Transcriptomic Profiling of Cotton Leaves in Response to Cotton Aphid Damage

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Research article

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Abstract

Background

Cotton aphids (*Aphis gossypii* Glover) are regarded as one of the most harmful insect pests for cotton production. They are usually capable of causing severe yield loss through sucking cotton liquids, secreting honeydew and transmitting plant viral diseases. However, the molecular mechanism of the interaction between cotton and cotton aphids remains unclear currently. Therefore, the RNA-Seq study of cotton leaves was performed in response to cotton aphid damage at different time points (0 h, 6 h, 12 h, 24 h, 48 h and 72 h).

Results

A total of 9, 103 new genes were identified, and 7, 510 of them were annotated functionally. Based on the comparison results, the gene expression was analyzed according to the expression amount of genes in different samples. 24,793 differentially expressed genes were authenticated in all and their functional annotation and enrichment analysis were conducted. Compared with 0 h (without aphid damage, CK), the amount of down-regulated DEGs was largely more than that of the up-regulated genes at different time points under cotton aphid attack except for 48h. As revealed by the functional annotation of DEGs, these genes were involved in all kinds of plant biological process, including various resistance to abiotic and biotic stress, hormone metabolism, signaling transduction and transcriptional regulation.

Conclusions

The results revealed the molecular mechanism of the interaction between cotton and cotton aphids and would facilitate the development of plant aphid resistant cultivars.

Background

Aphids are one of the common pests on almost all crops. They feed plant phloem sap with their piercing-sucking mouthparts, which can have a direct impact on the normal growth and development of crops and eventually cause a severe loss of crop yield and quality. Compared with chewing insects, aphid feeding only causes relatively little mechanical injury to crops. However, the everlasting feeding of a large quantity of aphids usually consumes a large amount of plant resources. Secondly, a lot of aphid species have the potential to spread plant viruses. For them, the detrimental effects caused by virus transmission are likely to outweigh the direct effects of aphid feeding. In addition, honeydew secreted by aphids also gives rise to sooty mould on the leaf surface to constrain leaf photosynthesis [1, 2]. To counter these damages, plants have developed a series of intricate mechanisms to combat against aphids. Plenty of studies have indicated that various changes occur in plants damaged by aphids, such as protein phosphorylation, calcium flux, reactive oxygen species (ROS) generation and phytohormone changes, which result in relevant transcriptional regulation in the early response to aphids. Finally, plants are capable of producing various defense compounds, including nutrient compounds, glutathione S

transferases (GSTs), peroxidases, and secondary metabolites for the sake of self-defense [3]. Overall, plant susceptibility or resistance to aphids is reliant on their ability to recognize aphid feeding and rapidly initiate defense response [4].

Plant resistance is considered as one of the most effective ways of achieving plant health management at the present time and in the near future [5]. Therefore, constantly exploring new aphid resistant genes has been a long-standing hot topic for scientists. *Mi-1.2* from the tomato was discovered to contribute to resistance to *Macrosiphum euphorbiae* and *Bemisia tabaci* [6, 7]. The melon gene *Vat* was reported to safeguard against virus aphid transmission (*Aphis gossypii* Glover) [8]. Both of them are the earlier known aphid resistance genes and fall into NBS-LRR family, indicating that NBS-LRR family members perform a significant part in plant aphid resistance. Lectins have also been proved to perform resistance to aphids. For instance, the *Galanthus nivalis* agglutinin (GNA) gene encoding a monocot mannose-binding was extensively and clearly documented to confer resistance to *Myzus persicae* [9]. *Amaranthus caudatus* agglutinin (ACA) gene conferred resistance to *Aphis gossypii* and *Myzus persicae* [10, 11]. In addition, the other aphid resistant genes, including rag, chitinase, β-amylase and on the likes, are researched actively and widely [12].

RNA-Seq is a robust technology for analyzing transcriptome and excavating new functional genes. Therefore, it is used in many fields including plant defense response to phytophagous insects [13]. For instance, the changes in gene expression in the whole genome of the cucumber aphid resistant cultivar 'EP6392' were monitored using an Illumina Genome Analyzer platform [14]. Furthermore, a comprehensive insect resistance response mechanism in cotton infested by the phloem feeding insect *Bemisia tabaci* was discovered through Transcriptome analysis [15]. In addition, the transcriptome analyses of *Gossypium hirsutum* at 24 h and 48 h in response to aphid and whitefly were conducted comparatively [16]. However, compared to other research areas, RNA-Seq analyses of plant defense response to phytophagous insects have been relatively sporadic up to now.

As an essential economic crop, the recent research on cotton transcriptome characterization is focused on comparative transcriptomic analysis at different growth stages and under all sorts of stresses. For instance, many new genes of cotton fiber were extensively analyzed from different angles using RNA-Seq method [17-21]. Furthermore, cotton RNA-Seq data were also analyzed under various abiotic stress, such as salt stress [22-25], dry stress [26-27]and high temperature stress [28]. Cotton is also attacked by all kinds of biotic factors, such as Cotton *Verticillium* wilt. As one of the most destructive cotton diseases, the study on Cotton *Verticillium* wilt using RNA-Seq is concerned widely. For example, RNA-Seq transcriptional analysis and histo-chemistry have illustrated that lignin metabolism plays an important role in the cotton resistance to *Verticillium dahlia* [29]. The gene families of resistance gene analogues in cotton and their response to *Verticillium wilt* were also studied [30]. The analysis of sea-island cotton and upland cotton in response to the damage of *Verticillium dahliae*was was conducted using RNA-Seq [31]. However, RNA-Seq has rarely been applied to research the interaction between cotton and herbivorous insects.

It is well known that using cotton aphid resistance to control aphids is both more effective and eco-friendly. Therefore, the analysis of the transcriptome dynamics of cotton defense response to cotton aphid damage was conducted using the RNA-Seq method for a better understanding of cotton aphid resistance mechanism. Our research will lay a solid foundation for further research into the molecular mechanism of the interaction between cotton and aphids, searching for new cotton aphid resistant genes and developing new cotton aphid resistant varieties.

Results

RNA-Seq outline of cotton leaves in response to cotton aphid attack

The damage caused by cotton aphids is rated as the serious at seedling stage during the growth and development of cotton. Therefore, the cotton leaves at the four-leaf stage were selected as the research materials. To excavate the crucial genes at the transcriptional level in cotton defense response to cotton aphid damage, the whole-transcriptome RNA sequencings of cotton leaves at 0 (without aphid damage, CK), 6, 12, 24, 48 and 72 h under sustained cotton aphid attack were performed respectively. After filtering out reads containing adapter or ploy-N, and low-quality sequences, there were 60.05, 58.70, 57.74, 65.73, 59.80, and 60.26 million clean reads with pair-end (Q_{30}> 85.03%, GC%=41.3-42.9%) respectively produced by RNA-Seq in all the six libraries (**Supplementary Table S2**). For the clean reads according to the single-end, 89.94–90.78% of them were mapped to the cotton reference genome sequences (**Supplementary Table S3**). The mapped reads were assembled with Cufflinks software, and compared with the original genome annotation information to explore the original unannotated transcription area and excavate the new transcripts and new genes. A total of 79,581 unigenes were identified by filtering out sequences that were comprised of less than 150 bp or only a single exon. To obtain the annotation information of the new genes, the databases were analyzed using a combination of BLAST [32], NR, GO, KEGG, COG and Swiss-Prot. A total of 9,103 new genes were discovered, of which 7,510 were functionally annotated (**Tab. 1**). The normalized FPKM was used to quantify the gene expression level [33]. The transcript expression level (FPKM) in this study ranged from 10^{-2} to 10^{4}, which was found to be coherent with most of the RNA-Seq results (**Fig. 1A**). FPKM box plot analysis demonstrated that the gene transcript expression levels varied in the six RNA-Seq data to some extent (**Fig. 1B**).

