant volatile emission was mainly regulated by the JA pathway with a likely minor role for the SA pathway. Specific volatile blends including MeSA consequently emitted from *A. lucorum*-infested cotton plants as well as synthetic MeSA alone were attractive to the parasitoid wasp *P. spretus*. Our results will help to elucidate molecular mechanisms of induced plant defense against sap-feeding mirids, but also to discover previously unknown defensive genes, for example, SA carboxyl methyltransferase, valuable for breeding new cotton cultivars with enhanced resistance to insects.

Author contribution

XH and YZ conceived and designed the experimental plan. XH, JK, XH, DL, SG, WJ and PS performed the experiments. YZ, XH and WS refined the manuscript. All authors approved the final manuscript.

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