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Genome-wide investigation and expression pattern of PHR family genes in cotton under low phosphorus stress

Yan Zhao [1], **Peiyu Li** [1], **Huarui Wang** [1], **Jiping Feng** [1], **Yuxin Li** [1], **Shanshan Wang** [1], **Yuanjie Li** [1], **Yanyan Guo** [1], **Lin Li** [1], **Yao Su** [Corresp., 1], **Zhengwen Sun** [Corresp. 1]

[1] State Key Laboratory of North China Crop Improvement and Regulation, Key Laboratory for Crop Germplasm Resources of Hebei, Hebei Agricultrual University, Baoding, China, China

Corresponding Authors: Yao Su, Zhengwen Sun
Email address: suyao@hebau.edu.cn, nxszhw@hebau.edu.cn

Phosphorus starvation response (PHR) protein is an important transcription factor in phosphorus regulatory network, which plays an important role in regulating the effective utilization of phosphorus. So far, the PHR genes have not been systematically investigated in cotton. In the present study, we have identified 22, 23, 41 and 42 PHR genes in *G. arboreum*, *G. raimondii*, *G. hirsutum* and *G. barbadense*, respectively. Phylogenetic analysis showed that cotton PHR genes were classified into five distinct subfamilies. The gene structure, protein motifs, and gene expression were further investigated. The PHR genes of *G. hirsutum* in the same subfamily had similar gene structures, all containing Myb_DNA-binding and Myb_CC_LHEQLE conserved domain. The structures of paralogous genes were considerably conserved in exons number and introns length. The cis-element prediction in their promoters showed that genes were not only regulated by light induction, but also were related to auxin, MeJA, abscisic acid-responsive elements, while some genes might be regulated by miRNA. The expression analysis showed that the *GhPHR* genes are differentially expressed in different tissues under various stresses. Furthermore, *GhPHR6*, *GhPHR11*, *GhPHR18* and *GhPHR38* were significantly changed after low phosphorus stress. The results of this study provide a basis for further cloning and functional verification of genes related to regulatory network of low phosphorus tolerance in cotton.

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State Key Laboratory of North China Crop Improvement and Regulation, Key Laboratory for Crop Germplasm Resources of Hebei, Hebei Agricultural University, Baoding 071001, China

*Correspondence: nxszhw@hebau.edu.cn

†These authors contributed equally to this work.

Abstract: Phosphorus starvation response (PHR) protein is an important transcription factor in phosphorus regulatory network, which plays an important role in regulating the effective utilization of phosphorus. So far, the PHR genes have not been systematically investigated in cotton. In the present study, we have identified 22, 23, 41 and 42 PHR genes in *G. arboreum*, *G. raimondii*, *G. hirsutum* and *G. barbadense*, respectively. Phylogenetic analysis showed that cotton PHR genes were classified into five distinct subfamilies. The gene structure, protein motifs, and gene expression were further investigated. The PHR genes of *G. hirsutum* in the same subfamily had similar gene structures, all containing Myb_DNA-binding and Myb_CC_LHEQLE conserved domain. The structures of paralogous genes were considerably conserved in exons number and introns length. The cis-element prediction in their promoters showed that genes were not only regulated by light induction, but also were related to auxin, MeJA, abscisic acid-responsive elements, while some genes might be regulated by miRNA. The expression analysis showed that the *GhPHR* genes are differentially expressed in different tissues under various stresses. Furthermore, *GhPHR6*, *GhPHR11*, *GhPHR18* and *GhPHR38* were significantly changed after low phosphorus stress. The results of this study provide a basis for further cloning and functional

verification of genes related to regulatory network of low phosphorus tolerance in cotton.

Keywords: cotton; PHR gene; transcription factor; low phosphorus stress; expression

Introduction

Phosphorus (P) is ~~one of the~~ necessary mineral nutrients for plant growth and development, playing an important role in plant cell energy metabolism, enzyme reaction and signal transduction. Plants mainly absorb inorganic phosphorus from soil solution through roots. However, about 95% ~ 99% of phosphorus ~~is easy to react~~ with iron, calcium and aluminum in acidic and alkaline soils to produce insoluble phosphorus, which is not conducive to plant absorption and assimilation, becoming an important constraint ~~on~~ global crop production(Péret et al., 2011). ~~In the agricultural production of crops~~, it is necessary to apply excessive phosphorus fertilizer to ~~make up for~~ the available phosphorus in the soil, but this process will cause serious environmental pollution. Therefore, ~~it is of great significance to the~~ sustainable ~~agricultural~~ production ~~of~~ phosphorus deficient soil ~~through exploring and analyzing~~ the molecular ~~mechanism~~ of high-efficiency utilization of ~~plant~~ phosphorus ~~using molecular biology~~.

~~In order to~~ adapt to ~~the~~ low phosphorus ~~ecological environment~~, plants have evolved a set of complex gene regulation networks, among which the most important members related to phosphate absorption and transport are PHR1 (phosphate startup response 1), IPS1 (induced by phosphate startup 1), miR399 (microRNA399), PHO2 (phosphate 2) and PT (phosphate transport). In this complex regulatory network, PHR protein is an important transcription factor in plant phosphorus regulatory network, which plays an important role in signal transduction and regulation induced by phosphate starvation. At present, PHR genes of *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Glycine max* and other species have been identified(Bustos et al., 2010;Woo et al., 2012;Lin et al., 2013;Guo et al., 2015;Xue et al., 2017;Xu et al., 2018). Arabidopsis PHR transcription factor directly regulates gene expression by binding to the sequence of phosphorus starvation induced gene P1BS (GNATATNC), and there is functional redundancy among members(Rubio et al., 2001). Arabidopsis PHR1 and PHL1 (PHR1-LIKE) transcription factors play a role in plant

response to phosphorus starvation. *PHR1* can bind to the promoters of many phosphorus starvation induced genes. The loss of *AtPHR1* gene function in Arabidopsis will reshape membrane lipid metabolism, primary and secondary metabolism and photosynthesis, which will affect the growth rate of Arabidopsis root and crown and the accumulation of anthocyanins. *PHR1* deletion mutation will affect the expression of some phosphorus starvation induced genes, resulting in the decrease of glucose, fructose, sucrose and starch contents in the mutants(Bustos et al., 2010;Nilsson et al., 2012;Pant et al., 2015). In rice, SPX family proteins are involved in phosphorus sensing and signaling by inhibiting the transcriptional activity of *OsPHR2*(Lv et al., 2014). Rice contains genes *OsPHR1*, *OsPHR2* and *OsPHR3* with MYB-CC domain. The loss of any gene function will inhibit the elongation of rice root hair, and then affect the effective absorption of phosphate by plants. MiRNAs regulate plant response to low phosphorus by down regulating gene transcription(Zeng et al., 2014). Mir399 and mir827 are involved in the response of plants to low phosphorus stress(Pant et al., 2008;Lin et al., 2010). miR399-PHO2 and miR827-NLA mediate the ubiquitination and degradation of phosphate transporters *PHT1* and *PHO1*, and participate in the systematic regulation of phosphorus balance(Chiou et al., 2006;Liu et al., 2014).