Differentially expressed genes from cotton leaves under cotton aphid attack

As gene expression has time specificity, it is quite significant to study the cotton differentially expressed genes (DEGs) at different time points under cotton aphid attack. Each sample damaged by cotton aphids was compared with the control (0 h) to identify differentially expressed genes (6 h vs. control, 12 h vs. control, 24 h vs. control, 48 h vs. control and 72 h vs. control). Fold change ≥ 2 and FDR < 0.05 were taken as the screening criteria. From these pairwise comparisons drawn with the control sample, we identified 5,790 (2,580 up- and 3,210 down-regulated) , 9,726 (3,452up- and 6,274 down-regulated), 5,279(1,836up- and 3,443 down-regulated), 4,549(2,477 up- and 2,072 down-regulated) and 8,683(2,769 up- and 5,914 down-regulated) DEGs respectively after 6 h, 12h, 24, 48h, and 72h under cotton aphid

attack（**Fig. 2A, Supplementary Table S4**). The differentially expressed genes were analyzed by means of hierarchical clustering, and the genes with the same or similar expression patterns were clustered (**Fig. 2B**). In addition, it was discovered that the DEGs between cotton aphid attack (48 h) and CK（0 h）is distinct from the other treats. The amount of up-regulated genes was higher than that of down-regulated genes between cotton aphid attack (48 h) and CK（0 h）(**Fig. 3A, B**).

DEG functional classification

The DEGs between cotton aphid attack (6 h, 12 h, 24 h, 48 h, and 72 h) and CK（0 h）were subjected to GO term enrichment analysis, involving cellular component, molecular function and biological function. The results indicated that plenty of DEGs were associated with biotic or abiotic stress, including response to salt stress (1734), response to chitin (1245), defense response to bacterium (1230), response to cold (1199), response to fungi (1196), response to wounding (1194), response to water deprivation (1349), response to abscisic acid (1292), and response to cadmium ion (1496) **(Fig. 4A，Supplementary Table S5)**. Additionally, it is noteworthy that two genes in the “response to insect (GO: 0009625)” and “defense response to insect (GO: 0002213)” were enriched during the biological process. To carry out a further investigation into the biological functions performed by these DEGs, pathway-based analysis was conducted using KEGG. Totally 126 pathways were identified that were significantly enriched in comparisons of cotton aphid attack (6 h, 12 h, 24 h, 48 h, and 72 h) versus CK (0 h), such as plant hormone signal transduction (378)，starch and sucrose metabolism (244), ribosome (236), carbon metabolism (307), biosynthesis of amino acids (275), pyruvate metabolism (125), carbon fixation in photosynthetic organisms (123), endocytosis (114), glutathione metabolism (119), pyrimidine metabolism (117), pentose and glucoronate interconversions (99), RNA degradation (94), fatty acid metabolism (93), arginine and proline metabolism (98), mRNA surveillance pathway (97), glycerophospholipid metabolism (98), RNA transport (103), cysteine and methionine metabolism (100), glyoxylate and dicarboxylate metabolism (106), phagosome (104), glycine, serine and threonine metabolism (104), peroxisome (103), photosynthesis (191), plant–pathogen interaction (176), Purine metabolism (169), amino sugar and nucleotide sugar metabolism (168), phenylpropanoid biosynthesis (141), oxidative phosphorylation (155), protein processing in endoplasmic reticulum (156), and glycolysis / gluconeogenesis (155) **(Fig. 4B)**. In conclusion, these results suggested that cotton's defense response to the damage of cotton aphids involved multiple genes and metabolic pathways, and cotton plants end up showing a comprehensive defense potential against the attack of cotton aphids.

Differentially expressed transcription factors involved in response to cotton aphid stress

Transcription factors (TFs) are a specific DNA-binding protein that has regulatory functions at transcription level and performs an essential role in plant growth and development. In this study, a total of 945 differentially expressed transcription factors were identified from cotton leaf response to cotton aphids. They were classed into 27 TF families respectively (**Supplementary Table S6**). A large majority of them encoded the members of the bHLH, MYB, WRKY, ERF, bZIP, TCP and NAC TF families (**Fig. 5A**). The bHLH family with 199 DEGs was rated as the largest TF family in cotton leaf response to cotton aphid

stress. In addition, a total of 152 DEGs were identified to belong to the MYB family. The expression levels for many of the differentially expressed TFs were up-regulated under cotton aphid attack, especially at 48 and 72 h under cotton aphid attack. However, the transcripts of some differentially expressed TFs were down-regulated, indicating that the functions carried out by cotton TFs were complex in cotton response to cotton aphid attack (**Fig. 5B**).

Differentially expressed aphid-resistant genes involved in response to cotton aphid stress

Aphid damage often triggers the differentially expressed genes related to plant resistance, involving plant morphological resistance, phytoprotective enzyme and SA, JA and ethylene signal pathway. Plenty of cotton aphid resistance genes were identified at first in this study. For example, 39 differentially expressed genes of NBS-LRR family were identified and 10 genes of them were reported at first. They were associated closely with the detection of bacteria, viruses, fungi, nematodes, insects and oomycetes (**Supplementary Table S7**). 106 DEGs of leucine-rich repeat receptor-like protein kinase were also found and 6 genes of them were novel (**Supplementary Table 8)**. Besides, 10 genes of 140 DEGs related to lectins were regarded as cotton new genes. Compared to the expression levels without cotton aphid attack（0 h), the expression levels of most lectin genes were up-regulated at 6, 12, 24, 48 and 72 h, and only transcription of two lectin genes were down-regulated (**Fig. 6A－B, Supplementary Table 9**). Additionally, in the functional analyses of differentially expressed genes, we also obtained many new other genes related to cotton aphid resistance, including 3 genes related to callose **(Supplementary Table S10)**, 3 genes related to trichomes **(Supplementary Table S11)**, 3 genes related to waxes **(Supplementary Table S12)**, 7 genes related to ca^{2+} **(Supplementary Table S13)**, 1 genes related to peroxidase **(Supplementary Table S14)** and 1 related to Phenylalanine ammonialyase (**Supplementary Table S15**).

