Genome-wide analysis of PRR gene family uncovers their roles in circadian rhythmic changes and response to drought stress in *Gossypium hirsutum* L.

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Background. The circadian clock not only participates in regulating various stages of plant growth, development and metabolism, but confers plant environmental adaptability to stress such as drought. Pseudo-Response Regulators (PRRs) are important component of the central oscillator (the core of circadian clock) and play a significant role in plant photoperiod pathway. However, no systematical study about this gene family has been performed in cotton.

Methods. *PRR* genes were identified in diploid and tetraploid cotton using bioinformatics methods to investigate their homology, duplication and evolution relationship. Differential gene expression, KEGG enrichment analysis and qRT-PCR were conducted to analyze *PRR* gene expression patterns under diurnal change and their response to drought stress.

Results. A total of 44 PRR family members were identified in four *Gossypium* species, with 16 in *G. hirsutum*, 9 in *G. barbadense* as well as in *G. arboreum* and 10 in *G. raimondii*. Phylogenetic analysis indicated that PRR proteins were divided into five subfamilies and whole genome duplication or segmental duplication contributed to the expansion of *Gossypium* PRR gene family. Gene structure analysis revealed that members in the same clade are similar, and multiple cis-elements related to light and drought stress response were enriched in the promoters of *GhPRR* genes. qRT-PCR results showed that *GhPRR* genes transcripts present four expression peaks (6 h, 9 h, 12 h, 15 h) during 24 hours and form obvious circadian waves. Transcriptome data with PEG treatment, along with qRT-PCR verification suggested that members of clade III (*GhPRR5a-d*) and clade V (*GhPRR3a* and *GhPRR3c*) may be involved in drought response. This study provides an insight into understanding the function of *PRR* genes in circadian rhythm and in response to drought stress in cotton.

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Abstract

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investigate their homology, duplication and evolution relationship. Differential gene expression, KEGG enrichment analysis and qRT-PCR were conducted to analyze *PRR* gene expression patterns under diurnal change and their response to drought stress.

Results. A total of 44 PRR family members were identified in four *Gossypium* species, with 16 in *G. hirsutum*, 9 in *G. barbadense* as well as in *G. arboreum* and 10 in *G. raimondii*. Phylogenetic analysis indicated that PRR proteins were divided into five subfamilies and whole genome duplication or segmental duplication contributed to the expansion of *Gossypium* PRR gene family. Gene structure analysis revealed that members in the same clade are similar, and multiple cis-elements related to light and drought stress response were enriched in the promoters of *GhPRR* genes. qRT-PCR results showed that *GhPRR* genes transcripts present four expression peaks (6 h, 9 h, 12 h, 15 h) during 24 hours and form obvious circadian waves. Transcriptome data with PEG treatment, along with qRT-PCR verification suggested that members of clade III (*GhPRR5a-d*) and clade V (*GhPRR3a* and *GhPRR3c*) may be involved in drought response. This study provides an insight into understanding the function of *PRR* genes in circadian rhythm and in response to drought stress in cotton.

Keywords: *Gossypium hirsutum*; PRR family; photoperiod; circadian rhythm; drought response

Introduction

The circadian clock is an autonomous endogenous biological rhythm that enables the living organisms to adapt to external daily and seasonal cycles, which play a significant role in plant growth and development for plant fitness (*Harmer, 2009*; *Hsu et al., 2014*; *Lee et al., 2005*; *McClung, 2006*; *Uehara et al.,* 2019) Although the circadian clock in different organisms is tissue-specific, most organisms have a conserved molecular mechanism - the core oscillator of positive and negative feedback loops formed at both the transcriptional and translational levels based on genome-wide gene expression regulation. (*Harmer, 2009*; *Hsu PY et al., 2014*; *Takata et al., 2009*; *Uehara et al., 2019*).Numerous studies have indicated that imperative roles for PRR gene family (PRR9, PRR7, PRR5, PRR3 and TOC1) in circadian clock (*Eriksson et al., 2003*; *Farre et al., 2005*; *Gould et al., 2006*; *Ito et al., 2009*; *Kaczorowski et al., 2003*; *Nakamichi et al., 2020*; *Salome et al., 2005*; *Yamamoto et al., 2003*).

In *Arabidopsis thaliana*, the gene expression and protein expression levels of PRR family members have obvious circadian rhythmic expression pattern (*Matsushika et al., 2000*). PRR proteins contain two domains,

the N-terminal contains a conserved REC (Response regulator receiver) domain, the C-terminus is a CCT domain, and CCT domain might interacts with CONSTITUITIVE PHOTOMOR-PHOGENIC 1 (COP1) to control CONSTANS (CO) protein stability (Jang *et al*., 2008). PRRs could interact with CO at specific times and stabilize CO expression during the day, which promoting the CO protein to bind the promoter of *FLOWERING LOCUS T* (*FT*), inducing FT expression and promoting flowering (*Hayama et al., 2017*; *Kobayashi et al., 1999*). Many studies showed that *PRRs* have circadian rhythmic expression levels in both transcriptional and protein levels, whether in continuous light or dark (*Más et al., 2003*; *Strayer et al., 2000*). Either in the *toc1* deletion mutant or *TOC1* overexpressing plants of *Arabidopsis thaliana*, the performance of the core oscillator has significant changes (*Huang et al., 2012*). Besides, PRR9, PRR7 and PRR5 could act as transcriptional repressors of CCA1 and LHY (*Nakamichi et al., 2010*).

At present, research mainly focuses on exploring the molecular mechanism of the photoperiod regulation pathway in *Arabidopsis thaliana*, and its regulation mechanism is becoming clear (*Song et al., 2013*; *Wang et al., 2013*; *Wickland et al., 2015*). Flowering time is an important factor affecting crop yield, thus dissection of photoperiod pathways regulating flowering time in crops and ornamental plants also becomes one of the hotspots in current researches (*Brambilla et al., 2017*; *Nakamichi et al., 2015*; *Yang et al., 2020*). However, molecular details concerning the photoperiodic control of crop flowering remain unclear. Only some studies on the cloning and function analysis of PRR genes have been carried out in crops currently, such as rice (*Oryza sativa*) (*Murakami et al., 2005*), wheat (*Triticum aestivum*) (*Nakahira et al., 1998*), barley (*Hordeum vulgare*) (*Turner et al., 2005*) and soybean (*Glycine max*) (*Liu et al., 2009*).