Cotton is an important cash crop and raw material for textile industry in China, and plays an important role in the national economy(Ma et al., 2021). Phosphorus is one of the three necessary nutrient elements for cotton growth and development. It can promote cotton budding and flowering in the middle growth stage, promote cottonseed maturation, increase boll weight and open boll early in the later growth stage, which directly affects the yield and fiber quality of seed cotton. Under low phosphorus stress, the adaptive change of root morphology is an important biological basis for crops to make efficient use of soil phosphorus. It has been found the total root length, total root surface area, lateral root length and lateral root number increase in varying degrees under low phosphorus stress in wheat, maize and rice. When low phosphorus stress occurs, different crops will form a set of adaptive mechanisms to deal with stress. There are few studies on the response of cotton to low phosphorus stress.

In order to explore the candidate gene of low phosphorus tolerance in cotton, we identified the

82 members of PHR gene family in the genome by bioinformatics, and analyzed their gene structure,
83 cis-acting elements and gene expression pattern based on the latest published genome sequence of
84 allotetraploid cotton. It provides a reference for further revealing the biological function of PHR
85 transcription factor and cultivating high-quality cotton varieties.
86
87 Material and Method
88 Identification and analysis of cotton PHR gene family members
89 The amino acid sequence of members of the PHR transcription factor family of *Arabidopsis*
90 *thaliana* was used as the reference sequence. The HMM model of *AtPHR* gene was established by
91 hmmbuild software, the homologous genes were searched in four cotton genome data(Paterson et
92 al., 2012;Li et al., 2014;Ma et al., 2021) to obtain the protein sequence and coding sequence of
93 PHR family genes. The obtained sequences with conserved domain Myb DNA-binding (PF00249)
94 and Myb_CC_LHEQLE (PF14379) of PHR transcription factor was identified using SMART
95 (http://smart.embl-heidelberg.de/) and CDD
96 (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The physical and chemical properties of
97 the protein were predicted using the online website ExPASY (https://web.expasy.org/protparam/).
98
99 Conserved domain and gene structure analysis of cotton PHR family members
100 The online website MEME (http://meme-suite.org/tools/meme) was used to predict the conserved
101 motif of cotton PHR gene, and the number of motifs was set to 10, other parameters are the default
102 settings. The gene structure of cotton PHR transcription factors were ~~draw~~ using online website
103 GSDS (http://gsds.cbi.pku.edu.cn/index.php) based on cotton genome annotations.
104
105 Phylogenetic relationship of cotton PHR gene family members
106 The protein sequences of *Arabidopsis thaliana* PHR family gene were downloaded from
107 Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html). The protein sequences of
108 PHR family genes in cotton and Arabidopsis were compared by MEGA 11.0 software(Koichiro et

al., 2021), and the phylogenetic tree was constructed by adjacency method (NJ). The bootstrap value was 1000, the model is Poisson model. Finally, we showed the results using the online tool iTOL(Letunic and Bork, 2021).

Analysis of cis-acting elements of promoters of gene family members

In order to understand the possible regulation and response mechanism of cotton PHR gene, the genome sequence of 1.5 kp upstream of each gene of GhPHR family was obtained, and submitted to PlantCARE online website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to predict cis-acting elements of promoters, and finally visualized by TBtools software(Chen et al., 2020).

Prediction of miRNA-target PHR gene

According to the principle of sequence complementarity, the regulatory miRNA of PHR gene was predicted. The *GhPHR* target gene of miRNA was predicted using psRNATarget online software (https://www.zhaolab.org/psRNATarget/).

Differential expression analysis of *GhPHR* family members in roots under low phosphorus stress

Tissue expression specificity of *GhPHR* genes were analyzed by using RNA sequencing data of *G. hirsutum* 'TM-1' during growth and development downloaded from the NCBI Sequence Read Archive (PRJNA490626). In order to further screen PHR genes in roots in response to low phosphorus stress, we analyzed the gene expression in roots based on transcriptome data. A P-resistant accession was selected for analysis under P deficient hydroponic conditions. First, seeds were grown in germination boxes containing quartz sand until the cotton cotyledons had fully expanded, and the seedlings were moved into half-strength Hoagland normal nutrient solution. The half-strength Hoagland normal nutrient solution was replaced with full-strength P deficient Hoagland nutrient solution after three days. The leaves were sampled at 0 day and 15 days after treatment under P-deficient and P-replete conditions and immediately frozen in liquid nitrogen and

stored at −80 °C for RNA-seq. The heat map of gene expression was drawn by Hemi 1.0 software (Deng et al., 2014) with log2(1 + FPKM) values after averaging three replicates. Six of the *GhPHR* genes were selected for gene expression analysis by qRT-PCR. All reactions were performed in three independent biological replicates, each with three technical replicates, using the Roche LightCycler96 RealTime PCR System. *GhUBQ14* expression was used as the internal control. Relative gene expression values were calculated using the $2^{-\triangle CT}$ method (Schmittgen and Livak, 2008).

Results

Identification of cotton PHR family gene members

To investigate the copy number variation in the PHR genes during cotton evolution, a comprehensive search was conducted for PHR genes across cotton lineages, including *G. arboreum*, *G. raimondii*, *G. hirsutum* and *G. barbadense*. The results were verified in the NCBI-CDD database. In total, 22, 23, 41, and 42 PHR genes were identified, respectively. A total of 128 PHR gene sequences were detected in the four cotton species, the detailed information of which is listed in Table S1. The results showed that the numbers of PHR genes in *G. arboretum* and *G. raimondii* were almost similar as were those in *G. hirsutum* and *G. barbadense*.

The PHR family genes in two diploid cotton species are basically half of the number in two tetraploid cotton species, which conforms to the known evolutionary relationship of cotton(Wang et al., 2018), indicating that the PHR family is conserved in the evolution of cotton.

