RNA-Seq and UHPLC-Q-TOF/MS Based Lipidomics Study in Lysiphlebia japonica

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Lipids play an important role in energy storage, membrane structure stabilization and signaling. Parasitoids are excellent models to study lipidomics because a majority of them do not accumulate during their free-living life-stage. Studies on parasitoids have mostly focused on the changes in the lipids and gene transcripts in hosts and little attention has been devoted to lipidomics and transcriptomics changes in parasitoids. In this study, a relative quantitative analysis of lipids and their gene transcripts in 3-days-old Lysiphlebia japonica larva (3 days after spawning) and pupae were performed using liquid chromatography, mass spectrometry and RNA-seq. Thirty-three glycerolipids and 250 glycerophospholipids were identified in this study; all triglycerides and the vast majority of phospholipids accumulated in the pupal stage. This was accompanied by differentially regulated lipid uptake and remodeling. Furthermore, our data showed that gene transcription was up-regulated in key nutrient metabolic pathways involved in lipid synthesis in 3-days-old larvae. Finally, our data suggests that larva and pupa of L. japonica may lack the ability for fatty acids synthesis. A comprehensive, quantitative, and expandable resource was provided for further studies of metabolic regulation and molecular mechanisms underlying parasitic response to hosts defense.

Lipids have diverse structure and composition1-2, enabling them to serve a variety of functions including as energy sources, structural components of cell membranes and organelles, and signaling molecules. In insects, lipids are grouped as internal or cuticular based on their organismal position3. Internal lipids are important for processes like oogenesis, metamorphosis, starvation, flight, and diapause4. Triacylglycerols (TAGs) serve as major energy sources for many insects during non-feeding periods and long distance flights5. Further, phospholipids (PLs), function as structural components of membranes6.

Parasitic wasps have developed a special developmental program when their larval growth and pupation occur as parasites of another insect species. Lysiphlebia japonica, the parasitoid wasp used in this study uses aphids as their preferred hosts. Numerous studies have demonstrated that some parasitoid species, such as Nasonia vitripennis, cannot synthesize lipids7-12.

Most key enzymes involved in the conversion of carbohydrates to triglycerides through pyruvate metabolism and the citrate cycle are functional in parasitoid wasps13. However, fatty acid synthase (FAS) is absent from the genome of the parasitic fungus Malassezia globosa14, and is therefore a prime candidate for studies on the lack of lipogenesis. A previous study showed that almost all genes involved in lipid synthesis pathways are up-regulated in Aphis gossypii following parasitic infection by L. japonica15. However, there are no data on the expression of lipid synthesis pathway genes in L. japonica itself.

Parasitic wasps are rapidly emerging as powerful model organisms to study lipid metabolism. Particular interest has been on understanding lipidome alterations during pupation. Until recently, it was not possible to address the genetic or nutritional influence on the lipidome. Mass spectrometry has become instrumental in identifying a vast number of different lipid species and is now recognized as a premier tool for lipidomics studies16.

In this study, we aimed to unravel the lipidomics and transcriptional profile during the developmental stages of the parasitic wasp, L. japonica, using mass spectrometry-based lipidomics that enabled us to simultaneously analyze changes in glycolipids and phospholipids. Transcriptome analysis by RNA sequencing was also performed to determine the molecular regulation of lipid metabolism genes under during the different developmental stages of L. japonica.

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Our data revealed unexpected shifts in lipid accumulation during larval growth and pupa development. Genes associated with key nutrient metabolic pathways in lipid synthesis were analyzed, and results suggest that TAG formation plays an important role in lipid homeostasis. The ability of fatty acid synthesis in *L. japonica* was also examined. These comprehensive datasets constitute a valuable resource for future research on changes in *L. japonica* lipid composition and gene expression.

Results

**UHPLC-Q-TOF/MS Analysis of Lipid Extracts in *L. japonica***. Samples were processed for chromatographic separation and mass spectrometry. Lipids identification was performed based on the existing literature and lipid map (http://www.lipidmaps.org/). From the original file, 2,056 and 3,757 peaks were detected in the positive (POS) and negative (NEG) modes. A total of 33 kinds of TAGs and 250 PLs (POS and NEG) were identified from the lipids extracts. Among these, the lipids exhibiting a significant increase or decrease were analyzed further.