Flowering at the appropriate period has an essential effect on the fiber yield and quality of cotton, and there were only a few studies on genes related to flowering regulation in cotton (*Gossypium spp*.) (*Cai et al., 2017*; *Zhang et al., 2016*). With the completion of the genome sequencing of *Gossypium* species (*Hu et al., 2019*; *Huang et al., 2020*; *Li et al., 2015*; *Wang et al., 2012*; *Wang et al., 2018*; *Yuan et al., 2015*; *Zhang et al., 2015*), the identification of new genes and the establishment of a new regulatory model would be helpful for studying the function of genes involved in cotton flowering pathway.

In addition, the biological clock plays a vital role in adapting to external environmental stress, such as drought stress. In soybeans, studies shown that drought stress affects the expression of circadian clock genes,

and the expression of drought-responsive genes also has circadian rhythm (*Gomes et al., 2014*). TOC1 has been shown to directly bind to the ABAR promoter region and regulate the periodic expression of ABAR, while ABA can up regulate TOC1.Therefore, TOC1 is considered to act as a molecular switch between the drought stress signaling pathway and the biological clock (*Legnaioli et al., 2009*).

Here, we identified 44 *PRR* genes from the four *Gossypium* species, and conducted basic bioinformatics analysis. We also investigated the periodic expression pattern of *PRR* family members at the transcriptional level during 24 hours. Further, we identified six *PRR* members respond to drought stress by analyzing transcriptome data with PEG treatment along with qRT-PCR verification. This study lays a foundation for studying the molecular mechanism of cotton photoperiod regulation and also provides an insight into understanding *PPRs* gene function in response to drought stress in cotton.

Materials & methods

Identification of PRR gene family in *Gossypium spp.*

The domain numbered PF00072 (Response regulator receiver domain) and PF06203 (CCT motif) in the Pfam database (*EI-Gebali et al., 2019*) are the domain of plant light signal transcription factor. Firstly, the genome-wide information files of *G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. raimondii* are downloaded from the cotton genome database (http://mascotton.njau.edu.cn/info/1054/1118.htm).Then, candidate sequences REC and CCT domains were aligned and searched in HMMER 3.0 (https://www.ebi.ac.uk/Tools/hmmer/) (*Jacob et al., 2007*) and the BLAST program. Next, the sequences obtained were searched and verified on the Conserved Domain database CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART (http://smart.embl-heidelberg.de/) (*Letunic et al., 2002*), to determine its conserved domain.

Finally, the whole genome sequence of *Arabidopsis thaliana* (*Initiative et al., 2000*), Cocoa (*Theobroma cacao*) (*Argout et al., 2011*) and rice (*Oryza sativa*) (*Yu et al., 2005*) were loaded from the Arabidopsis database TAIR10 (https://www.arabidopsis.org/), the plant genome database Phytozome 12 (http://phytozome.jgi.doe.gov/pz/portal.html) and EnsemblPlants (http://plants.ensembl.org/index.html), respectively. Finally, the online site ExPASy Proteomics Server (http://www.expasy.org/) and Softberry(http://linux1.softberry.com/berry.phtml?topic=protcomppl &group=programs&subgroup=proloc) were used to analyze the physicochemical properties of the identified cotton PRR gene family, including

amino acid number, nucleotide data, molecular weight, isoelectric point prediction and subcellular localization.

Chromosomal locations, duplications, and synteny analysis of PRR gene members

Chromosomal location information for *PRR* genes was obtained from a gff3-file of each cotton genomic databases and genes were mapped on the chromosomes using TBtools (https://github.com/CJ-Chen/TBtools). Then MCScanX (*Wang et al., 2012*) was used to determine and analyze cotton *PRR* duplication and collinearity, Circos (http://circos.ca/) and Adobe illustrator CS5 software were used to conducted image showing gene location and gene homology relationship.

Phylogenetic analyses and gene structure organization of the PRR proteins in *Gossypium spp.*

To analyze evolutionary relationship, multi-protein sequence alignment of the PRR proteins were aligned using MEGA7 (*Sudhir et al., 2016*), and constructed a phylogenetic tree with neighbor-joining (NJ) method, with the bootstrap 1000. Finally, the evolutionary tree is visualized and beautified by the online software iTOL (https://itol.embl.de/) (*Letunic et al., 2019*).

SeqHunter1.0 (*Ye et al., 2010*) and TBtools software were used to extract *PRR*s gene families GFF3 files from upland cotton genome sequencing file. Location information and the predicted CDS sequence of the *PRR* members were obtained**,** and presenting the gene structures in the online software Gene Structure Display Server (GSDS 2.0) (http://gsds.cbi.pku.edu.cn/index.php) (*Guo et al., 2007*), Then we performed motifs analysis by the online software MEME (http://meme-suite.org/) (*Bailey et al., 2009*) with following parameters: the maximum number discovered for the motif is set 1 to 10, and the other parameters are default values. The graphic display is based on the Amazing optional gene viewer section in the software TBtools.

To identify the cis-elements in the promoter sequences of the 16 PRR family genes in *G. hirsutum*, the 2000 bp of genomic sequences upstream of the start codon of each *PRR* gene were submitted to the online site PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to predict the cis-element type, and the results are displayed by the Simple Bio Sequence Viewer in TBtools.

Plant materials and treatment

The upland cotton (*G. hirsutum*) accession (Lumianyan 19, LMY 19) selected in this study were kept in our laboratory, planted in growth chamber (day/night temperature cycle of 28°C light/25 °C dark with a 12-photoperiod), and samples were picked every 3h from leaf in three-true-leaves stage. Germinated TM-

1 cotton seeds were planted in the same photoperiod and temperature environment as LMY19, and treated with 400 mM PEG at the three-leaf stage, samples collected at 0, 1, 3, 6 h respectively. Three biological replicates of each sample were performed and all samples were freezed with liquid nitrogen immediately and stored at -80 °C for qRT-PCR.

RNA isolation and qRT-PCR analysis

The RNA was extracted from the samples using the Rapid Universal Plant RNA Extraction Kit (Huayueyang Biotechnology Co. Ltd.), and the Prime Scrip First Strand cDNA Synthesis Kit (Takara) used for reverse transcription, SYBR Premix Ex Taq II. (Takara) kit used for real-time PCR experiment, qRT-PCR analysis was carried out using SYBR Green on the Roche LightCycler® 480 II. The primers of PRR gene family were designed using Primer Premier 5.0 software and listed in table S1, and the actin gene (AF059484) was selected as the internal reference gene (*Zhang et al., 2013*). The volume of the qRT-PCR reaction was 20 μL, and the amplification procedure was as follows: pre-denaturation at 95 ℃ for 30 s; denaturation at 95 ℃ for 5 s, annealing at 60℃ for 30 s, 40 cycles. Three biological and technical replicates were performed for the qRT-PCR tests. The relative gene expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method (*Livak et al., 2001*).