The names of the PHR genes were determined according to the gene information of Arabidopsis and the locations on the chromosomes. The encoded protein of the PHR family genes in *G. hirsutum* contains 236~494 amino acid residues. The relative molecular mass is between 26.53 and 54.30 kDa, and the theoretical isoelectric point is between 5.50 and 9.82. Each of the family members contains Myb DNA-binding and Myb_CC_LHEQLE domain. Fourty-one PHR genes were distributed on 21 chromosomes except (A05, A07, D01, D03 and D07) of *G. hirsutum* (Table 1). Subgenome A and subgenome D contained 22 and 19 sequences, respectively. The number of

163 *GhPHR* genes in subgenome A was consistent with the number of GaPHR genes, and four of the
164 PHR genes were missing from subgenome D compared to the number of *GrPHR* genes. This result
165 indicated that subgroup D might have lost genes due to redundant gene functions during cotton
166 evolution.
167 Three sequences were observed on chromosomes A09 and A13, while chromosomes D09 and D13
168 contained two sequences. The A01 and A03 chromosomes contained one sequence, but the
169 *GhPHR* gene sequence was not contained in D01 and D03 chromosomes. This result showed that
170 the PHR genes might have been lost and duplicated in the process of evolution. However, there
171 was a strong correlation between subgroup A and subgroup D, which was also in line with the
172 evolutionary relationship in cotton(Wang et al., 2012;Wang et al., 2018).
173
174 Phylogenetic analysis of the PHR gene family in cotton
175 To explore the phylogenetic relationship of the cotton PHR genes, we constructed a phylogenetic
176 tree with the neighbor-joining method using 41 *G. hirsutum*, 42 *G. babardence*, 22 *G. arboreum*,
177 23 *G. raimondii* and 13 Arabidopsis PHR amino acid sequences (Fig. 1). All the PHR proteins can
178 be divided into five subgroups. The number of PHR genes in each subgroup of *G. hirsutum* and
179 *G. barbadense* was basically twice the number in each subgroup of *G. arboreum* and *G. raimondii*.
180 This was consistent with the results of the previous analysis and conforms to the evolutionary
181 relationship in cotton. The results showed that the PHR genes were relatively conserved in
182 evolution in cotton. Among these subfamilies, the largest subgroup Ⅴ consisted of 12 *GhPHRs*,
183 12 *GbPHRs*, 7 *GaPHRs*, 7 *GrPHRs* and 6 *AtPHRs*, showing that has expanded considerably in
184 allotetraploid cotton. In contrast, subgroup Ⅲ only included 4 *GhPHRs*, 4 *GbPHRs*, 2 *GaPHRs*, 2
185 *GrPHRs* and 1 *AtPHRs*, indicating a highly-conserved ancient clade.
186
187 Analyses of gene structures and protein motifs of PHR genes in *G. hirsutum*
188 ~~Through~~ gene structure analysis of PHR gene family members~~, it is found~~ that the ~~gene~~ structure
189 of *GhPHR* members in the same subgroup ~~is~~ similar, and there ~~is~~ little difference in the number of

exons of *GhPHR* members among different subgroups. Except ~~that~~ the *GhPHR* gene of class Ⅴ contain 7 exons, the other ~~most~~ *GhPHR* genes contain 6 exons (Fig. 2). Furthermore, these PHR protein sequences were submitted to MEME to discover conserved motifs. The adjacent clades carried similar motifs. Analysis of the conserved domains of *GhPHR* gene family members showed that Myb_DNA-binding and Myb_CC_LHEQLE were present~~ed~~ in all GhPHR proteins and other motifs ~~are~~ functionally unknown motifs (Fig. 2). Among them, motif 4 ~~exists~~ in class I, class II and class IV subgroups, motif 6 ~~is~~ unique to class II subgroup, motif 10 ~~are~~ unique to class IV subgroup, and motif 5 ~~is~~ unique to two of class I class II subgroup. It is worth noting that the GhPHR protein motif types of class II ~~are~~ different. Among them, there ~~are~~ eight motifs in GhPHR15, GhPHR35 and GhPHR2, only six motifs in GhPHR5 and GhPHR26, and seven motifs in other five GhPHR. These special conserved motifs may be the main factors ~~for different~~ GhPHR to participate in different biological functions.

Analysis of cis-acting elements in the promoter of *GhPHR* gene family

~~In order to~~ further clarify the possible regulatory mechanism of *GhPHR* family genes under abiotic stress, the promoter sequence was analyzed by using PlantCARE database. The results showed ~~that~~ 13 types of cis acting elements ~~were identified~~, including light responsive, salicylic acid responsiveness, gibberellin-responsive, MeJA-responsiveness, anaerobic induction, auxin-responsive, abscisic acid responsiveness and so on (Fig. 3). In terms of composition and quantity, *GhPHR* gene contains an average of 18 cis-acting elements, all of which ~~contain~~ light responsive elements. Among them, *GhPHR6* and *GhPHR19* contain the most types of response elements (10 kinds), while *GhPHR3*, *GhPHR5*, *GhPHR7* and *GhPHR40* contain the least cis-acting elements (3 kinds). In terms of element types, 17 genes containing gibberellin-responsive elements include *GhPHR1*, *GhPHR12*, *GhPHR34*, etc. 31 genes containing anaerobic induction elements include *GhPHR5*, *GhPHR19*, *GhPHR24*, etc, and 11 genes containing auxin-responsive elements include *GhPHR16*, *GhPHR20*, *GhPHR36* and so on. The results showed that the expression of *GhPHR* gene was not only regulated by light induction, but also played a role in drought, anaerobic and

other stress resistance. Among them, the promoter regions of *GhPHR3*, *GhPHR5*, *GhPHR7* and *GhPHR40* contain less cis elements, which ~~are~~ only related to light, MeJA, abscisic acid and anaerobic induction.

Prediction of the regulatory miRNA of PHR gene family

The online software psRNATarget was used to predict and analyze the regulatory miRNAs of PHR gene. The regulatory combinations of 33 miRNAs and PHR gene were predicted (Table 2). It was found that 12 miRNAs could regulate 16 PHR genes. A lower expected value indicates that the miRNA matches the target gene sequence well. Unpaired energy (UPE) is the energy required to unlock the secondary structure of the target gene miRNA target site. A lower UPE value indicates that miRNA is more likely to bind or cleave the target gene. This study shows some of the predicted results with an expected value less than or equal to 5. *GhPHR21* and *GhPHR32* can be recognized by miR396 and miR7510a, miR2949 and miR7491 at the same time, respectively, which may be regulated by these two miRNAs. *GhPHR19* may be regulated by the sequence cleavage of miR482, *GhPHR4* and *GhPHR24* may be regulated by the transcriptional inhibition of miR827, ~~and~~ *GhPHR17* and *GhPHR37* may be regulated by the sequence cleavage of miR2948-5p, and *GhPHR23*, *GhPHR25* and *GhPHR40* may be regulated by the transcriptional inhibition of miR2948-5p. In this study, the regulation modes of different interaction combinations ~~are~~ different. About 2/3 of the regulation modes belong to sequence cleavage and 1/3 belong to transcriptional inhibition (Table 2).