Verification of RNA-Seq data by qPCR

To validate the results of the RNA-Seq, ten random DEGs including five up-regulated and five down-regulated genes were selected to perform qPCR. The selected genes were successfully amplified and the products were of the expected size, indicating the reliability of the assembly work. The qPCR results demonstrated that the relative expression levels of ten selected genes were consistent with the results of RNA-Seq despite the differential expression folds, suggesting that the RNA-Seq data were highly reliable (**Fig. 7**).

Discussion

RNA-Seq presents an effective way for the identification of DEGs and their regulatory mechanisms at the transcriptome level [34]. It could provide new insights into the molecular basis for plant response to all kinds of abiotic and biotic stress [35-37].

RNA-Seq in cotton response to cotton aphid damage

Aphids are the largest group of the phloem-feeding insects and often cause severe economic loss in crop production. However, the mechanism for the interplay between plants and aphids remains unclear at the moment [1]. Therefore, there is great significance for understanding their interaction mechanism to study the cotton response to cotton aphid damage using RNA-Seq method. Dubey et al. (**2013**) carried out the comparative transcriptome analysis of *Gossypium hirsutum* in response to aphids and whiteflies [16]. However, only the transcriptomes of cotton infested with aphids and whiteflies for 2 h and 24 h were sequenced and analyzed. In this study, the RNA-Seq data of cotton leaves at 0, 6, 12, 24, 48, and 72 h under the attack of cotton aphids were analyzed. The sequencing results suggested that a total of 9, 103 new genes were discovered and 7, 510 were annotated functionally at different time points. Based on the comparison results, the gene expression was analyzed in accordance with the expression amount of genes in different samples. 24,793 DEGs were authenticated in all and their functional annotation and enrichment analysis were performed. The number of down-regulated DEGs was found out to be largely higher than that of the upregulated genes at different time under cotton aphid attack, which was in line with the previous studies that infestations of whiteflies and aphids drove transcriptional suppression over induction [38, 16]. However, the number of down-regulated DEGs was slightly smaller than that of the upregulated genes at 48 h under cotton aphid attack in this study. Functional annotation of DEGs led to a finding that these genes were involved in a lot of plant biological processes, including all sorts of resistance to abiotic and biotic stress, hormone metabolism, signaling transduction and transcriptional regulation. However, only two genes in the “response to insect (GO: 0009625)” and the “defense response to insect (GO: 0002213)” were enriched in biological process, which suggested that the study of biological information on plant response to insects should be enforced. In addition, the study on cotton transcriptome in response to the damage of aphids and whiteflies displayed significantly enrichment of the amino acid biosynthesis pathway [16]. We got similar results in our study, suggesting the amino acid biosynthesis pathway was important very much.

Plant aphid resistant genes

Plant defenses against aphids are initiated at various levels of their interplay with aphids [39]. Firstly, plant surface is the first barrier of aphid feeding and activities, such as thorn and glandular trichomes on the surface of plant [40-42]. In our study, 3 new DEGs (Protein WAX2 or Protein WAX2 -like protein) were identified, and these DEG expressed levels varied at 6, 12, 24, 48 and 72h after cotton aphid attack in comparison with the CK Control (0 h). Trichomes on the surface of plants could also exert impacts on aphid activities by hindering aphid movement. Furthermore, glandular trichomes are usually an origin of sugar esters and secondary metabolites that are harmful to insects [43, 44]. Some glandular trichomes even have the potential to release the aphid alarm pheromone, such as (E)−β-farnesene [45]. Our data analysis showed a total of 3 differentially expressed genes on trichomes were identified at first, suggesting that trichomes were crucial to plant aphid resistance. In addition, plants inhibit the afflux of phloem inclusion into the aphid by facilitating sieving element occlusion (SEO). SEO includes two processes: a rapid formation of proteinaceous plugs that transiently seal sieve plates and a slower callose deposition that causes long-term occlusion of sieve tubes [46]. A total of 3 new DEGs on callose

deposition were identified in this study. These results indicated that callose was involved in cotton response to cotton aphid attack.

Some loci with specific aphid resistance have been stated in many plants, but only the *Mi-1.2* gene from tomato and the *Vat* gene from melon have been cloned. They contribute to resistance against *Macrosiphum euphorbiae* and *Aphis gossypii* respectively [6, 47, 48]. They belong to coiled-coil nucleotide-binding site -leucine-rich repeat type proteins (CC-NBS-LRR). In our study, 10 new NBS-LRR DEGs were found and 6 Leucine-rich repeat receptor-like protein kinase were identified at first. The carbohydrate-binding protein lectins exist in many plants and perform important functions in guarding plants against insect pests. At the moment, many lectin genes from plants have been transferred to plants to improve resistance to many phloem insects [49]. The agglutinin gene is the most widely studied for plant resistance to aphid as the carbohydrate-binding proteins currently.10 genes were considered as cotton new lectin genes in our RNA-Seq data.. In addition, Plenty of other aphid resistant genes were also involved in this study, including peroxidase, phenylalanine ammonialyase, polyphenol oxidase and on the likes.

Transcription factors involved in response to cotton aphid stress

Some transcription factors in plants have been validated to act as a key role in the face of the attack by herbivorous insects. For example, overexpressing *CmMYB19* improved aphid tolerance in *Chrysanthemum* by facilitating lignin production [50]. Similarly, three MYB genes were related to the wheat defense against English grain aphid [51]. In addition, overexpression of *GsMYB15* from the wild Soybean R2R3 enhanced resistance to *Helicoverpa Armigera* in *Arabidopsis* [52]. However, *myb102* from *Arabidopsis* increased plant susceptibility to aphids by substantial activation of ethylene biosynthesis [53]. A total of 152 DEGs belonging to the MYB family were identified in this study. The functions of them need to further be verified through relative experiments. Some members of the WRKY family are reported for aphid resistance. For instance, the overexpression of a chrysanthemum WRKY transcription factor enhanced aphid resistance [54]. However, *AtWRKY22* from *Arabidopsis* increased *Arabidopsis* susceptibility to aphids and modulated SA and JA signaling [55]. In addition, the rice *WRKY53* suppressed herbivore-induced defenses by acting as a negative feedback modulator of map kinase activity [56]. In short, the functions of transcription factors in plant response to aphids are relatively complex. Studies of cotton transcription factors are primarily focused on fiber development and the response to all kinds of abiotic stress at present. Therefore, studies of their aphid resistance should be performed continuously in the future.

Plant defense signaling in cotton response to cotton aphids

The plant hormones (salicylic acid, jasmonic acid, ethylene and abscisic acid) lead to signaling related to plant-aphid interaction. Despite the aphid effectors remain to be identified, important advances have been acquired in comprehending the signaling machinery related with *Mi-1.2* in tomato. Some genes in ETI to microbes are also required for *Mi-1.2*-conferred resistance to *Macrosiphum euphorbiae*. Such as Heat shock protein 90 and Suppressor of G-two allele of Skp1 [57]. A receptor-like kinase encoded by the

tomato *SERK1*, a mitogen-activated protein kinase cascade and the transcription factors WRKY70 and WRKY72 are also required for *Mi-1.2* resistance to aphids [58-60]. Ca^{2+} functions as a secondary messenger in eukaryotes. It is universally thought that Ca^{2+} could influence callose deposition, promote phloem protein aggregation and result in phloem occusion [61-63]. 7 new DEGs on Ca^{2+} were identified in this study, suggesting that ca^{2+} was essential in cotton response to cotton aphids. However, aphids conquered this defense by secreting Ca^{2+}-binding protein, which suggested that the interaction between plants and aphids was relatively complicated [16].