Expression patterns and pathway enrichment analysis of PRR members

The transcriptome data sets of *G. hirsutum* TM-1 were downloaded from SRA database (PRJNA490626) in NCBI (https://www.ncbi.nlm.nih.gov/sra/) (*Zhang et al., 2015*), and the accession numbers are SRS824360, SRS824361, and SRS824362 respectively. The gene expression pattern of *PRR* genes were displayed by R/heatmap with the expression values normalized by $\log_2$(FPKM+1) and the differentially expressed genes (DEGs) were identified using the R/mpoly (*Kahle et al., 2013*). KEGG analyses of DEGs were conducted in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for enrichment (*Kanehisa et al., 2014*), KEGG enrichment of DEGs was evaluated with KOBAS2.0 software (*Xie et al., 2011*) and bubble graph was displayed by R/ggplot2.

Results

Genome-wide identification of PRR family genes in *Gossypium spp.*

Based on multiple sequence alignment analysis, complete *PRR* genes were identified in four *Gossypium* species, including 16 in *G. hirsutum* (AD_1), 9 in *G. arboretum* (A_2), 10 in *G. raimondii* (D_5), and 9 in *G.*

barbadense (AD_2). Additionally, we proceeded with *PRR* genes retrieved from plant genome database, with 5 in *Arabidopsis* (dicots), 5 in *rice* (monocots), and 6 in *cocoa* (dicot). All of them were renamed based on the homologous genes in Arabidopsis (Table S2). The number of *PRR* gene family in *G. hirsutum* was about twice as that in *G. arboreum* (A group) or *G. raimondii* (D group), consistent with they are tetraploid and diploid genomes respectively. The basic information of *PRR* genes including protein sequence length, isoelectric points, and molecular weight in cotton were listed in Table S3. The predicted GhPRR proteins ranged from 553 (GhPRR1b) to 796 (GhPRR3a) amino acids, with isoelectric points changed from 5.16 (GhPRR9a) to 8.54 (GhPRR7c) and molecular weight from 61.87 kDa (GhPRR1a) to 85.93 kDa (GhPRR3a).

Chromosomal locations, duplications, and synteny analysis of PRR gene members

In order to display the chromosome distribution of *PRR* genes, mapping them on the corresponding chromosome. Eight of *GhPRR* genes were located on chromosomes of At sub-genome while five of *GhPRR* genes were on that of Dt sub-genome and three *GhPRR* genes were present in different scaffolds (Fig.S1). We further conducted whole genome collinearity analysis of 44 identified *PRR* genes in cotton, and explored the locus relationships between At and Dt sub-genomes as well as with A and D diploid cotton genomes (Fig.1A, Table S4). There are 34 orthologous gene pairs were resulted from whole genome duplication or segmental duplication among *Gossypium spp.*.Whole Genome duplication or segmental duplication was suggested to be the main causes of PRR gene family expansion in cotton (Table S5).

Phylogenetic analyses and gene structure organization of the PRR proteins in *Gossypium spp.*

To investigate the evolutionary relationship of GhPRR proteins among mentioned seven species, phylogenetic tree was constructed (Fig.1B). The PRR family of *Gossypium* was divided into 5 subgroups (clade I-V). There were 13 PRRs in Clade III (three GaPRRs, GbPRRs and GrPRRs respectively, four GhPRRs) and 11 PRRs (one GrPRR, two GaPRRs, four GbPRRs and GhPRRs individually) in clade IV. Clade I consisted of 9 PRRs (one GaPRR, two GbPRRs and GhPRRs singly, four GrPRRs), Clade V contained 7 PRRs (one GrPRR, two GaPRRs and four GhPRRs) and Clade II had 4 PRRs (one GaPRR and GrPRR respectively, two GhPRRs). GhPRRs were distributed throughout five subgroups (clade I-V), clade-I, clade-II and clade-IV containing PRRs from monocots and dicots simultaneously, illustrating that evolution of *GhPRR* genes in three clades occurred before the separation of monocots and dicots.

PRRs protein in *G. hirsutum* was also divided into five subgroups (Fig. 2A), consistent with phylogenetic analyses. The motif distribution indicated that the order, size, and location of the motifs in the same subgroup were similar, but there were significant variety between different subgroups. Among them, 37.5% of the family members have the same sequence of motif structure: motif 4_9_3_1_7_5_6_10_8_2, while Clade-I contains the least number of motifs with only 5 motifs. All members of the PRR gene family contain motif1, motif2, motif3, motif4 and motif6, which are the conserved motifs of PRR family. In addition, the gene structure analysis exhibited that the distribution of introns and exons were similar among different subgroups, and the functional elements REC and CCT were distributed in both end side of each gene. All of member contained three REC structure elements, and most member contain two CCT domains, except that two members of the Clade-I subgroup contain one CCT domain.

To further analyze the transcriptional regulation and potential function of the *PRR* genes, the cis-elements in the promoter region were predicted (Fig. 2B).The results displayed that there are abundant regulatory elements existing in the promoter region, mainly focused on light response elements (G-Box, GT1-motif and TCT-motif, etc.), hormone responsive elements: abscisic acid response (ABRE), MeJA-response (CGTCA-motif and TGACG-motif), gibberellin-responsive element (TATC-box, P-box and GARE-motif), and stress responsive elements: drought-inducibility (MBS), low-temperature response (LTR), etc. These results implied that the expression of *GhPRR* genes may be induced by light, abscisic acid and drought stress.

The periodic expression pattern of PRR members under diurnal change

A feature shared by many clock gene transcripts is that their abundance is subject to diurnal oscillation. To analyze the peak transcripts of *GhPRR*s under diurnal cycle, the relative expression levels of *GhPRRs* together with its related genes (*GhFT* (*Gh_A08G2015*), *GhCO* (*Gh_D12G0543*), *GhLHY* (*Gh_D11G1068*) and *GhCCA1* (*Gh_A05G2124*)) under circadian rhythm was detected by qRT-PCR (Fig. 3), the results presented that the expression of *GhPRR* genes reached the peak every 3 hours after 6 hours of light condition, and there were multiple members at each peak. *GhFT*, *GhCO*, and *GhLHY* had the peak expression at 3 hours of light. Subsequently, members include-II (*GhPRR9a* and *GhPRR9b*) and clade-IV (*GhPRR7a*, *GhPRR7b*, *GhPRR7c* and *GhPRR7d*) reached the expression peak after 6 hour of light condition, and then members in clade III (*GhPRR5a*, *GhPRR5b*, *GhPRR5c* and *GhPRR5d*) and clade-V (*GhPRR3a* and *GhPRR3c*) at 9 hour, another two members of clade-V (*GhPRR3b* and *GhPRR3d*) at 12 hour. Finally, members (*GhPRR1a* and *GhPRR1b*)

in clade-I reached expression peak at night. Additionally, the expression of *GhLHY* and *GhPRR1b* always showed an opposite trend during 24 hours, it can be speculated that a mutual inhibition maybe exist between the two genes. This results indicated that expression of *GhPRR* genes has obvious circadian waves during 24 hours.