Tissue expression analysis of *GhPHR* family genes

The expression of *GhPHR* family genes ~~were~~ investigated across different tissues and developmental stages of upland cotton ~~from~~ transcriptome sequencing data(Zhang et al., 2015). Most of these genes were expressed at varying levels across different tissues and developmental stages (Fig. 4). It was found that 5 *GhPHR* genes were ~~almost~~ expressed in all tissues. 13 *GhPHR* werehighly expressed in all tissues, indicating that these genes play an important role in all

morphogenesis of cotton. Among them, *GhPHR5* and *GhPHR6* were highly expressed in leaves, while *GhPHR13*, *GhPHR14*, *GhPHR26* and GhPHR27 were highly expressed in both roots and leaves. Combined with the cis element structure of *GhPHR* promoter, it is speculated that they may play an important role in leaf photosynthesis. 8 *GhPHR* were expressed across different tissues except fiber developmental stages, and the expression of the other 6 *GhPHR* genes were low in different tissues. Altogether, the expression profiles of *GhPHR* gene shows that plays a role in all tissues of cotton, among which *GhPHR2*, *GhPHR5*, *GhPHR6* and *GhPHR13* have obvious tissue expression specificity.

Expression pattern of *GhPHR* gene in roots under low phosphorus stress

The expression analysis of 41 *GhPHR* genes in roots showed that most *GhPHR* genes ~~was~~ affected under low phosphorus treatment, except that *GhPHR4*, *GhPHR7*, *GhPHR12*, *GhPHR19*, *GhPHR24*, *GhPHR33* and *GhPHR39* were not detected (Fig. 5). The expression of *GhPHR1* and *GhPHR11* decreased ~~after~~ low phosphorus stress~~. The expression~~ of *GhPHR3*, *GhPHR6*, *GhPHR17*, *GhPHR18*, *GhPHR27*, *GhPHR30* and *GhPHR38* increased ~~after low phosphorus stress~~. Among them, expression level of *GhPHR17*, *GhPHR30* and *GhPHR30* was significantly higher than that before stress treatment. In addition, *GhPHR5*, *GhPHR13*, *GhPHR15* and *GhPHR26* maintained ~~at~~ a high expression level. It should be noted that the expression of *GhPHR11* was significantly lower ~~after~~ low phosphorus stress than ~~that of~~ normal phosphorus treatment, and ~~the expression~~ of *GhPHR18* was significantly higher than that of normal phosphorus treatment (Fig. 5). Further, we verified six *GhPHR* genes by qRT-PCR (Fig. 6). This provides further evidence that the six putative genes were closely associated with low-phosphorus tolerance.

Discussion

Phosphorus deficiency is a major factor limiting crop yield. Plants have evolved a series of morphological, physiological and molecular strategies to adapt to ~~the symptoms of~~ phosphorus

deficiency(Veneklaas et al., 2012), including symbiosis with mycorrhizal fungi, secretion of organic acids, remodeling of root structure, and improving the expression of phosphorus transporters(Chiou and Lin, 2011;Sawers et al., 2017). Most of these strategies improve the utilization efficiency of phosphorus by enhancing the mobility of phosphorus in soil or the acquisition of phosphorus by roots. In recent years, genes and proteins related to low phosphorus stress have been found and identified. Among them, PHR is a MYB transcription factor, which plays an important role in plant response to low phosphorus stress(Bustos et al., 2010). It has been reported that PHR 1 and PHR1-like genes play a key role in the phosphorus signal regulation network of plants such as Arabidopsis(Karthikeyan et al., 2007), rice(Guo et al., 2015), soybeans(Xue et al., 2017), wheat(Chiou and Lin, 2011), maize(Lin et al., 2013;Sawers et al., 2017) and rape(Ren et al., 2012). In addition, genome-wide transcriptional analysis of Arabidopsis and rice showed that most phosphorus starvation response genes were induced and activated by *AtPHR1* and *OsPHR2* and their homologous genes *AtPHL1*, *AtPHL2*, *OsPHR1* and *OsPHR3*(Guo et al., 2015;Sun et al., 2016).

Cis-acting elements regulate gene transcription by responding to different external signals, and then affect plant growth and development(Schmitz et al., 2022). ~~It has been found that~~ phosphorylation signal transduction and phosphorus starvation response are affected by light, sugar, plant hormones (auxin, ethylene, cytokinin and gibberellin), as well as oxygen(Karthikeyan et al., 2007;Lei et al., 2011;Klecker et al., 2014). For example, the expression of *AtPHR1* is regulated by light and ethylene, and the response to phosphorus starvation is regulated by the promoter of *AtPHR* gene(Liu et al., 2017). In this study, 13 types of cis-acting elements were identified in the promoter of PHR gene. A large number of light response elements and hormone elements showed that the expression and regulation of PHR gene were affected by light and hormone. MiRNAs regulate plant response to low phosphorus by down regulating gene transcription(Zeng et al., 2014). MiR399 and miR827 are involved in the response of plants to low phosphorus stress(Pant et al., 2008;Lin et al., 2010). In this study, 12 cotton miRNAs such as miR396, miR482 and miR827 have the potential to regulate *GhPHR* genes, which may play a role

~~in the process of~~ phosphorus absorption and transport in cotton.

~~It has been reported that most~~ PHR genes in maize, rice and sorghum are continuously expressed in all tissues, indicating that they may play an important role in regulating phosphorus uptake and transport(Lin et al., 2013;Xu et al., 2018). This study analyzed the tissue expression of cotton PHR family genes in roots, stem, leaves and so on, and found that there was tissue-specific expression of cotton PHR family genes, which was similar to that of other crops(Lin et al., 2013). In tissue expression analysis, it was found that the expression of *GhPHR* in roots was high, and there were gibberellin and auxin response elements related to stress resistance in the cis-acting elements of promoter. In addition, the expression of *GhPHR* exceeded the expression level before stress after low phosphorus stress, so it is speculated that *GhPHR* may be related to the remodeling of root morphology under abiotic stress.

In conclusion, 128 PHR genes were identified in cotton, ~~which~~ 41 in *G. hirsutum*. There are great differences in the number of amino acids and isoelectric point characteristics of these *GhPHR* genes. In addition, *GhPHR* has many cis-acting elements related to light response, ~~biological~~ and abiotic stresses in the promoter region. Further analysis of the differential expression of gene showed that *GhPHR11* and *GhPHR18* were significantly highly expressed in roots ~~after~~ low phosphorus stress. This study ~~will lay~~ a foundation for ~~the~~ subsequent functional study of PHR gene and the breeding of new cotton varieties.