Conclusions

In the present study, RNA-Seq was applied for detection of the global transcriptional changes in seedling cotton leaves in response to cotton aphid attack. A total of 9, 103 new genes were identified and 7, 510 were annotated functionally. Crucially, 24,793 DEGs were commonly identified in response to cotton aphids, suggesting that there were plenty of common and unique molecular mechanisms in relation to the response to cotton aphid stress in cotton. This study extends the understanding of the molecular mechanisms for cotton leaf resistance to cotton aphids in the seedling stage and facilitates the development of plant aphid resistant cultivars.

Methods

Plant materials

Red-leaf cotton (*Gossypium hirsutum*), an aphid-resistant cotton cultivar, was provided by the Institute of Cotton from Chinese Academy of Agricultural Sciences. At first its seeds were sown directly in 30-cm-diameter plastic pots filled with nutrient soil until the seedling grew to 4 leaves in a growth room (25°C, 16 h light/8 h dark). The leaves were collected respectively without cotton aphid damage (0 h, CK) and at 6, 12, 24, 48 and 72 h under the attack of cotton aphids (*Aphis gossypii*), transferred quickly into liquid nitrogen for 1 min and stored in the ultra-low temperature freezer.

Library construction and sequencing

Total RNA from each sample was isolated using Trizol method. 1.5 μg RNA from each sample was used as input material for the removal of rRNA utilizing the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA). The libraries for sequencing were established with NEBNext^{R} Ultra^{TM} Directional RNA Library Prep Kit for Illumina^{R} (NEB, Ipswich, USA). The index codes were appended to attribute sequences to each sample. The quality of the library was estimated using the Agilent Bioanalyzer 2100 and qPCR method. The clustering of each index-coded sample was executed on acBot Cluster Generation System using TruSeq PE Cluster Kitv3-cBot-HS (Illumia). After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 150-200 paired-end reads were derived.

Transcriptome assembly

Raw reads were pre-processed for quality filtering where reads including that (1) unknown (N) bases are more than 10 %, (2) contain adaptor sequences, and (3) contain low quality bases (Q ≤20) more than 50 % were removed through in-house perl scripts. According to the reference genome, the mapped reads were assembled using Cufflinks software. The original unannotated transcription area was searched using BLAST software that was used to conduct sequence alignment with databases of NR[64], swiss-prot [65], GO[66], COG[67] and KEGG[68] and all parameters are default values, and new transcripts and new genes were obtained to supplement and perfect the original genome annotation information. The sequences that are less than 150 bp or that contain only a single exon were filtered out. The sequencing data were deposited in the NCBI Short Read Archive database with the accession number **PRJNA576973**.

Differentially expressed gene analysis

Gene FPKMs (fragments per kilo-base of exon per million fragments) were calculated by summing the FPKMs of transcripts in each gene group. FPKMs of coding genes in each sample were computed using StringTie (1.3.1). EBSeq was employed for different analysis [69]. During the course of differentially expressed gene detection, Fold change ≥2 and False Discovery Rate（FDR）< 0.05 served as the screening criteria. Fold change represents the ratio of expression quantity between two treats. FDR is determined through the p-value correction of different significance.

Functional annotations of differentially expressed genes

The functions of differentially expressed genes were annotated with NR, swiss-prot, GO, COG and KEGG. In order to obtain the annotation information about the new genes, the reference *Gossypium hirsutum* genome and the annotation files were downloaded from the CottonGen database (http://www.cottongen.org). Gene Ontology (GO) enrichment analysis of DEGs was implemented by the topGO R packages. KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathways.

Quantitative real-time PCR

To ascertain the expression level of ten genes, quantitative real-time PCR (qPCR) was carried out based on the manufacturer's guides for the Bio-Rad CFX 96 Real-Time Detection System (Bio-Rad, Hercules, CA, USA) and the SYBR premix ex Taq II system (Takara perfect real time). Cotton leaves were firstly collected without cotton aphid damage (0 h, CK) and at 6, 12, 24, 48, and 72 h under cotton aphid attack at the four-leaf stage. Total RNA was isolated from each sample and reversely transcribed into cDNA as qPCR template. The expression profiles of the relative genes were examined through qPCR method. All reactions were conducted in triplicate, and controls were included. The $2^{-\triangle\triangle Ct}$ method was applied to the calculation of relative gene expression values [70]. The prime sequence was presented in **Supplementary Table S1**. The difference between each treatment (**6, 12, 24, 48 or 72 h**) and the control (0 h) was statistically analyzed with the t test for independent samples for qPCR data. Two significance levels were used (*, P<0.05 and **, P<0.01).

Abbreviations

DEG: Differentially expressed gene; TF: Transcription factor; RNA-Seq: RNA sequencing; qPCR: Real-time quantitative polymerase chain reaction; FPKMs: Fragments per kilo-base of exon per million fragments; ROS: Reactive oxygen species; GST: Glutathione S transferases; FDR: False Discovery Rate

Declarations

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Availability of data and materials

The datasets of this publication are included within the article and its Additional files.

Authors' contributions

ZJM and YYZ conceived and designed the research. ZX, FP, MQQ performed the experiments, ZJM analyzed the data and wrote the manuscript. SQ and WXP revised the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Additional Files

Additional files 1: Primer sequences

Additional files 2: The evaluation analysis on RNA sequencing data

Additional files 3: Summary of RNA-seq data and reads mapped to the cotton reference genome sequence

Additional files 4: The number of DEGs betweent CK and cotton aphid treatment

Additional files 5: Classification statistical analysis of GO annotation of differentially expressed genes

Additional files 6: The differentially expressed transcription factors in six RNA-Seq data

Additional files 7: DEGs of NBS-LRR in six RNA-Seq data

Additional files 8: DEGs of Leucine-rich repeat receptor-like protein kinase in six RNA-Seq data

Additional files 9: DEGs related to lectins in six RNA-Seq data

Additional files 10: DEGs related to callos in six RNA-Seq data

Additional files 11: DEGs related to trichomes in six RNA-Seq data

Additional files 12: DEGs related to waxes in six RNA-Seq data

Additional files 13: DEGs related to Ca^{2+} in six RNA-Seq data

Additional files 14: DEGs related to peroxidase in six RNA-Seq data

Additional files 15: DEGs related to phenylalanine ammonialyase in six RNA-Seq data

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Table

Due to technical limitations, table 1 is only available as a download in the supplemental files section.