Identification of drought-stress related PRR genes in *G. hirsutum*

To investigate the roles for PRR genes in response to drought, we investigated the expression profile of *GhPRRs* under polyethylene glycol (PEG) treatment at 1, 3 and 6 h from the published transcriptome data sets. We selected the group data at 6 h treated with PEG for KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis for it having the largest number (848) of significantly up-regulated genes (Fig.4A).The results revealed that the up-regulated genes are mainly involved in circadian rhythm, photosynthesis, starch and sucrose metabolism, etc. (Fig.4B). Six of *GhPRR* genes including four members in clade III (*GhPRR5a-d*) and two in clade-V (*GhPRR3a* and *GhPRR3c*) were involved in circadian rhythm pathway. The expression pattern of *PRR* genes under PEG treatment showed that these six members have same expression trend and high expression level at 6 h with PEG treatment (Fig. 4C and Table S6), suggesting these *PRR* genes are significantly induced by PEG treatment.

To further prove the expression changes of these genes at different time of PEG treatment (1h, 3h, 6 h), the expression level of all member of PRR family were detected by qRT-PCR (Fig.5). All PRR genes displayed almost the similar expression changes compared with transcriptome data sets. The expression of the above mentioned genes (*GhPRR5a-d*, *GhPRR3a* and *GhPRR3c*) at the sixth hour after PEG treatment was significantly higher than that of the blank control. Especially, the expression level of *GhPRR3c* gene is almost nine times higher than that of the blank control, suggesting these genes may be involved in drought response.

Discussion

Light, one of the vital environmental factors, plays a significant role in promoting plant growth and development. Especially, with the alternating of sunrise and sunset, plants form a unique biological clock to regulate the growth and metabolic activities, like regulation of flowering time (*Hayama et al., 2017*; *Song et al., 2015*), hypocotyl elongation (*Seaton et al., 2015*; *Soy et al., 2016*; *Zhu et al., 2016*), biotic (*Bhardwaj et al., 2011*; *Korneli et al., 2014*; *Zhang et al., 2013*) and abiotic (*Keily et al., 2013*; *Nakamichi et al., 2009*) stress

response, and so on. Circadian clock related genes regulate many vital agronomic traits, especially in the photoperiod pathway of plants. There are a few studies about *PRR* genes in cotton (*Gossypium spp*.), however, advances in cotton genomics and genetics recent years allowed us to perform a systematic study on *PRR* genes and to probe their potential functions in circadian clock. This study will provide basic information and for further investigation of cotton *PRR* gene functions.

Here, 16 *GhPRR* genes were identified totally in *G. hirsutum*, which are homologous with 5 PRR genes in *Arabidopsis* (*PRR1* (*TOC1*), *PRR3*, *PRR5*, *PRR7*, *PRR9*). According to chromosomal localization and genomic collinearity analysis, it can be speculated that due to the hybridization of A and D subgenome in the *G. hirsutum*, the gene amplification is carried out by tandem repeat and fragment replication, resulting in more family members (*Jackson et al., 2010*). There is a high degree of collinearity between the genes of the A subgenome and the D subgenome of the tetraploid *G. hirsutum* (*Li et al., 2015*). In this study, 14 (7 pairs) of 16 PRR members are orthologous genes, indicating that *G. hirsutum* has undergone large-scale gene rearrangement at the genomic level during species formation, which is consistent with the results of the allotetraploid *G. hirsutum* genome (*Wang et al., 2018*; *Li et al., 2015*; *Zhang et al., 2015*).

A large number of experimental studies have been carried out about circadian clock in *Arabidopsis* (*Alabadí et al., 2001*; *Más et al., 2003*; *Legnaioli et al., 2009*). PRRs proteins interact with CCA1 and LHY through complex mechanisms, playing a vital role in the growth and development, flowering induction and metabolic regulation of plants (*Harmer, 2009*; *Legnaioli et al., 2009*; *Mizuno et al., 2005*). In addition to confirming the regulation of flowering by the circadian clock in model plants, the study of circadian clock mechanism has been carried out in crops recently. The function of some circadian clock-related genes has been cloned and verified based on gene homology in major crops, such as rice, soybean (*Gome et al., 2014*; *Xue et al., 2012*; *Yang et al., 2013*). In rice, overexpressing *OsPRR37* weakens the transcriptomic rhythms and alters the phases of rhythmic genes. So far, circadian clock regulation mechanism in cotton is still a mystery, only one study has identified *Gh_D03G0885* (*GhPRR1b*) as a candidate gene for cotton early maturity traits using genotyping-by-sequencing (*Li et al., 2017*). TOC1 (known as Pseudo Response Regulator, PRR1) is an important component of the core oscillator and closed positive and negative feedback loop with LHY (Late elongated hypocotyl) and CCA1 (circadian clock associated 1), formulating the basic framework of the *Arabidopsis* circadian clock core

oscillator (*Alabadí et al., 2001*; *Gendron et al., 2012*; *Huang et al., 2012*). As an inhibitor of circadian clock gene expression, TOC1 gene can inhibit the expression of most circadian clock core genes, and affect flowering pathway of photoperiod regulation by controlling the function of circadian clock (*Strayer et al., 2000*; *Pokhilko et al., 2012*). However, the function and regulatory pathways involved of this gene have not been reported in cotton.

Further, qRT-PCR analysis revealed that the relative expression of PRR members had apparent circadian waves among 24 hours, which similar with that of PRR members *(PRR1/TOC1, PRR3, PRR5, PRR7, PRR9)* in *Arabidopsis*. Transcript expression peaks appear in the order of *PRR9*, *PRR7*, *PRR5*, *PRR3* and *TOC1* (*PRR1*) in *Arabidopsis* (*Matsushika et al., 2000*), while four expression peaks appeared in this study and there were multiple members at each peak, speculating that it is related to chromosome doubling in the process of forming allotetraploid in *G. hirsutum* (*Jackson et al., 2010*). The *PRR1a* gene had the last peak of expression and highly expressed at night, which consistent with that of *APRR1* in *Arabidopsis* (*Caluwé et al., 2016*), while *GhPRR1b* has two peak of expression at night in this study. Therefore, detailed study should be carried out about this the gene in cotton.