AUTHOR CONTRIBUTION STATEMENT Z.S. designed the study; Y.Z., P.L., H.W., J.F., Y.L., S.W., Y.J.L. and L.L prepared samples and ~~generated~~ the experiments; Z.S., Y.Z. and P.L. collected data and wrote the manuscript. Y.G., Y.S and Z.S. provided suggestions and revised the paper. All authors read the final manuscript and approved submission. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors and available from the corresponding author on reasonable request.

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Table 1 Information of PHR gene family in *G. hirsutum*.

A subgenome of *G. hirsutum*					A subgenome of G. hirsutum				
Gene name	Gene ID	Protein length	MV(Da)	pI	Gene name	Gene ID	Protein length	MV(Da)	pI
GhPHR1	GhM_A01G2399	380	42304.2	7.76	GhPHR23	GhM_D02G1490	364	40435.4	6.85
GhPHR2	GhM_A02G0277	303	33075.2	6.52	GhPHR24	GhM_D04G2388	346	38667.5	9.37
GhPHR3	GhM_A03G1365	364	40578.5	6.85	GhPHR25	GhM_D05G1302	494	54301.4	6.23
GhPHR4	GhM_A04G1918	346	38712.5	9.34	GhPHR26	GhM_D06G2662	298	32958.0	5.88
GhPHR5	GhM_A06G2674	298	32865.9	6.11	GhPHR27	GhM_D06G2663	333	35805.0	8.65
GhPHR6	GhM_A06G2675	333	35829.1	8.65	GhPHR28	GhM_D08G0228	411	46409.8	7.05
GhPHR7	GhM_A08G0240	417	47059.5	6.86	GhPHR29	GhM_D08G1976	279	31618.2	9.82
GhPHR8	GhM_A08G2024	279	31659.3	9.78	GhPHR30	GhM_D08G2683	372	41765.9	8.19
GhPHR9	GhM_A08G2740	411	46419.1	8.69	GhPHR31	GhM_D08G2924	357	39650.8	8.62
GhPHR10	GhM_A08G2986	348	38638.6	8.77	GhPHR32	GhM_D09G1508	316	36391.6	8.20
GhPHR11	GhM_A09G1611	315	36353.5	8.05	GhPHR33	GhM_D09G1943	267	30049.4	8.20
GhPHR12	GhM_A09G2040	267	29998.3	8.20	GhPHR34	GhM_D10G0017	399	44484.4	5.78
GhPHR13	GhM_A09G2485	253	27983.4	5.97	GhPHR35	GhM_D10G1602	302	33108.2	6.78
GhPHR14	GhM_A10G0026	433	48090.4	5.50	GhPHR36	GhM_D11G1558	478	52511.1	5.69
GhPHR15	GhM_A10G1517	302	33070.1	6.25	GhPHR37	GhM_D11G3020	448	49301.5	6.20
GhPHR16	GhM_A11G1564	478	52622.2	5.69	GhPHR38	GhM_D11G3158	387	43073.3	7.41
GhPHR17	GhM_A11G3088	418	46179.0	5.97	GhPHR39	GhM_D12G2463	236	26531.7	8.43
GhPHR18	GhM_A11G3236	387	43196.4	6.88	GhPHR40	GhM_D13G0849	356	39260.4	8.15
GhPHR19	GhM_A12G2578	236	26730.9	8.43	GhPHR41	GhM_D13G1605	347	38580.7	8.01
GhPHR20	GhM_A13G0904	309	34021.4	8.07					
GhPHR21	GhM_A13G1449	374	41288.3	8.17					
GhPHR22	GhM_A13G1727	347	38480.6	8.33					

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464 **Table 2 Bioinformatic analysis of partial miRNAs target sites.**

miRNA	Target genes	Expectation		Start	Sequence	End	Inhibition mode
miR827a	GhPHR4	5.0	miRNA	1	UUAGAUGACCAUCAACAAACA	21	Translation
					: ::::::: :::::: : :		
			Target	952	GGAUUGUUGA-GGUCAUUUGA	971	
miR827b	GhPHR4	5.0	miRNA	1	UUAGAUGACCAUCAACAAACA	21	Translation
					: ::::::: :::::: : :		
			Target	952	GGAUUGUUGA-GGUCAUUUGA	971	
miR827c	GhPHR4	5.0	miRNA	1	UUAGAUGACCAUCAACAAACA	21	Translation
					: ::::::: :::::: : :		
			Target	952	GGAUUGUUGA-GGUCAUUUGA	971	
miR7491	GhPHR11	4.0	miRNA	1	UGGGAUCUUCGAGAGGAUUGAGCC	24	Translation
					:::::: : ::::::: :		
			Target	324	CCAGAAAUCCUUUGAAAGAUCCUA	347	
miR2949b	GhPHR12	4.0	miRNA	1	UCUUUUGAACUGGAUUUGCCGA	22	Translation
					:. :::: ::::::::		
			Target	497	AGUCUGAGUCCAAUUCAAAAGA	518	
miR2949c	GhPHR12	4.0	miRNA	1	UCUUUUGAACUGGAUUUGCCGA	22	Translation

					..:.::::: :::::::::		
			Target	497	AGUCUGAGUCCAAUUCAAAAGA	518	
miR2949a-5p	GhPHR12	5.0	miRNA	1	ACUUUUGAACUGGAUUUGCCGA	22	Translation
					..:.::::: ::::::::		
			Target	497	AGUCUGAGUCCAAUUCAAAAGA	518	
miR482a	GhPHR19	5.0	miRNA	1	UCUUUCCUACUCCUCCCAUACC	22	Cleavage
					::::: ::::: :::::::		
			Target	620	AUUAUGGAGGGAGAUGGAGAGA	641	
miR7510a	GhPHR21	4.5	miRNA	1	AAGGUCAUGAUCUUUAGCGGCGUU	24	Cleavage
					::: : : ::..::::. ::.:::::		
			Target	88	AAUGGCACUGGAGAUUCUGGCCUU	111	
miR396a	GhPHR21	5.0	miRNA	1	UUCCACAGCUUUCUUGAACUG	21	Cleavage
					:: :::::: ::.. :::::		
			Target	560	AAGCUCAAGAGAGUCUUGGAA	580	
miR396b	GhPHR21	5.0	miRNA	1	UUCCACAGCUUUCUUGAACUG	21	Cleavage
					:: :::::: ::.. :::::		
			Target	560	AAGCUCAAGAGAGUCUUGGAA	580	
miR2948-5p	GhPHR40	4.5	miRNA	1	UGUGGGAGAGUUGGGCAAGAAU	22	Translation
					::: ::::. ::::: .:::		
			Target	46	UUUCCUGCCUGCCUCUCUUACA	67	

Figure 1 Phylogenic tree of the PHR family members in *G. arboreum*, *G. raimondii*, *G. hirsutum*, *G. barbadense* and *Arabidopsis thaliana*. The unrooted phylogentic tree was

constructed using MEGA 11.0 by Neighbor-Joining method. Numbers on branches were bootstrap portions from 1000 replicates. Percentage bootstrap scores of <50% were hidden. The specific color indicated different families.