Figures

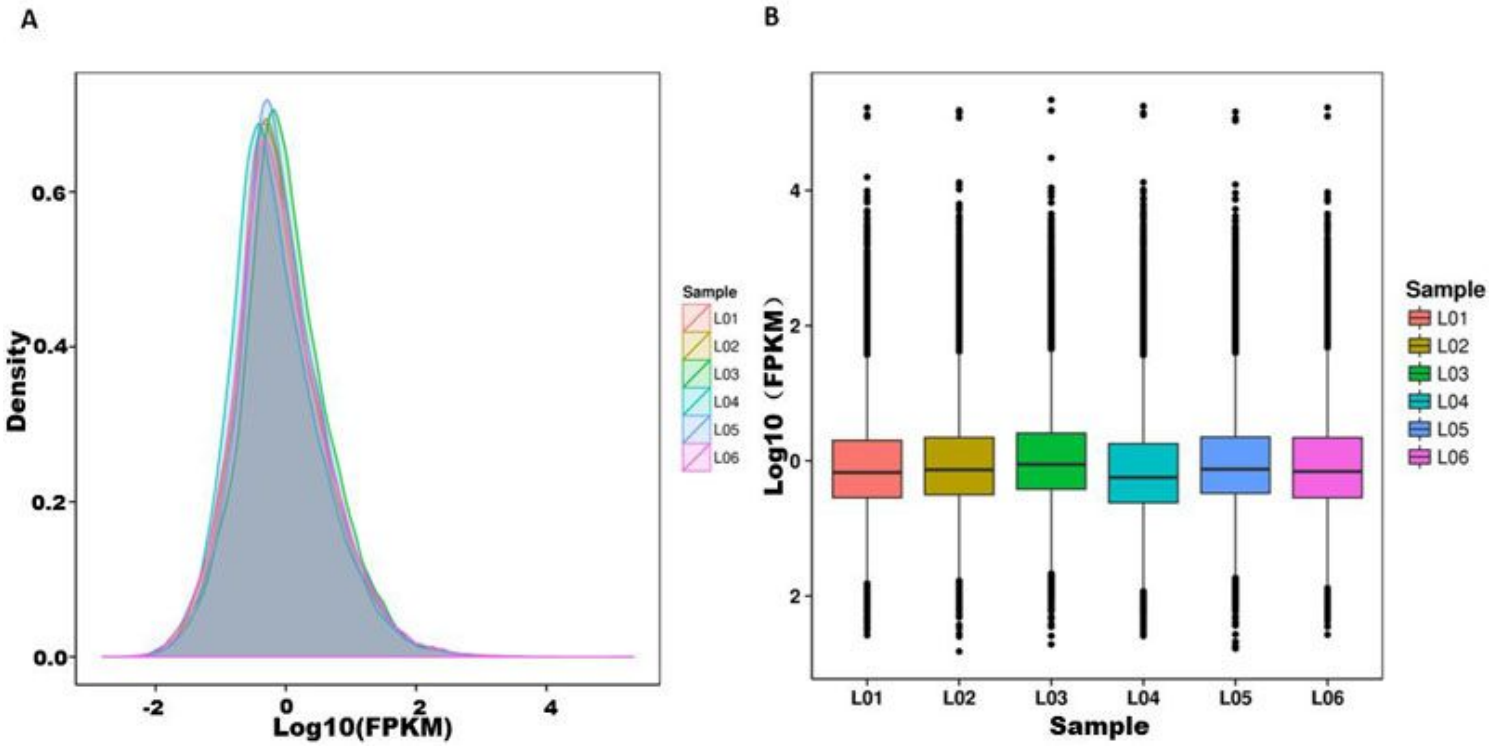

Figure 1

FPKM analysis in six sample RNA-Seq. （A） Comparison of FPKM density distribution. （B） FPKM box plot. L01. 0 h: CK (without cotton aphid damage), L02: 6 h, L03: 12 h, L04: 24 h, L05: 48 h. L06: 72 h.

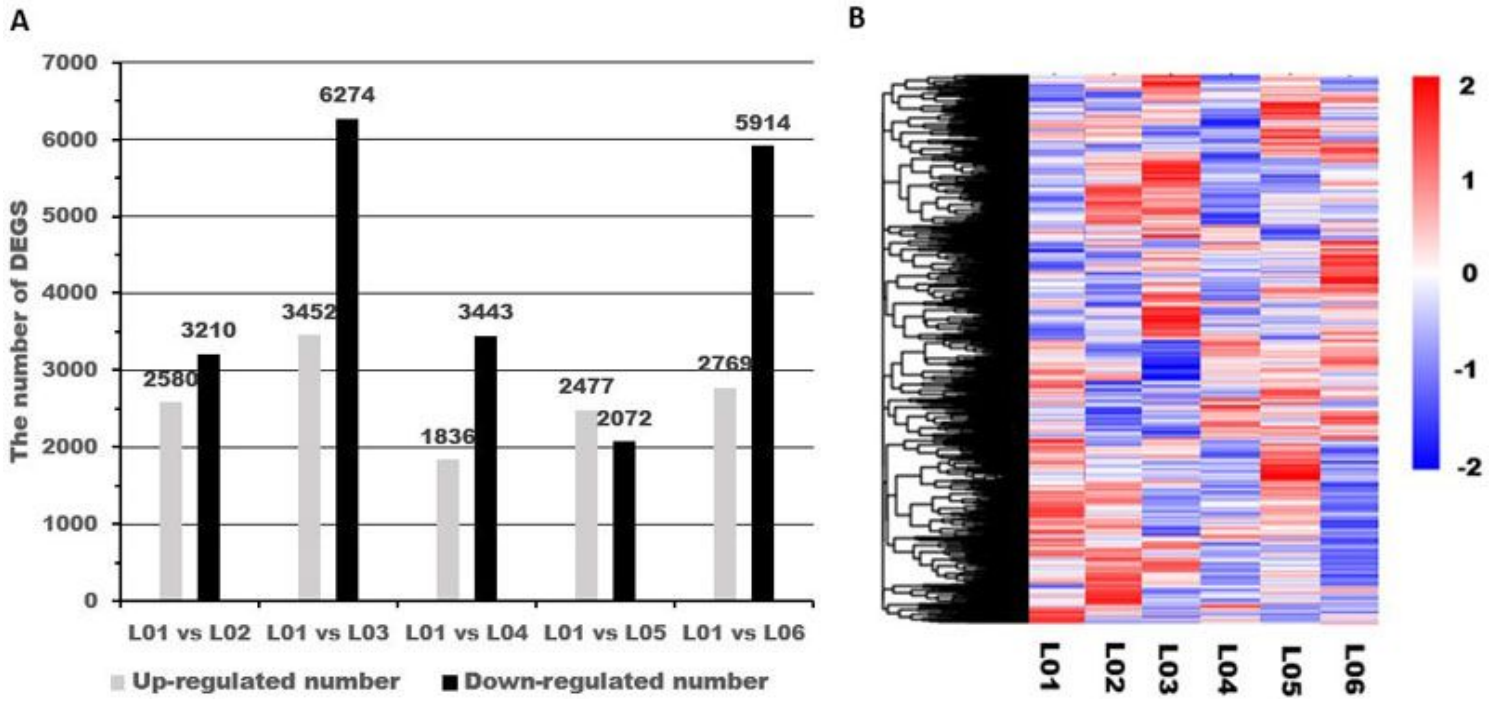

Figure 2

Analysis of differentially expressed genes of cotton leaves between cotton aphid damage and CK（L01） at the different time points. (A) Analysis of the number of differentially expressed genes. (B) The cluster

diagram of differentially expressed genes. Different columns in the Fig. B represent different samples, and different rows represent different genes. Color represents the level of gene expression in the sample. L01: CK, L02: 6 h, L03: 12 h, L04: 24 h, L05: 48 h, L06: 72 h.