In order to eliminate false positives and negatives, six independent pairwise comparisons were performed thus identifying statistically significant changes in lipid composition. In PCA, samples are classified based on a data matrix without prior information about class membership. PCA is a useful tool for classifying data, detecting outliers, and validating the stability and reproducibility of an analytical method. Graphical representation of the analytical PCA results is presented in Fig. 1(A,B), where lipids from groups J and Y are clearly separated into two clusters. A higher level of group separation and a better understanding of classification variables were applied by Supervised OPLS-DA. Table 1 and OPLS-DA scores plots. Figure 1C,D show the characteristics of the generated models. Groups J and Y achieved good separation of lipid extracts. A loading plot was constructed based on the OPLS-DA, which showed the contribution of variables to the differences observed among samples (Additional file 1: Fig. S2).
TAGs Diversity and Fatty Acyl Chains Associated with TAG Under Pupal State. Significant metabolic changes among lipid species are represented as heat maps in Fig. 2 (VIP > 1, p < 0.05). In most analyzed lipid classes, relative differences revealed significant changes in the abundance of specific lipid species (Fig. 3). As shown in Fig. 2, the lipid composition is dynamic between the two tested stages. In the pupal stage (group J), TAGs increased significantly compared with the larval stage (group Y). More detailed classification for all lipid classes presented in Fig. 3A reveals significant changes in the abundance of most lipid classes analyzed in this study. However, there were numerous changes in species composition (Fig. 3; Additional file 2) and in the total concentration of individual fatty acyl chains associated with TAG (Fig. 4F). All TAGs (e.g., (TAG (16:0/18:1/18:2), TAG (16:0/16:0/18:2), TAG (18:1/16:0/18:1)) (Fig. 3D,E) in the pupal stage increased markedly relative to the larval stage. For example, TAG (18:1/16:0/18:1) increased 5.8-fold, and TAG (18:2/18:2/18:2) increased 3.8-fold. Conversely, no increases or decreases were observed in the levels of fatty acyl chains associated with TAGs. Furthermore, C16:0, C16:1, C18:1, and C18:2 (Fig. 3F) of major lipid classes were only marginally affected in the pupal stage compared to the larval stage.

Glycerophospholipids Up-regulated in L. japonica Pupal stage. Changes of the composition on different glycerophospholipid classes were analyzed in the pupal stage. Significant changes in PLs are shown in a heat map (Fig. 2 VIP > 1, p < 0.05). Although pupation did not alter the abundance of the different glycerophospholipid classes, the numbers of major and middle glycerophospholipid species decreased (Fig. 4, Additional file 1: Fig. S3).

Lipidomics also revealed complex specificity in lipid metabolism. Compared to the larval stage, the vast majority of phospholipids significantly increased in pupae (e.g., PC (phosphatidylcholines (14:0/22:5)), 4.9-fold; PI (phosphatidylinositols (18:2/18:2)), 3.5-fold; SM (sphingomyelins (d15:1/22:0)), 4.0-fold; LPC (lysophosphatidylcholines (18:0/0:0)), 2.9-fold; PE-P (alkenyl-linked PE (P-18:0/0:0)), 8.0-fold). However, almost all phosphatidylethanolamines (PES) (e.g., PE (18:2/16:1), 0.1-fold) and the majority of phosphatidylinerines (PSs) (e.g., GPser (18:0/18:1), 0.3-fold) significantly decreased in the pupal stage relative to the larval stage (Fig. 4A). Additionally, when analyzing fatty acyl chains associated with glycerophospholipids, a marked reduction in C18:2 and only marginal increases in C16:0 and C16:1 (Fig. 4) were detected. Increases in C16:0 and C16:1 corresponded with higher LPC and LPE (lysophosphatidylethanolamines) (Fig. 4E,F). However, C18:2 levels decreased with elevated levels of LPE, LPC, PI and PG (Fig. 4A,E,F,G,H), and with decreased levels of PS and PE (Fig. 4B,L). In these

| Model | Type | A    | N  | R2X (cum) | R2Y (cum) | Q2 (cum) | Title      |
|-------|------|------|----|-----------|-----------|----------|------------|
| M5    | OPLS-DA | 1 + 1 + 0 | 12 | 0.701     | 0.986     | 0.958    | J VS Y (POS) |
| M5    | OPLS-DA | 1 + 1 + 0 | 12 | 0.805     | 0.992     | 0.977    | J VS Y (NEG) |

Table 1. OPLS-DA Model Summary for the discrimination between groups J and Y, using cross-validation. The R2X and R2Y values show the total variation in the X and Y matrix explained by the models, respectively. The Q2 value represents the model predictability and statistical validity.