Figure 2 Distributions of gene structure and conserved protein motifs in *GhPHR* genes. The red boxes and gray lines represented the exon and intron, respectively. The lengths of the boxes and lines were scaled based on the length of the genes. Conserved motifs in the GhPHR proteins are indicated by colored boxes.

Figure 3 Cis-acting element analysis of PHR family members in *G. hirsutum*.

Figure 4 Expression profiles of *GhPHR* transcription factor genes in different tissues. The color scale of heat map indicates the relative expression levels where blue indicates low and red indicates high.

Figure 5 Expression pattern of *GhPHR* gene in roots under low phosphorus stress. The color scale of heat map indicates the relative expression levels where blue indicates low and red indicates high.

Figure 1

Figure 1

Phylogenic tree of the PHR family members in *G. arboreum*, *G. raimondii*, *G. hirsutum*, *G. barbadense* and *Arabidopsis thaliana*.

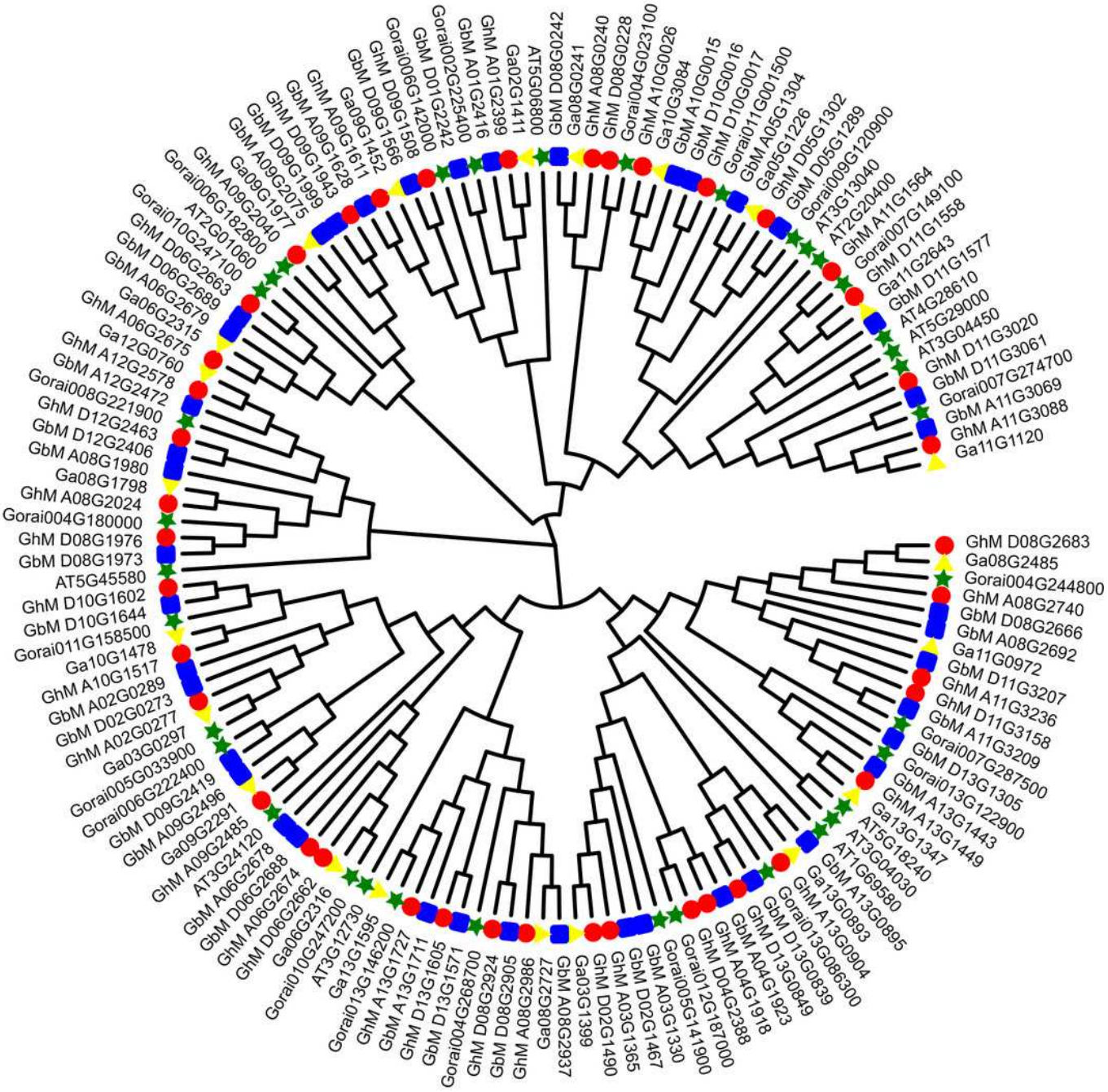

Figure 2

Figure 2

Distributions of gene structure and conserved protein motifs in *GhPHR* genes.