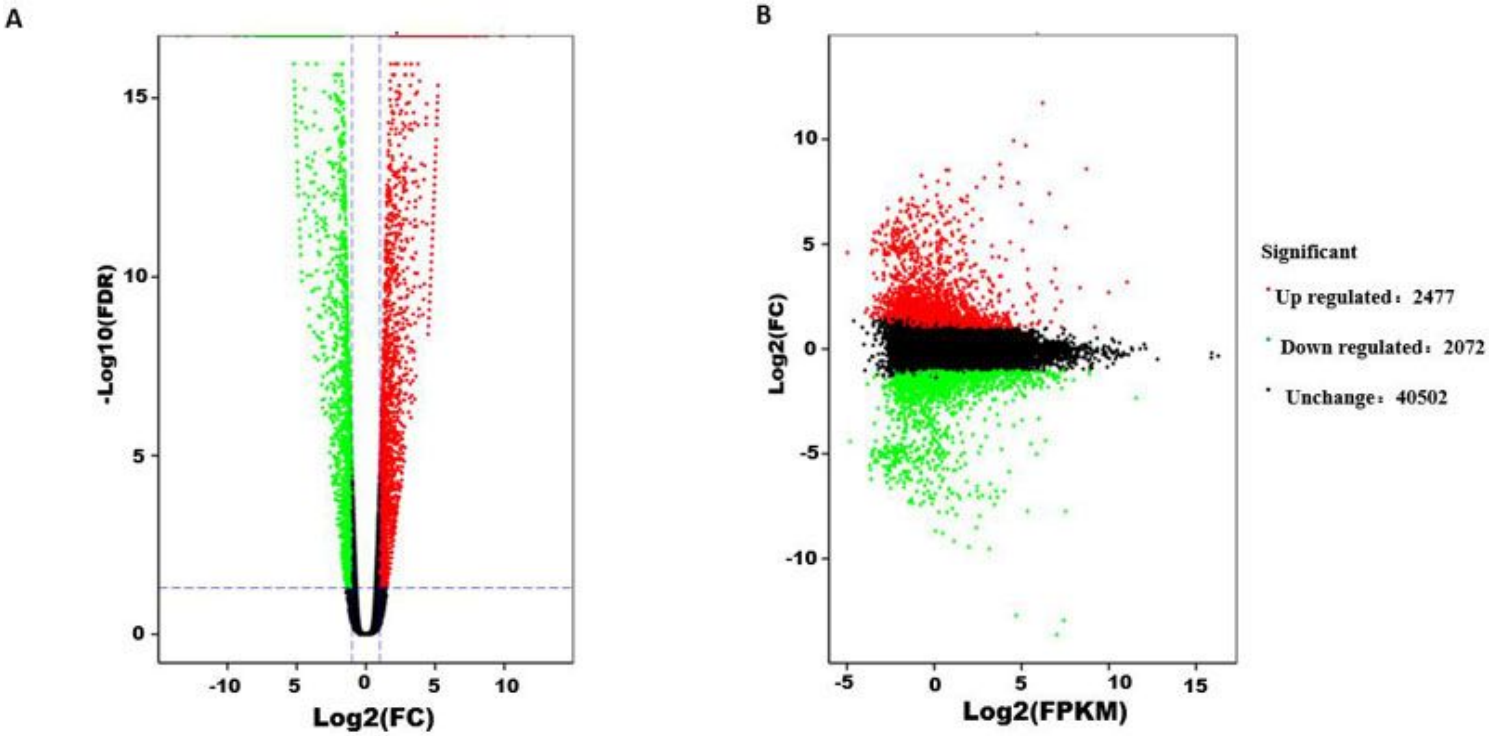

Figure 3

Analysis of differentially expressed genes of cotton leaves between cotton aphid damage（48h）and CK （0h）. (A) The Volcano plot. (B) MA plot. The green dots represent down-regulated differentially expressed genes, the red dots represent up-regulated differentially expressed genes, and the black dots represent non-differentially expressed genes.

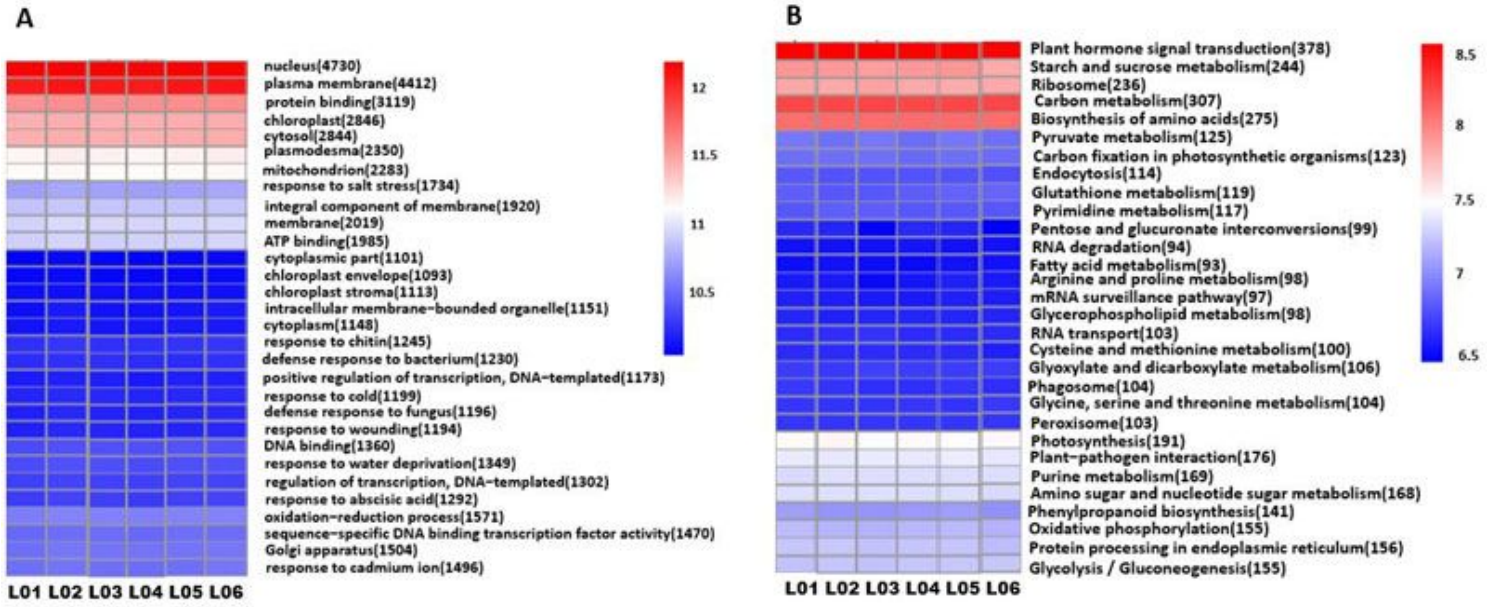

Figure 4

Analyses of DEGs under cotton attack at different time points. (A) GO rich clustering class diagram of the total differentially expressed genes. red indicates the classification of high expression function, while blue indicates the term with relatively low expression. The parenthesis after each term label is the number of genes containing significant differences for that term. (B) The KEGG rich cluster class diagram of the total differentially expressed genes. Red indicates high expression functional classification, while blue indicates relatively low expression metabolic pathways. Each metabolic pathway is labeled with the number of genes containing significant differences within the metabolic pathway in parentheses.