In cotton, the expression peak of the members of the *GhPRR* gene family follows the expression peak of *GhLHY*, which is consistent with the results in *Arabidopsis* (*Mizuno et al., 2005*). *GhPRR1b* and *GhLHY* have opposite expression trends among 24 hours, and the *GhPRR1b* gene has high homology with *PRR1* in Arabidopsis thaliana by alignment, so it is speculated that *GhPRR1b* is the core component of the circadian clock in *G. hirsutum*, and there is maybe a mutual inhibition between *GhPRR1* and *GhLHY*. As an important factor in the export pathway of the circadian clock, CO protein has been proved in *Arabidopsis* to confirm the stability of PRRs protein-mediated CO expression, and can enhance the binding of CO to *FT* promoter, then *FT* start transcribe and promote flowering (*Jang et al., 2008*). The pathway of PRRs family members mediate the stability of CO expression still needs further experiments in cotton.

In addition, there are many studies focus on the response of circadian clock to abiotic stress in crops (*Flowers et al., 2004*; *Lu et al., 2017; Zhang et al., 2020*). TOC1 can bind to the *ABAR* promoter of ABA-related genes and regulate its circadian rhythm expression, and can be thought to act as molecular switches between drought stress signaling pathways and circadian clocks in *Arabidopsis* (*Legnaioli et al., 2009*). In

soybeans, studies have also shown that drought stress affects the expression of circadian clock genes, and the expression of drought-responsive genes also has circadian rhythm (*Gomes et al., 2014*). Based on these researches, this study identified 16 PRR members in cotton and analyzed the expression pattern of PRR genes among 24 hours and in response to drought stress, the result showed that PRR members expression display obvious circadian waves and six of them may be involved in responding to drought stress, which is helpful to understand the evolution and function of the PRRs gene family, and provide thoughts and clues for further study the function of the PRR gene family in cotton.

Conclusions

In this study, we identified 44 *PRR* genes in cotton (*Gossypium spp.*) and classified them into 5 subgroups based on the phylogenetic tree. Then comprehensively and systematically analyzed PRRs in cotton (*Gossypium spp*.), including the domains, the gene structure, promoter cis-acting element, chromosome localization distribution and collinearity analysis. In addition, we also investigated the evolutionary relationship of PRRs among *G. hirsutum, G. barbadense, G. arboreum* and *G. raimondii*, *Arabidopsis thaliana*, *Theobroma cacao* and *Oryza sativa*. Moreover, qRT-PCR results showed that the expression of members of PRRs family has obvious circadian waves, and Gene differential expression and KEGG enrichment analysis of the transcriptome data with PEG treatment, along with qRT-PCR verification altogether demonstrated members of clade III (*GhPRR5a-d*) and two members of clade-V (*GhPRR3a* and *GhPRR3c*) are significantly induced by PEG treatment, so it is speculated that these *GhPRR* genes may be involved in drought response. This study will provide a theoretical basis for studying the function of *PRRs* in cotton.

Availability of data and materials

All related data are available within the manuscript and its additional files. The RNA sequences raw data was downloaded from the SRA database, National Center for Biotechnology Information (NCBI) under the accession numbers (PRJNA490626).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1 Phylogenetic and collinearity analysis of PRR proteins in cotton.

(A) Phylogenetic tree of the *PRR* gene family; (B) Gene duplication and collinearity analysis among cotton *PRR* genes (green lines and brown indicates paralogous genes in *G. hirsutum* and *G. barbadense*, orange lines indicates orthologous genes between *G. arboreum* and *G. hirsutum*, black lines indicates orthologous genes between *G. arboreum* and *G. raimondii*, blue lines indicates orthologous genes between *G. barbadense* and *G. hirsutum*, seagreen indicates orthologous genes between *G. arboreum* and *G. barbadense*, lightsteelblue indicates orthologous genes between *G. barbadense* and *G. raimondii*, yellow indicates orthologous genes between *G. raimondii* and *G. hirsutum*).

Figure 2 Genetic structure and motif prediction of PRR members.

(A) Genetic structure of *GhPRR* genes and motif prediction of GhPRR proteins; (B) Cis-elements prediction of *GhPRR* promoters.

Figure 3 Periodic xpression pattern of PRR gene family and related genes during 24 hours in LMY19.

Figure 4 Expression analysis and KEGG enrichment of *PRR* genes.

(A)Differential expression genes analysis; (B) KEGG pathway enrichment of DEGs PEG_6 h group;

(C)Expression pattern of *GhPRR* genes with PEG treated at 1, 3 and 6 h.

DEGs: differentially expressed genes.

Figure 5 qRT-PCR analysis of *PRR* genes under PEG treatment at 1, 3, 6 h. The different lowercase letters indicate significant differences at $p<0.05$. Differences analysis were compared using one-way ANOVA and Duncan test.

Supplemental Information

Figure S1 Distributions of the PRR family genes on chromosomes in *Gossypium spp*. (A) Distributions of *GaPRR* genes on chromosomes in *G. arboretum*; (B) Distributions of *GbPRR* genes on chromosomes in *G. barbadense*; (C) Distributions of *GhPRR* genes on chromosomes in *G. hirsutum*; (D) Distributions of *GrPRR* genes on chromosomes in *G. raimondii*.

Table S1 qRT-PCR primers for *GhPRR* and related genes used in this study.

Table S2 Rename information of *PRR* genes.

Table S3 The general information of the PRR gene family.

632 **Table S4 Orthologous relationships among four *Gossypium* species.**

633 **Table S5 The duplication of PRR gene pairs in *Gossypium spp*.**

634 **Table S6 FPKM values of *GhPRR* genes.**

635

636

Figure 1

Phylogenetic and collinearity analysis of PRR proteins in cotton.

(A) Phylogenetic tree of the *PRR* gene family; (B) Gene duplication and collinearity analysis among cotton *PRR* genes (green lines and brown indicates paralogous genes in *G. hirsutum* and *G. barbadense*, orange lines indicates orthologous genes between *G. arboreum* and *G. hirsutum*, black lines indicates orthologous genes between *G. arboreum* and *G. raimondii*, blue lines indicates orthologous genes between *G. barbadense* and *G. hirsutum*, seagreen indicates orthologous genes between *G. arboreum* and *G. barbadense*, lightsteelblue indicates orthologous genes between *G. barbadense* and *G. raimondii*, yellow indicates orthologous genes between *G. raimondii* and *G. hirsutum*).