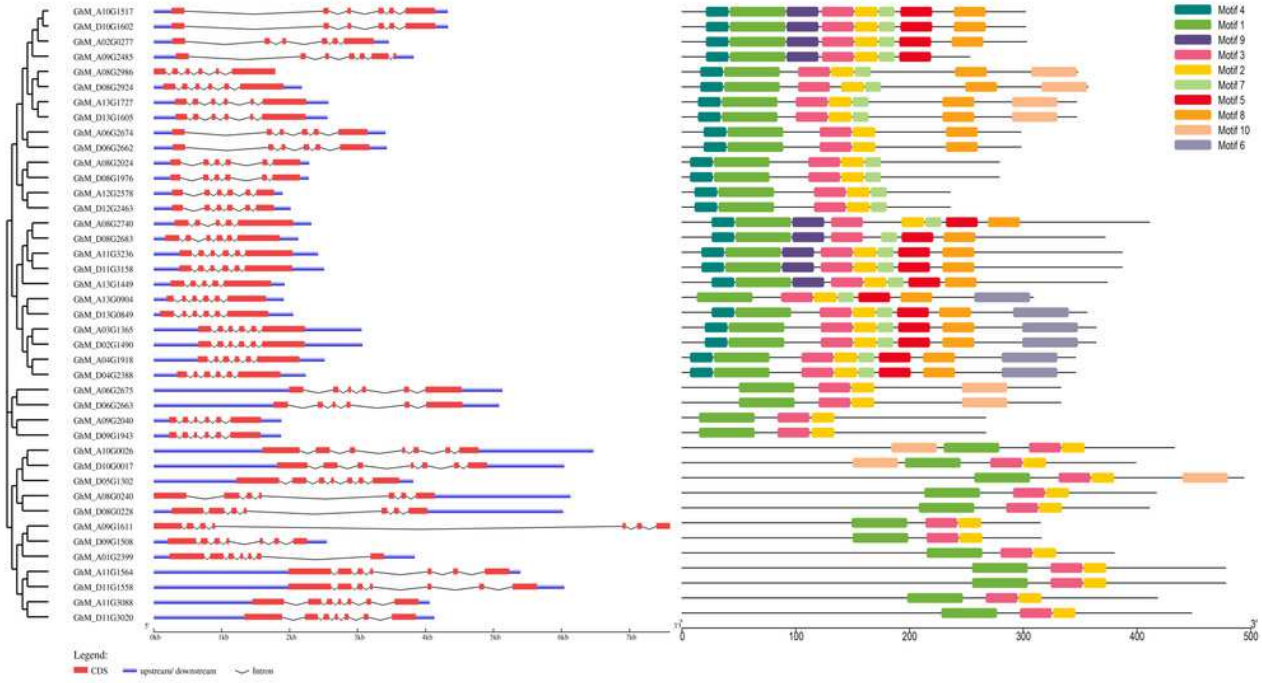

Figure 3

Figure 3

Cis-acting element analysis of PHR family members in *G. hirsutum*.

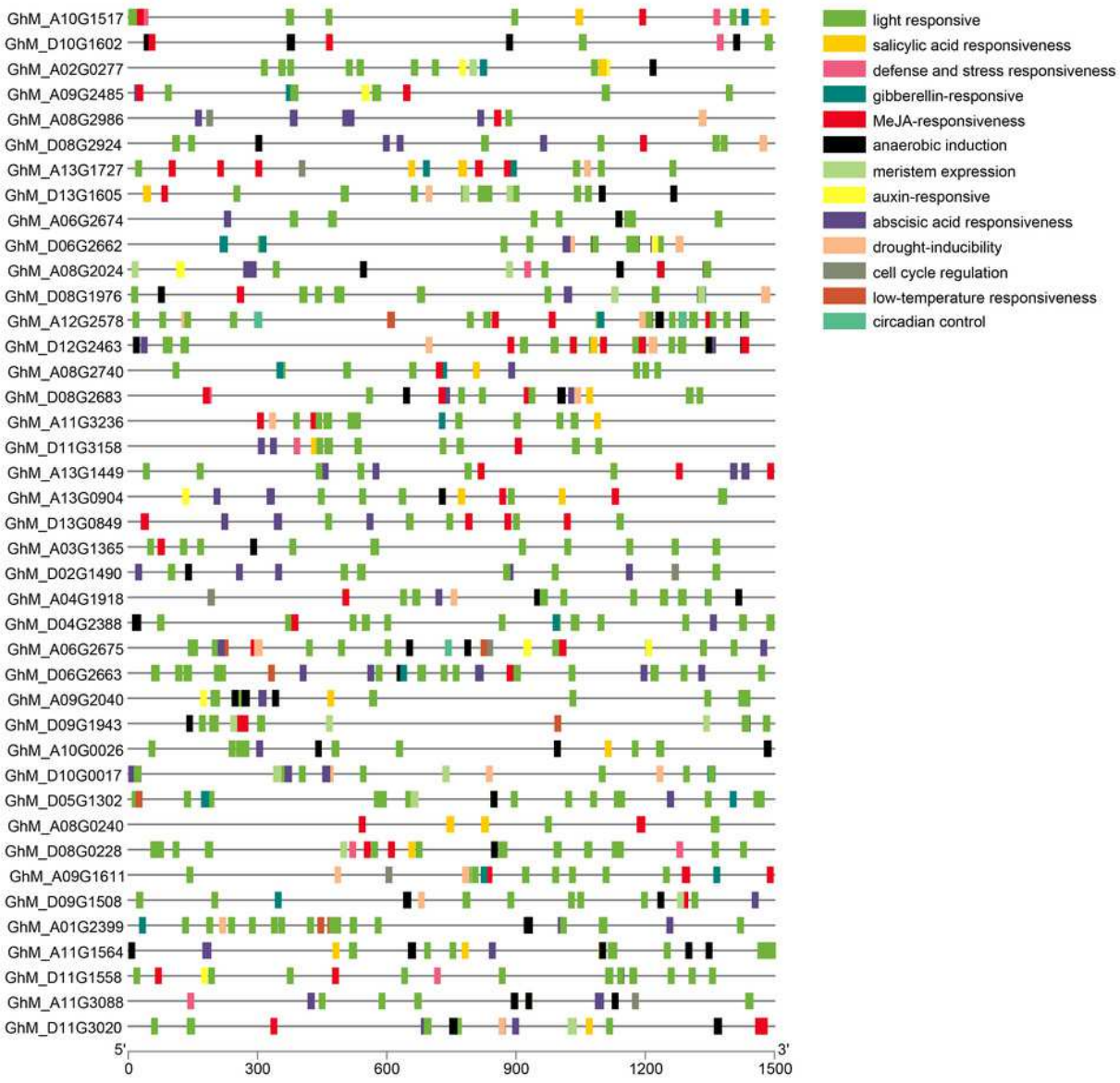

Figure 4

Figure 4

Expression profiles of *GhPHR* transcription factor genes in different tissues.