Figure 2. Integration of intermatrix lipid correlations between J and Y. Significantly changed metabolites in the lipid species of groups J and Y from GC-MS were analyzed by heat map. Heat maps were applied to display the relative quantity of each lipid species, in which a gradient of red colors represents higher quantity and a gradient of blue colors represents lower quantity in J compared with Y. (A) Heat map of J vs Y (POS). (B) Heat map of J vs Y (NEG). (VIP > 1, p < 0.05).
analyses, the direct effects of fatty acyl chains remodeling during pupation could not be excluded. When quantified for individual glycerophospholipid subclasses, a marked reduction in C18:1 and increase in C18:0 in species PE and PS (Fig. 4B,L) were detected and significant increases in C18:1 in species PI and PG (phosphatidylglycerols) were observed. Based on these results we concluded that the changes in acyl chain composition in glycerophospholipids were associated with distinct L. japonica subclasses. Thus, these changes confirmed that not only the content of glycerophospholipid subclasses changed, but glycerophospholipid fatty acyl chains remolded for new tissue and cell membrane construction.

Figure 3. Changes in lipid composition and TAG fatty acyl chains during L. japonica pupation (middle or minor were determination by abundance). (A) Lipid classes identified in lipodomics experiments and their abbreviations as used in this article. (B,C) The relative percentage differences among all quantified lipid species between pupae and 3-days-old larvae (POS and NEG). Each dot represents a lipid species, and dot size indicates significance. Different colors represent different lipid classes. n = 6 for both groups. (D,E) The relative peak area of quantified lipid classes in major TAG (D) and middle TAG (E) in pupae and 3-days-old larvae. Data is presented as means ± SEM; n = 6 for both groups. Significance level: fold change > 1.5 or < 0.5, and p < 0.05, *p < 0.05, **p < 0.01. The relative peak area of quantified lipid classes in minor TAG is shown in Fig. S3. (F) Glycerolipid base chemistry and fatty acyl chains changes among major TAG in pupae and 3-days-old larvae. Data is presented as means ± SEM; n = 6 for both groups.
Gene Expression Related to Lipid Metabolism. Using a significance level of \( p < 0.05 \), a total of 1,159 differentially expressed genes, of which 463 show up-regulated (>2-fold) and 696 down-regulated (<0.5-fold) expression (Fig. 5A) were detected. All differentially expressed genes are shown in Additional file 3. This suggests an effect of pupation on lipid gene expression.
Analyses of the differentially expressed genes (DEG) in 3-days-old larvaes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways \(^{13,17}\) revealed significant enrichment in lipid metabolic pathways and key nutrient metabolic pathways involved in lipid synthesis. These include glycerophospholipid metabolism, glycolipid metabolism and fat digestion and absorption (Fig. 5C, D, E). The most highly enriched pathway for down-regulated genes was the MAPK pathway (Additional file 1, Fig. S1C). Moreover,
we found extensive changes in the 3-days-old larval transcriptome compared to the pupal transcriptome. These changes included up-regulation in the expression of genes involved in lipid metabolism.

**Gene Transcription in Key Nutrient Metabolic Pathways Involved in Lipid Synthesis.** At the transcription level genes involved in the nutrient metabolic pathways in *L. japonica* were significantly different following pupation compared to 3-days-old larvae (Fig. 6, Additional file 1). Pupation reduced the transcription levels of phosphoglucone isomerase (*pgi*) (Fig. 6) involved in carbohydrate metabolism, and located at the beginning of the glycolytic pathway. Enolase (*eno*), a metalloenzyme responsible for catalyzing the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), showed a decrease in transcript levels (Fig. 6). Further support is provided by the lower abundance of glucose-6P dehydrogenase (*g6pd*) of the pentose phosphate pathway in pupae, compared with 3-days-old larvae of *L. japonica*. Furthermore, the rate-limiting enzyme, *pk*, was expressed less in pupae than in 3-days-old larvae, indicating a decrease in acetyl-CoA levels from sucrose. Conversely, 5 genes significantly decreased in pupae compared to 3-days-old larvae of *L. japonica*. These were: *atpcl* (phosphoenolpyruvate carboxykinase) and ATP citrate lyase (*atpcl*), the FPKM values of which were below 1. Additionally, LSD (lipid storage droplet), which is specifically involved in the activation of triglyceride lipolysis in fat body adipocytes 18, was up-regulated in 3-days-old larvae relative to pupae. Finally, neither 3-days-old larvae nor pupae expressed any of the key genes involved in fatty acid synthesis in abundance (e.g., *acc* (acetyl-CoA carboxylase), *fas* (fatty acid synthase), *scd* (long-chain fatty acyl-CoA synthetase)) (Fig. 6). Based on these data, we suggest that *L. japonica* does not possess the ability for de novo fatty acids synthesis.