Figure 1

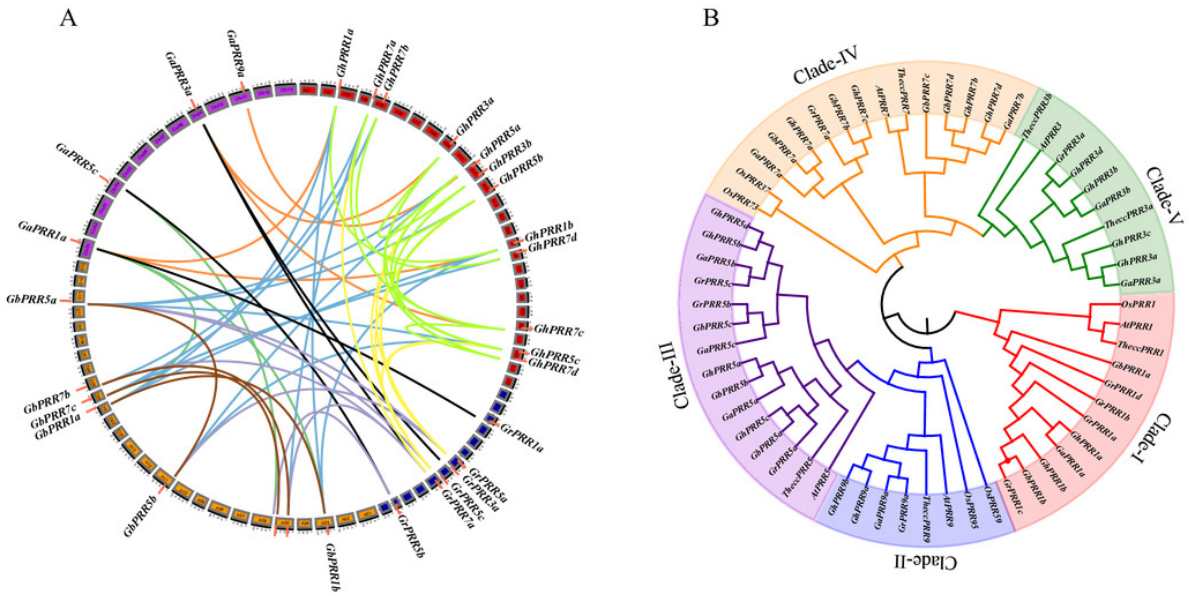


Figure 2

Genetic structure and motif prediction of PRR members.

(A) Genetic structure of *GhPRR* genes and motif prediction of GhPRR proteins; (B) Cis-elements prediction of *GhPRR* promoters.