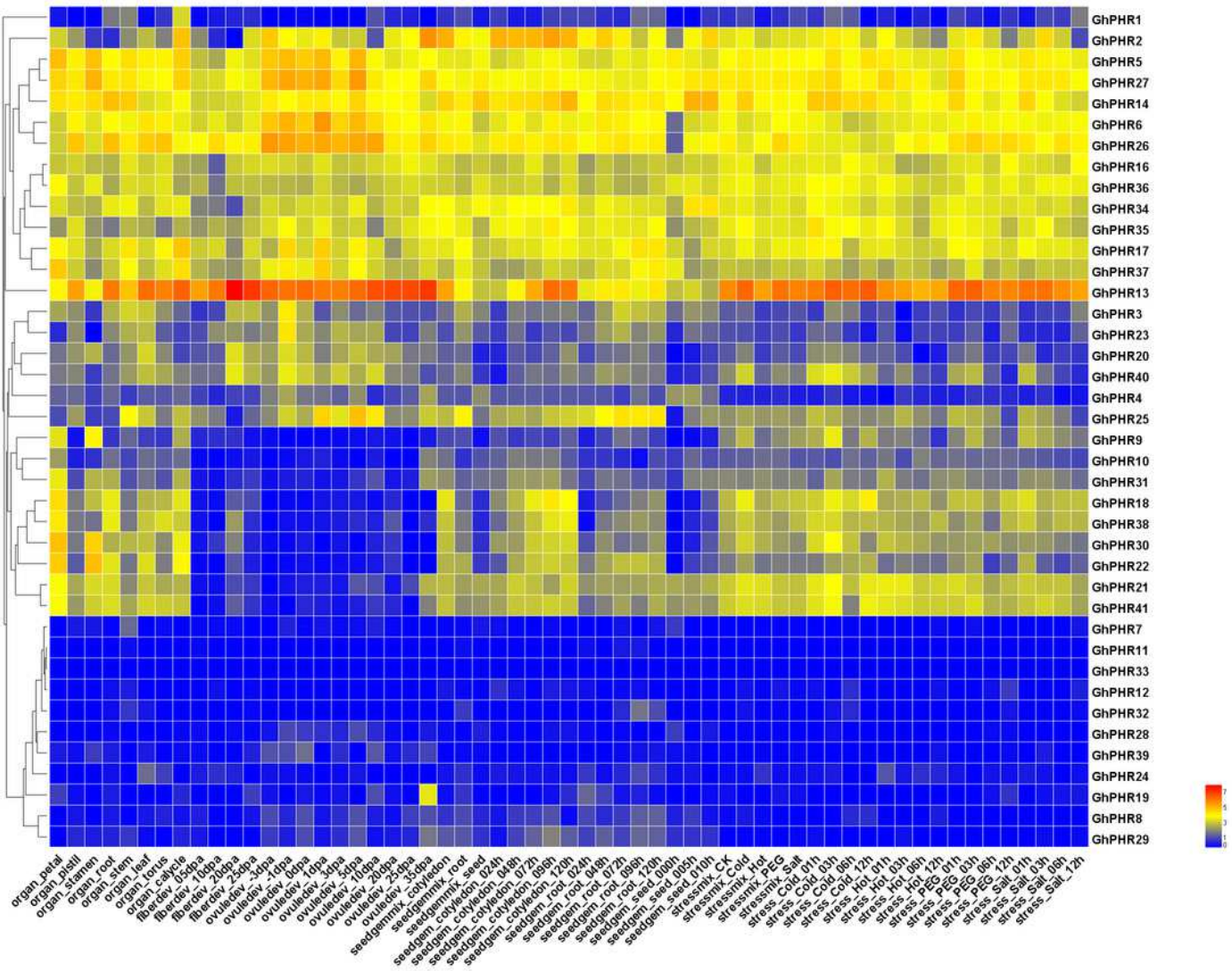

Figure 5

Figure 5

Expression pattern of *GhPHR* gene in roots under low phosphorus stress.

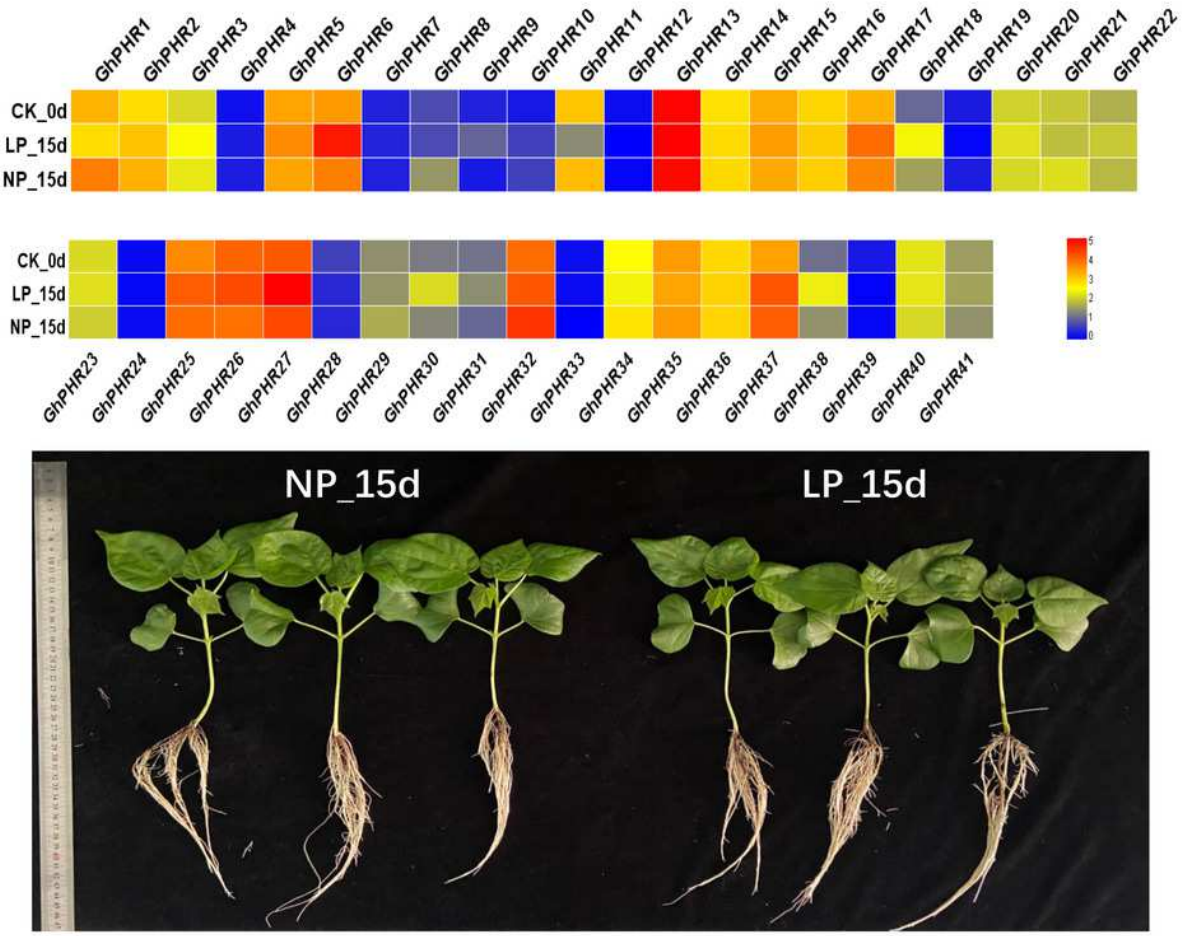

Figure 6

Figure 6

Relative expression levels of six representative *GhPHR* gene by qPCR.