**Discussion**

Research suggests that parasitoid wasps do not synthesize lipids 7–10,19–22. Studies have also shown that mortality to wasps in highly resistant aphids typically occurs <72 h after oviposition, the host aphid is killed on day 6 or 7, and the adult parasitoids emerge 12 days after oviposition 23–25. We observed that *L. japonica* had shorter developmental periods than the other parasitoids; 72 h after oviposition, *L. japonica* was only in the 2–3 instar larval stage. Although studies conducted on parasitoids have primarily focused on life-history and the physiological and biochemical changes in hosts 15,28,27, little is known about the changes in lipids and transcriptional regulation in *L. japonica*. Lipids change in *L. japonica* is a multistep process that entails a complex interplay between metabolic and transcriptional signaling pathways. In this study, mass spectrometry-based lipidomics and RNA-seq were applied to provide a resource to study these changes at the level of the lipidome and transcriptome.

Our data demonstrated that TAGs and glycerophospholipids are not only modulated differentially, but phospholipid subspecies are also selectively remodeled during pupation. Due to their roles in various metabolic processes and being integral to maintaining metabolic homeostasis, differences in lipid content and fatty acyl chain composition during *L. japonica* development are valuable resources. Previous studies reported that the total amount of lipids increased during the pupal stage relative to the larval stage 22–28. This study showed that *L. japonica* has a significant effect on *A. gossypii* lipid synthesis 15. The abundance of TAGs in mummified aphids was significantly higher than in 3-days-old larvae. During the pupal stage metabolism was slow with almost no life activities, increased material metabolism, and signal transduction was not required 7. The higher amount of TAGs in pupae suggested that TAGs were not utilized as a source of energy via beta oxidation. Instead, deposited TAGs may be required for tissue remodeling during pupal development 42. Furthermore, this increase likely enhanced changes in membrane composition 21. Our data also demonstrated that almost all phospholipids increased during the pupal stage, except for PE and the majority of PS (Figs 3B,C and 4A,B). Changes in lipid stores occurred because of flight, mating activities, and oviposition 30,33–35. These changes closely correlate with the physiological needs of insects and differ considerably between species, sex, age, and developmental stage, and are influenced by environmental factors 36–39. However, it is possible that these changes also reflect the differential effect of *L. japonica* developmental stages on the affinities of enzymes involved in remodeling and glycerophospholipid and TAG synthesis 40.

We also observed significant glycerophospholipid remodeling in pupae compared to larvae. Similar findings were reported for RAT 51. In the analysis of fatty acyl chains associated with TAG, we detected only the effect on TAG species. However, when analyzing the total pool of fatty acyl chains associated with glycerophospholipids, a marked reduction in C18:2 (Fig. 4) were detected. Furthermore, differential retention of acyl chains in different phospholipid subclasses and subspecies occurred during different developmental stages. Therefore, one key finding of this study is that pupation adaptation entails the remodeling of TAG and glycerophospholipids in a highly subspecies-selective manner. Future studies are required to determine the functional consequences of this remodeling in *L. japonica*.