Figure 2

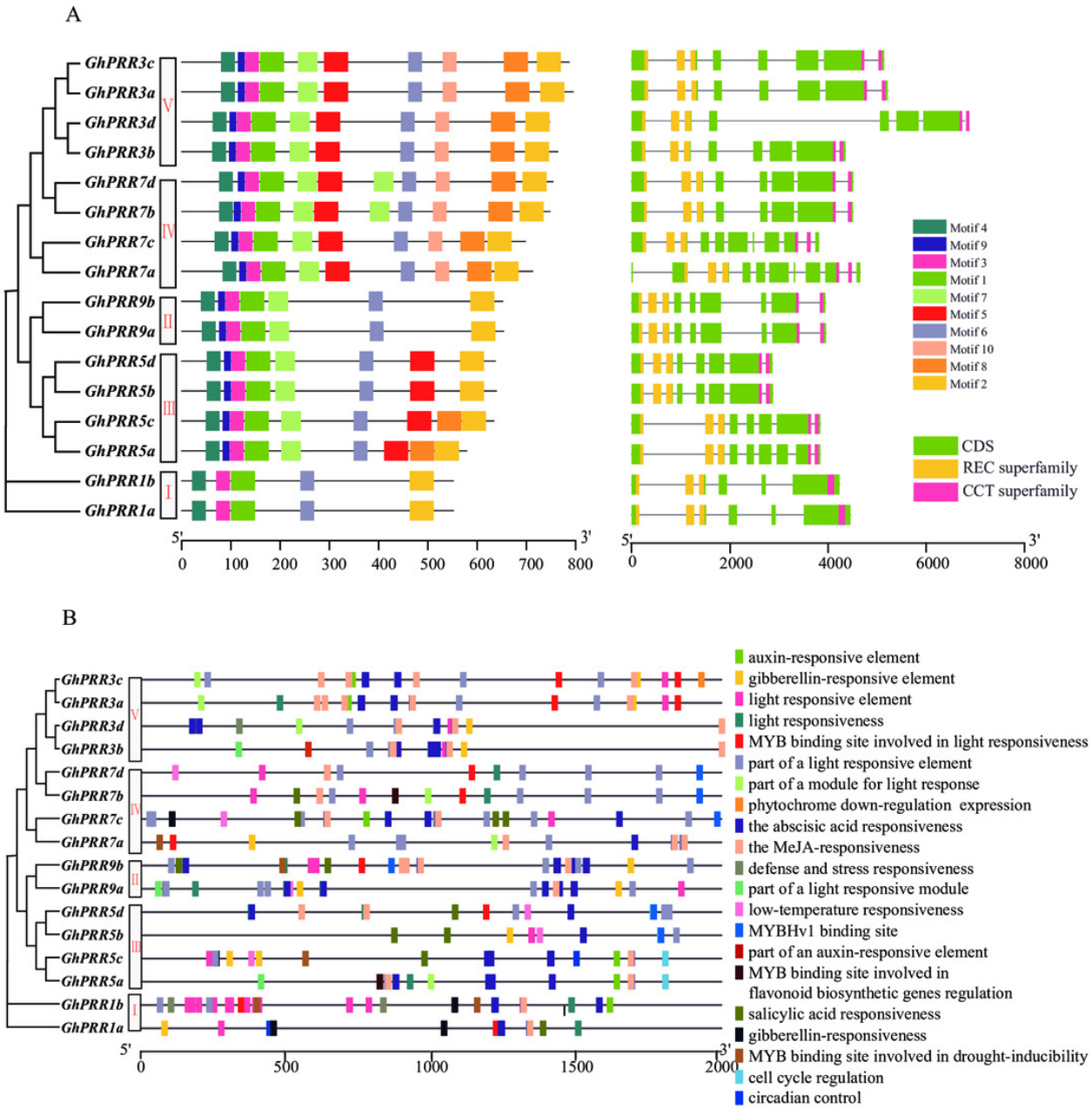

A
GhPRR3c
GhPRR3a
GhPRR3d
GhPRR3b
GhPRR7d
GhPRR7b
GhPRR7c
GhPRR7a
GhPRR9b
GhPRR9a
GhPRR5d
GhPRR5b
GhPRR5c
GhPRR5a
GhPRR1b
GhPRR1a
V
IV
II
III
I
5'
3'
0 100 200 300 400 500 600 700 800
0 2000 4000 6000 8000
Motif 4
Motif 9
Motif 3
Motif 1
Motif 7
Motif 5
Motif 6
Motif 10
Motif 8
Motif 2
CDS
REC superfamily
CCT superfamily
B
0 500 1000 1500 2000
auxin-responsive element
gibberellin-responsive element
light responsive element
light responsiveness
MYB binding site involved in light responsiveness
part of a light responsive element
part of a module for light response
phytochrome down-regulation expression
the abscisic acid responsiveness
the MeJA-responsiveness
defense and stress responsiveness
part of a light responsive module
low-temperature responsiveness
MYBHv1 binding site
part of an auxin-responsive element
MYB binding site involved in flavonoid biosynthetic genes regulation
salicylic acid responsiveness
gibberellin-responsiveness
MYB binding site involved in drought-inducibility
cell cycle regulation
circadian control

Figure 3

Periodic xpression pattern of PRR gene family and related genes during 24 hours in LMY19.

Figure 3

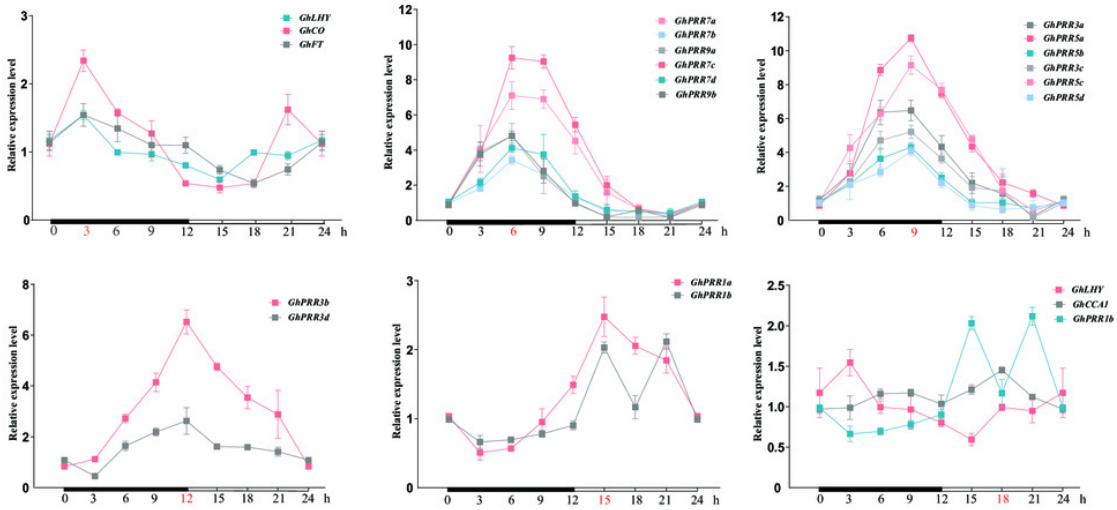

Relative expression level
GhLHY
GhCO
GhFT
GhPRR7a
GhPRR7b
GhPRR9a
GhPRR7c
GhPRR7d
GhPRR9b
GhPRR3a
GhPRR5a
GhPRR5b
GhPRR3c
GhPRR5c
GhPRR5d
GhPRR3b
GhPRR3d
GhPRR1a
GhPRR1b
GhLHY
GhCCA1
GhPRR1b
0 3 6 9 12 15 18 21 24 h

Figure 4

Expression analysis and KEGG enrichment of *PRR* genes.

(A)Differential expression genes analysis; (B) KEGG pathway enrichment of DEGs PEG_6 h group; (C)Expression pattern of *GhPRR* genes with PEG treated at 1, 3 and 6 h.