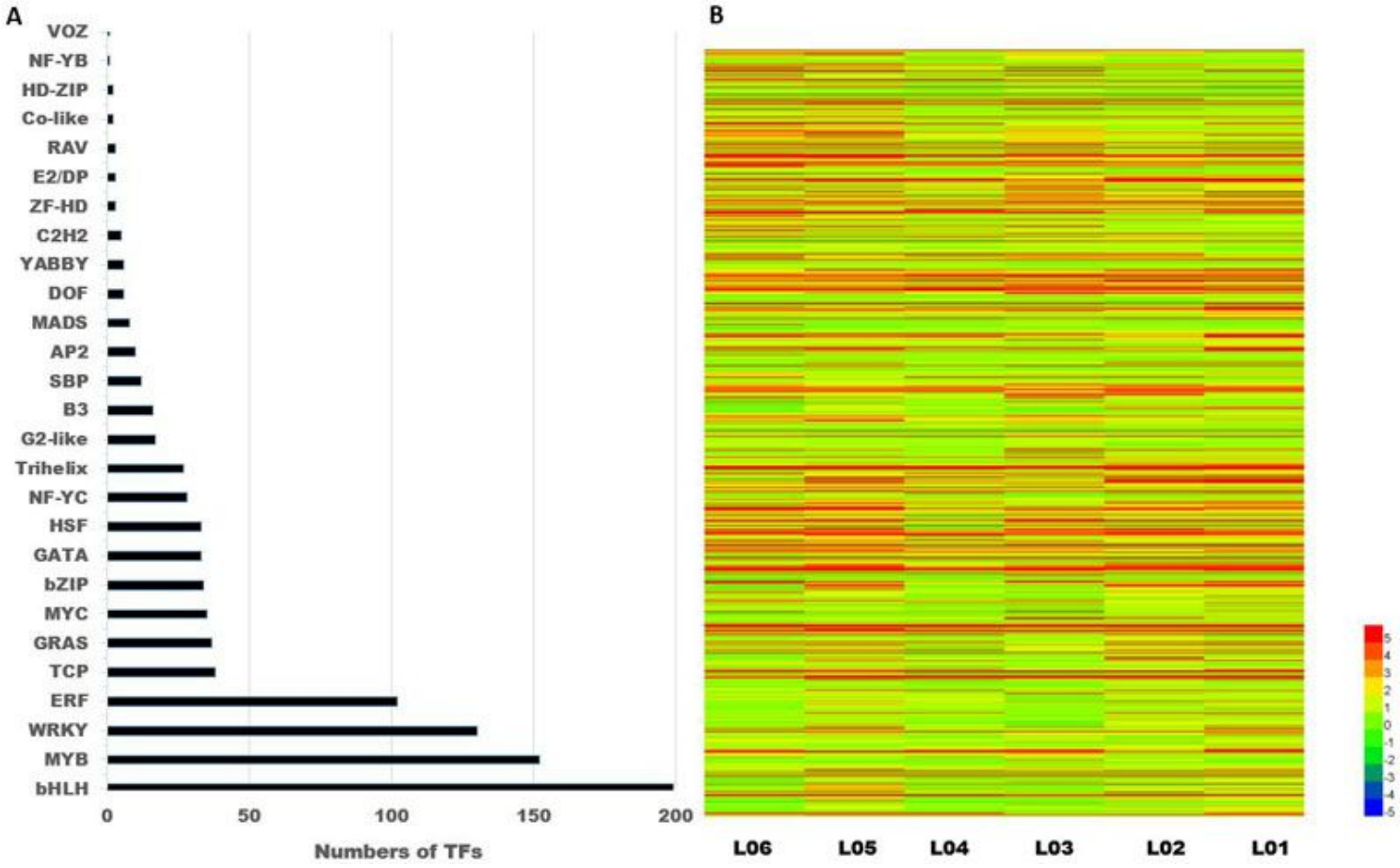

Figure 5

Number distribution and expression of differentially expressed transcription factors from different families. (A) Number distribution of the differentially expressed transcription factors. (B) Expression values of differentially expressed transcription factors that were more than 30. Their expression values are presented as FPKM-normalized log2-transformed counts. L01: CK, L02: 6 h, L03: 12 h, L04: 24 h, L05: 48 h, L06: 72 h.