Based on significant lipidome changes between stages, we focused our transcriptome analyses on key lipid synthesis metabolic pathways. In *Rhodnius prolixus*, the glycerol-3-phosphate pathway is the only route for triacylglycerol synthesis 49. *Pgi, fbp, and gapdh* are involved in glycolysis, which consumes GAP to produce pyruvate, and were significantly up-regulated in 3-days-old *L. japonica* larvae (Fig. 6). This result synchronizes with parasitized 3-days-old aphids 43. Additionally, *g6pd*, a TCA cycle transcript, producing the reducing agent nicotinamide adenine dinucleotide phosphate (NADP) in the pentose phosphate pathway, was down-regulated in pupae (Fig. 6). Transcription levels of these key nutrient metabolic pathways genes were in line with the high abundance of TAG in pupae. In conclusion, we have demonstrated that relative to pupae, 3-days-old *L. japonica* larvae have...
up-regulated expression of key nutrient metabolic pathway genes involved in lipid synthesis. Furthermore, genes involved in the MAPK signal pathway, which is essential for lipid homeostasis\(^\text{42}\), were up-regulated in pupae (Fig. S1C).
Our data showed a robust accumulation of numerous TAGs in pupae. This conversion did not change the lipid class, but changed lipid content and the fatty acyl chains associated with TAG species (Fig. 3), and increased expression of genes encoding enzymes involved in TAGs synthesis. Some enzymes that catalyze reversible reactions in the glycerolipid synthesis pathway, such as gpat (glycerol-3-phosphate O-acyltransferase) and dgat, can be regulated at the transcriptional level in mammals. Among the analyzed genes involved in the glycerolipid pathway, gk, ppp2r2, agoapt, and dgat were significantly down-regulated in pupae relative to 3-days-old larvae (Fig. 6). We presume that TAG synthesis and accumulation primarily occurred in larvae and then broke-down in the pupal stage. Studies have shown that insect lipids increase because of absorption or synthesis from food material during larval stages. Many insects were also shown to deposit large lipids, and metabolize them during pupal-adult transformation for new synthesis, cellular changes, and tissue remodeling. Further support for this was provided by the higher transcription of genes involved in fat digestion and absorption in 3-days-old L. japonica larvae, relative to pupae. Furthermore, higher transcript abundance of isl, a gene specifically involved in the activation of triglyceride lipolysis in fat body adipocytes, was found in pupae than in 3-days-old larvae. With the increase in triglyceride synthesis in 3-days-old larvae, isl was significantly up-regulated in pupae.

In this study, the transcript abundance of lipid synthesis genes indicates that fatty acid synthesis may not occur in L. japonica, at least not in the larval or pupal stages. Detectable transcript levels were observed for atpe, dlat, acc, fas, scd, and lefas3 in either larvae or pupae (Fig. 6). This result is in contrast to the high expression of these genes in parasitized aphids. Acc and fas are 2 key genes for which active gene transcription is crucial for fatty acid synthesis. The most important function of Acc is to provide the malonyl-CoA substrate for fatty acid biosynthesis, while fas encodes the enzyme that performs the majority of steps involved in fatty acids synthesis. Many factors could have resulted in parasitoids losing the ability to synthesize fatty acids. For example, mutations accumulating in the fas promoter or genes encoding transcription factors could impair the lipogenic ability of parasitoids. Alternatively, this may be an evolutionary choice stemming from lifestyle. Our findings provide strong evidence that L. japonica neither synthesizes fatty acids nor balances increased catabolism of fatty acids. However, we cannot conclude that L. japonica is unable to perform lipid synthesis, as more studies are needed to investigate the molecular mechanisms underlying the lack of fatty acids synthesis, and to determine whether L. japonica has the capability to synthesize lipid and fatty acids in adult life.

Conclusions
In summary, the changes in the lipidome and transcriptome of 3-days-old L. japonica larvae and pupae using advanced omics techniques were first determined. The results showed large amounts of TAGs being absorbed and synthesized in 3-days-old larvae, resulting in the accumulation of TAGs and PLs in pupae. This was accompanied by highly subspecies-selective TAG and glycerophospholipids remodeling, and a marked increase in the expression of genes involved in TAG synthesis and key nutrient metabolic pathways involved in lipid synthesis. Results also showed that neither larvae nor pupae possess the ability for fatty acid synthesis. Together, our data showed that lipidome and transcriptome changes between L. japonica pupae and 3-days-old larvae constitute a comprehensive and valuable resource for further studies on metabolic regulation and the molecular mechanisms underlying parasitic responses.

Methods
Insects. Adult L. japonica were collected from the Institute of Cotton Research of Chinese Academy of Agricultural Sciences, and reared in the laboratory on cotton-melon aphids at 24 ± 1 °C, a 14:10 h light/dark (LD) photoperiod, and 75 ± 5% relative humidity (RH). Third instar cotton-melon aphids from the same mother were exposed to wasps, and the parasitized aphids were collected to separate wasp larvae after 3 days. Other parasitized aphids were reared on cotton leaves and collected 5 days later, when they became mummified alates. After dissection, the 3-days-old larvae (named group Y) and mummified (named group J) L. japonica were cleaned with ultrapure water and stored at −80 °C.