Figure 4

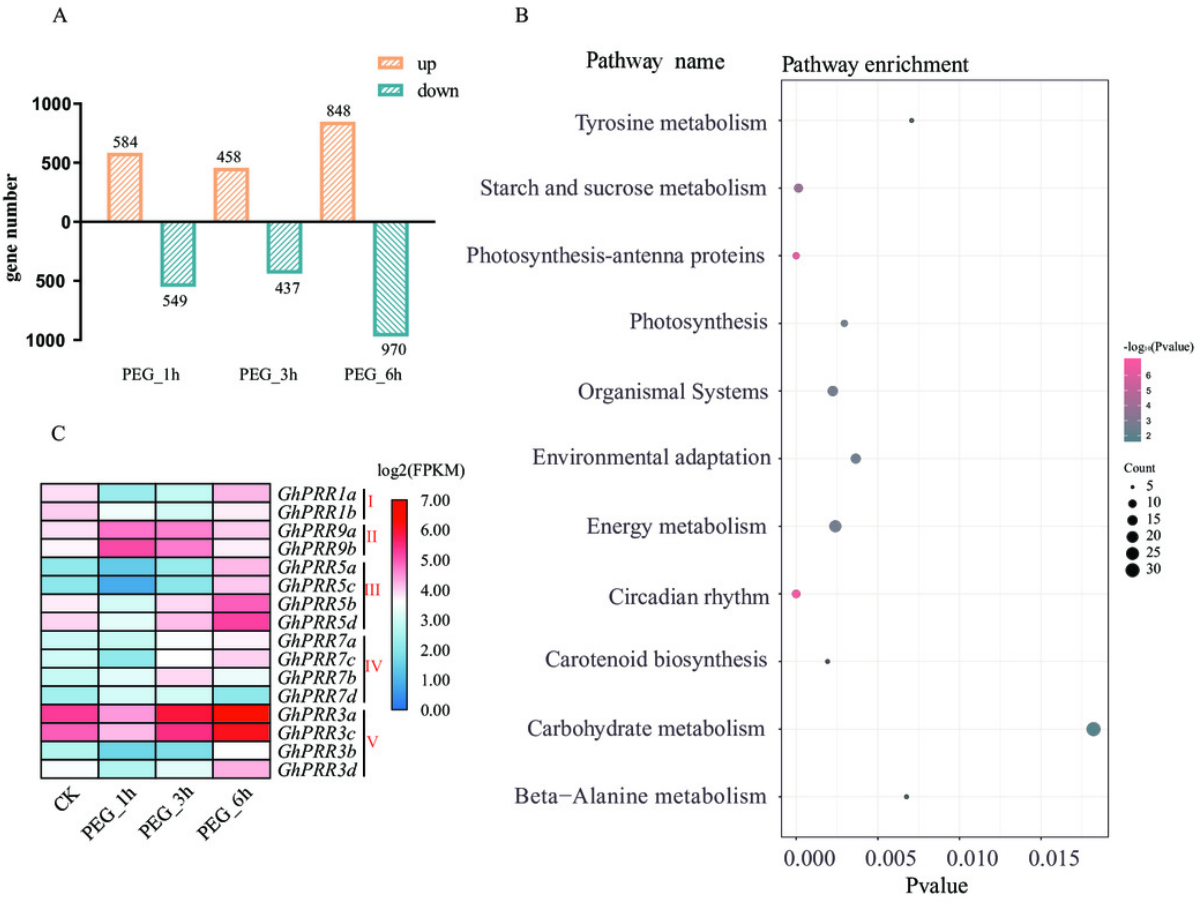
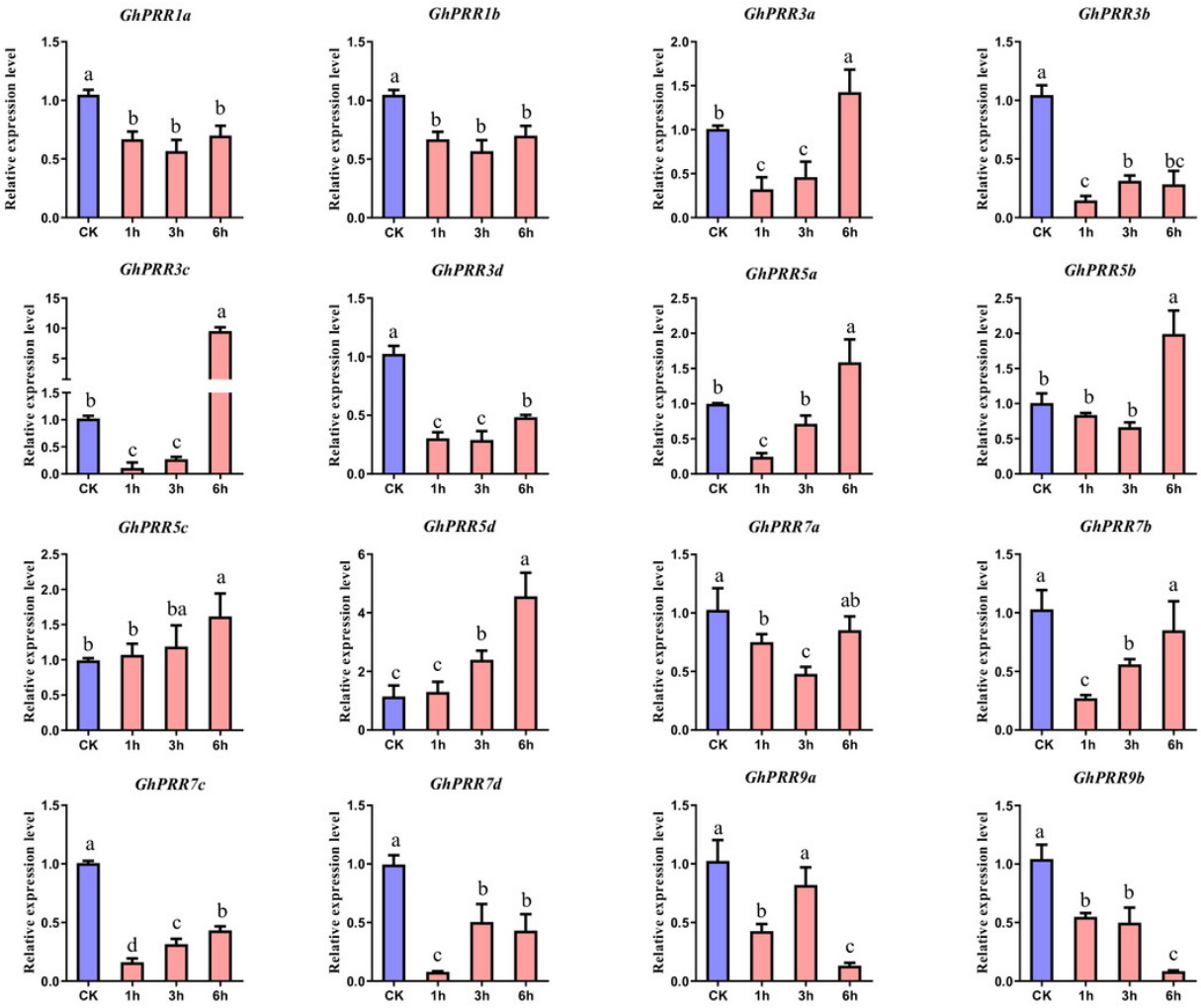

A
up
down
gene number
1000
500
0
500
1000
584
549
458
437
848
970
PEG_1h
PEG_3h
PEG_6h
B
Pathway name
Pathway enrichment
Tyrosine metabolism
Starch and sucrose metabolism
Photosynthesis-antenna proteins
Photosynthesis
Organismal Systems
Environmental adaptation
Energy metabolism
Circadian rhythm
Carotenoid biosynthesis
Carbohydrate metabolism
Beta−Alanine metabolism
0.000
0.005
0.010
0.015
Pvalue
-log10(Pvalue)
Count
5
10
15
20
25
30
C
log2(FPKM)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
GhPRR1a
GhPRR1b
GhPRR9a
GhPRR9b
GhPRR5a
GhPRR5c
GhPRR5b
GhPRR5d
GhPRR7a
GhPRR7c
GhPRR7b
GhPRR7d
GhPRR3a
GhPRR3c
GhPRR3b
GhPRR3d
I
II
III
IV
V
CK
PEG_1h
PEG_3h
PEG_6h

Figure 5

qRT-PCR analysis of *PRR* genes under PEG treatment at 1, 3, 6 h.

The different lowercase letters indicate significant differences at $p<0.05$. Differences analysis were compared using one-way ANOVA and Duncan test.

Figure 5


GhPRR1a
GhPRR1b
GhPRR3a
GhPRR3b
GhPRR3c
GhPRR3d
GhPRR5a
GhPRR5b
GhPRR5c
GhPRR5d
GhPRR7a
GhPRR7b
GhPRR7c
GhPRR7d
GhPRR9a
GhPRR9b
Relative expression level
CK
1h
3h
6h