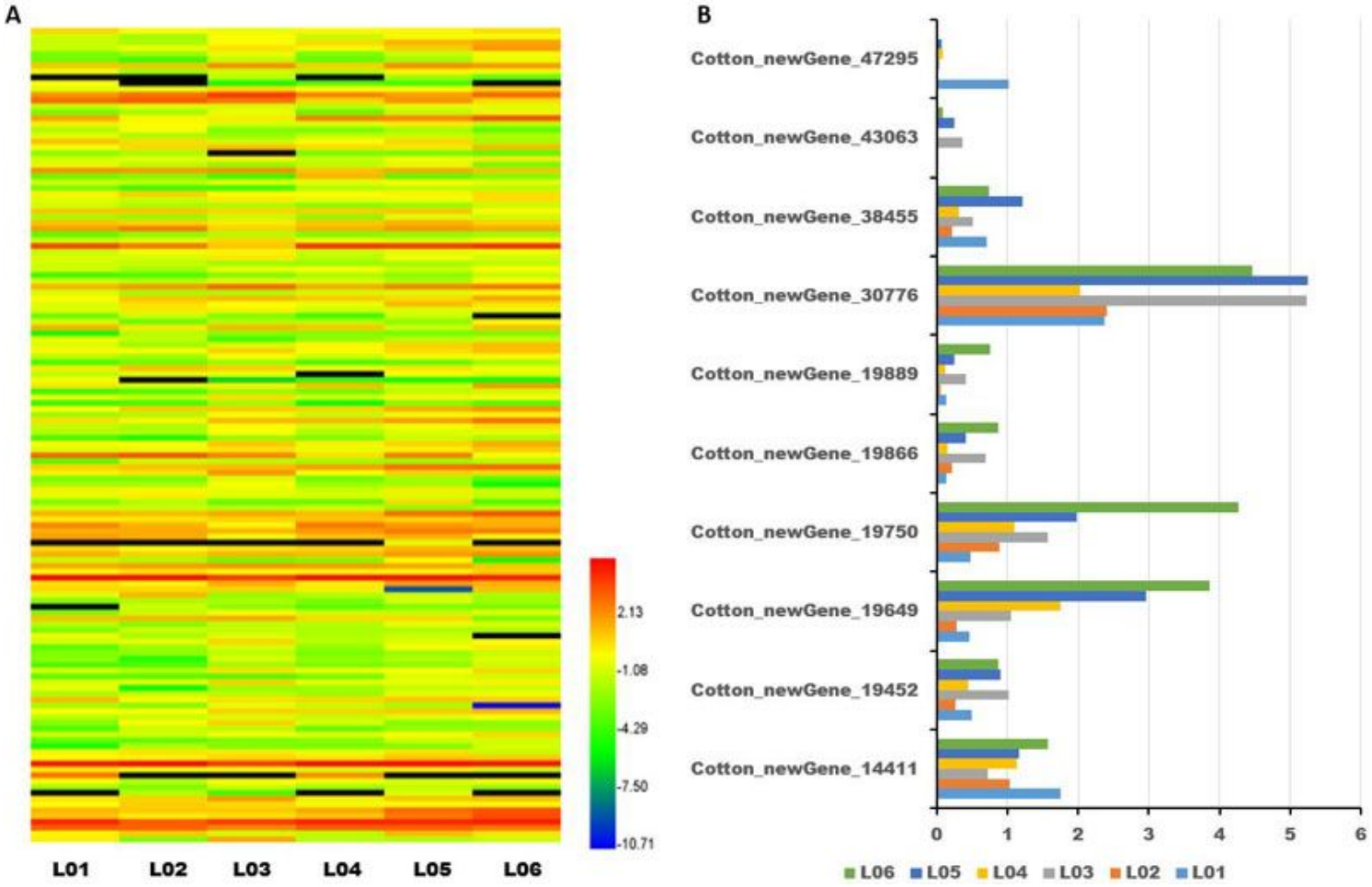

Figure 6

Expression of differentially expressed lectins at different time points under cotton aphid attack. (A) Expression analysis of all differentially expressed lectins. (B) Expression values of 10 differentially expressed new lectins. Their expression values are presented as FPKM-normalized log2-transformed counts.

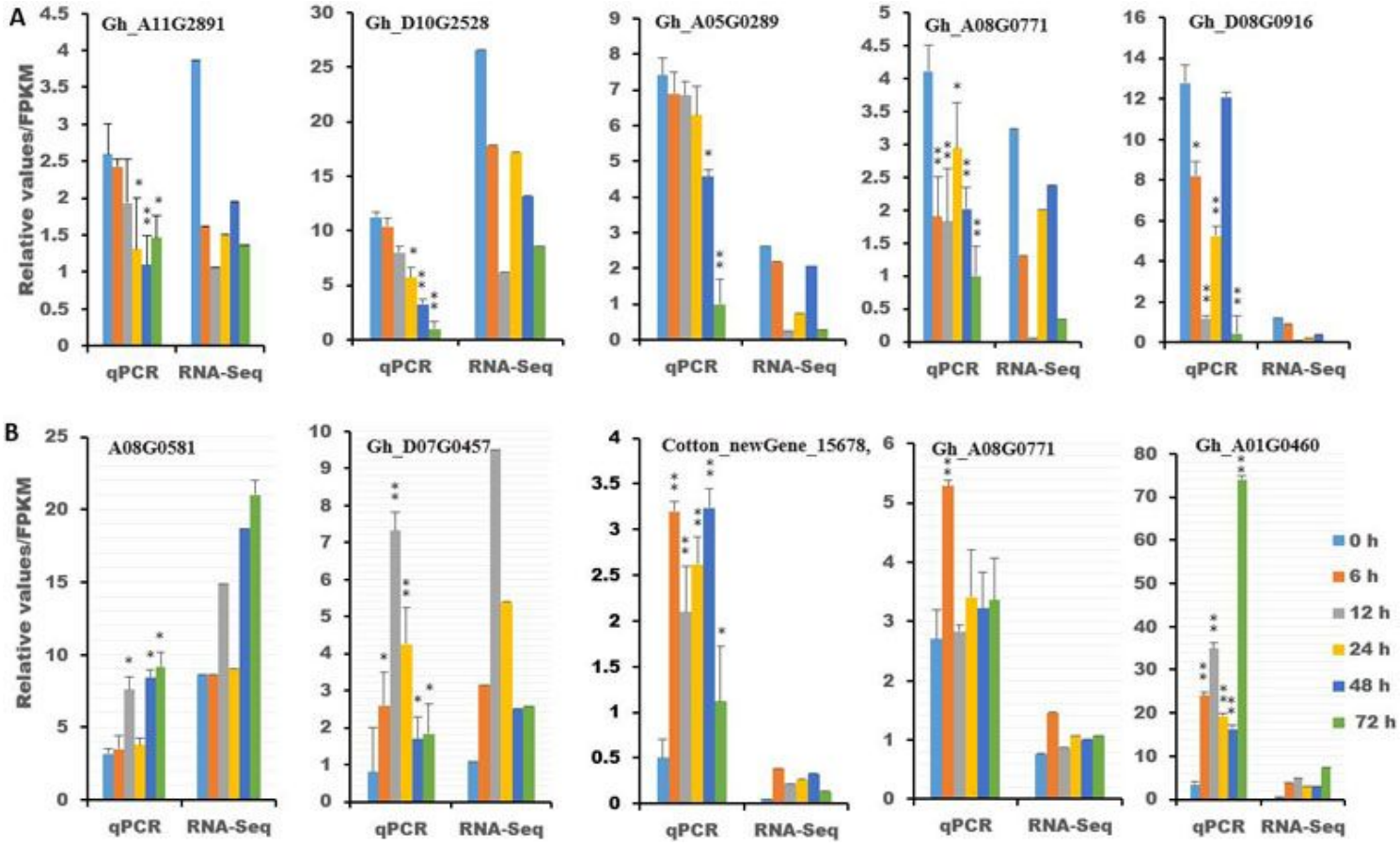

Figure 7

Expression level of ten selected DEGs in cotton response to aphid infestation over time (0, 6, 12, 24, 48 and 72 h) using qPCR and RNA-Seq methods. A Expression level of five up-regulated DEGs. B Expression level of five down-regulated DEGs. The 0 h time point (no aphid infestation) serves as a control. Relative values was calculated based on the 2-△△Ct method using GhUBI as the reference gene for qPCR, and three biological replicates and three technical replicates were performed. Five down-regulated DEGs are as follows: Gh_A11G2891, Gh_D10G2528, Gh_A05G0289, Gh_A08G0771, Gh_D08G0916. Five up-regulated DEGs are as follows: Gh_A08G0581, Gh_D07G0457, Gh_A03G0199, Cotton_newGene_15678, Gh_A01G0460. The difference between each treatment （0, 6, 12, 24, 48 or 72 h ）and the control （0 h） was statistically analyzed with the t test for independent samples for qPCR data. Two significance levels were used (*, P<0.05 and **, P<0.01).

Supplementary Files

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