Lipid extraction, identified and quantified. The 3-days-old and mummified L. japonica were prepared to extract lipid metabolites, each group had 6 biological repetitions with a total of 12 samples. 20 mg of each L. japonica sample was used. Lipids were extracted as described in our previous study. During the LC/MS experiment, to acquire MS/MS spectra on an information-dependent basis (IDA), TripleTOF mass spectrometer was used. In this mode, the acquisition software (Analyst TF 1.7, AB Sciex) continuously evaluates and triggers the acquisition of MS/MS spectra. ProteoWizard was used to convert the raw data into mzXML and further analysis was performed using XCMS. For metabolite identification, accurate mass search (<30 ppm) and an in-house standard MS/MS library was used for MS/MS spectral match. Using the interquartile range denoising method, peaks were detected and metabolites were isolated. Half of the minimum detectable value was filled with the missing values. Molecular lipids were analyzed in both positive and negative ion modes using multiple precursor ion scanning-based methods. Molecular lipid species were identified and relatively quantified (TAG, PC, PE, PS, LPC, LPE, PG, SM, PI, PA, PC-P, and PE-P). Lipids are referred to according to LIPID MAPS lipid nomenclature recommendations.

Lipidomics Statistical Analysis. We used the area normalization method, with the total area of each sample as the denominator, the sample of the test substance divided by this total area. To account for group separation and classification variables, principal component analysis (PCA) and orthogonal projections to latent structures-discriminate analysis (OPLS-DA) were performed using the SIMCA14 software package (Umetrics, Umea, Sweden). A loading plot was constructed based on OPLS-DA, which showed the contribution of variables to differences between the groups. Heat maps were used to intuitively illustrate differences among these components. To refine this analysis, the first principal component of variable importance projection (VIP) was
obtained. Changed metabolites were determined by the Student’s t test (p > 0.05) and VIP values exceeding 1.05859. Additionally, another criterion for compounding differences (fold change > 1.5 or < -0.5, and p < 0.05) was used. The relative difference (percent) of samples was conducted by Hodges-Lehmann estimator. A complete list of the lipidomics data is provided in Additional file 2. The Metabolights number is MTBLS406.

**RNA Extraction.** Transcriptome analysis was performed to investigate the changes in lipid composition at the level of gene expression. Since *L. japonica*’s pupation period is about 5 days, consistent with lipidomics, we chose an intermediate transition period of 3 days and the early stage of pupation to verify gene transcriptional changes. Each group had 3 biological repetitions. Total RNA samples were extracted from 3-days-old wasp larvae and mummified *L. japonica* using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Illumina sequencing was performed using the Illumina HiSeq. 2500 system at the Beijing Genomics Institute in Shenzhen, China.

**RNA-Seq Data Analysis.** The sequencing reads were first processed to remove low-quality, adaptor-linked, and high content of unknown base (N) reads, prior to downstream analyses. Transcriptome de novo assembly was carried out with the short read assembling program Trinity10,49, with min_kmer_cov set to 2 by default, and all other parameters also set to default. Resulting assembled sequences were considered unigenes. Unigenes larger than 150 bp were compared using BLASTX to protein databases including NCBI-nr, Swiss-Prot, KEGG, and COG (e-value < 10^−5). Sequences were screened for preliminary identification and further functional annotation by BLASTX in NCBI-nr and Swiss-Prot and BLASTN in NCBI-nt (e-value < 10^−5). Then, the Blast2GO program15 was used to obtain GO (Gene Ontology) annotation for the unigenes, and WEGO software29 to obtain GO functional classification. HOMER64 was used for functional enrichment analysis and KEGG database was used to annotate genes to metabolic pathway13,17. The reads per kilobase per million mapped reads (RPKM) method was used to estimate the abundance of each unigene, eliminating the influence of gene length and sequencing discrepancies in the calculations. Differential expression was determined using a cutoff significance level of p < 0.05 under a fold change >2 or < -0.5. The sequencing data is submitted to the ArrayExpress database and has the accession number E-MTAB-5227.

**Availability of data and materials.** The raw sequence datasets supporting the results of this article are available in the sequence read archive repository (http://www.ebi.ac.uk/arrayexpress/ and http://www.ebi.ac.uk/metabolights/) under the accession numbers E-MTAB-5227 and MTBLS406. Additional supporting tables are included as Additional files.

**Ethics approval and consent to participate.** Samples in the study were collected from the Institute of Cotton Research of Chinese Academy of Agricultural Sciences. This article did not contain any studies with human participants or animals performed by any of the authors. No specific permits were required.

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Author Contributions
Z.S., G.X.K. and C.J.J. conceived and designed the experiments; Z.S. and G.X.K. performed the experiments; G.X.K., Z.S., L.J.Y., L.L.M. and Z.L.J. analyzed the data; Z.L.J. and Z.S. evaluated the conclusions; and G.X.K., Z.S. and C.J.J. wrote the paper. All authors read and approved the final manuscript.

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