

Article

Phosphorus Regulates Cotton Fiber Development Through GhPHR1-GhGCS1 Module

Zhiqing Qiao ^{1,2}, Junli Ding ^{1,2}, Qiaoling Wang ^{1,2}, Xingying Yan ^{1,2}, Yinghui Gao ^{1,2}, Pingting Tan ^{1,2}, Ning Liu ^{1,2}, Fei Liu ^{1,2} and Ming Luo ^{1,2,*} 

¹ College of Agronomy and Biotechnology, Southwest University, Chongqing 400715, China; qiao1qiao_8@163.com (Z.Q.); 15340423358@163.com (J.D.); wql19980513@163.com (Q.W.); yxingying@swu.edu.cn (X.Y.); yinghuigao2022@163.com (Y.G.); 15922605913@163.com (P.T.); 18290409878@163.com (N.L.); blur2000@163.com (F.L.)

² Engineering Research Center of South Upland Agriculture, Ministry of Education, Southwest University, Chongqing 400715, China

* Correspondence: luomingyuan@swu.edu.cn

Abstract

Cotton is an important cash crop globally. Cotton fiber is the main economic product of cotton plants. Phosphorus, as one of the essential nutrients, plays an important role in plant growth and development. However, few studies focus on phosphorus regulating fiber elongation. In this study, we used the cotton ovule culture system in vitro to explore the effects of various phosphorus levels on fiber and ovule growth, and screened for phosphorus-responsive factor, as well as revealed its action mechanism. The results indicated that fiber elongation was more sensitive than ovule growth to phosphorus deficiency. GhPHR1, a homolog of phosphate starvation response 1 (PHR1) in upland cotton, was significantly upregulated in fibers and ovules under phosphorus-deficient conditions. GhPHR1 directly binds to the promoter of the glucosylceramide synthase gene in cotton (*GhGCS1*) and positively regulates its expression. Overexpressing *GhGCS1* enhanced phosphorus uptake and transport in cotton, increased phosphorus content in fiber cells, and promoted fiber cell elongation. Conversely, downregulating *GhGCS1* reduced phosphorus content in fiber cells and suppressed fiber elongation. These findings demonstrate the importance of the GhPHR1-*GhGCS1* molecular module in regulating fiber cell elongation and elucidate the molecular mechanism by which phosphorus influences fiber elongation.

Keywords: sphingolipid; GhPHR1; *GhGCS1*; phosphorus metabolism; regulatory mechanism; cotton fiber



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1. Introduction

Cotton is an important economic crop globally and cultivated in more than 100 countries, with China, India, and the USA leading in the global production ranking [1–3]. The global cotton market value is estimated at about USD 44 billion. The cotton industry employs about 7% of total labor in developing economies, and more than 350 million people are directly and indirectly employed globally [1,4]. This indicates that cotton is important to the global economy. Fiber is the main economic product of cotton plants and finds uses in a lot of textile products production, healthcare, and minting of diverse financial securities, etc. [2]. A fiber cell is one of the longest single cells in a plant and originates from the epidermal cells of the cotton ovule outer integument [5]. There are five stages in the growth and development of fiber cells: initiation, elongation (primary wall synthesis),

transition, secondary cell wall synthesis, and dehydration maturation [5]. Among these, the elongation and secondary cell wall synthesis stages are two critical periods that determine fiber quality (length, strength, and fineness) and also influence fiber yield [6,7]. Therefore, studying the key genes and regulatory mechanisms governing fiber cell elongation and secondary cell wall formation is of great importance. On the other hand, as one of the longest plant cells, cotton fibers also serve as an ideal model for studying plant cell growth and development [8]. The development of new varieties is identified as one of the most significant advancements in cotton agronomy [1]. Hence, in recent years, with the publication of the cotton genome sequence, progress has been made in identifying key genes and regulatory mechanisms involved in fiber cell growth and development [9–15]. However, the regulatory mechanisms underlying fiber development are still not fully understood. Especially, the understanding of the function and action mechanism of plant nutrients such as phosphorus in fiber cell growth is very limited.

Phosphorus, as one of the vital macronutrients for plant growth, plays a crucial role in the process of plant development [16,17]. It not only promotes plant growth but is also involved in energy storage and supply, respiration, the biosynthesis of nucleic acids, membrane lipid metabolism, signal transduction, and photosynthesis [18–20]. Most plants grown in phosphorus-deficient environments exhibit stunted growth, reduced seed setting rates, decreased plant height, stem diameter, and leaf size [21–25]. As an indispensable nutrient, phosphorus participates in key biological processes and directly determines crop yield and quality [26–29]. Cotton is sensitive to phosphorus deficiency [30]. By supporting overall plant growth and development, phosphorus promotes root development, enhances nutrients and water absorption capacity of roots, encourages early budding and flowering, improves pollination quality, and increases the number of effective bolls, finally indirectly regulating fiber quality and yield, which in turn influences the efficiency of cotton industrial processing [31–33]. Studies have shown that as soil phosphorus content increased, the boll weight of cotton gradually rose, while phosphorus deficiency reduced the lint percentage of cotton. Appropriate phosphorus levels help to improve cotton fiber length and strength. Under phosphorus stress conditions, as phosphorus content increases, both fiber length and strength generally show an upward trend [30,32,34]. Recently, Sun et al. demonstrated that reducing soil available phosphorus (AP) content decreased the contents of osmotically active solutes, including potassium ions, malate, soluble sugars, and sucrose in cotton fibers, as well as reduced the activity of enzymes involved in fiber elongation, such as plasma membrane H^+ -ATPase, thereby inhibiting fiber elongation via osmotic regulation mediated by vacuoles [35]. Similarly, Li et al. found that phosphorus fertilization promoted fiber elongation by enhancing sucrose conversion, K^+ accumulation, and malate synthesis in cotton fibers [33]. In another study, Sun et al. showed that low phosphorus stress reduced fiber strength by inhibiting carbohydrate metabolism. Specifically, soil AP deficiency declined the activity of enzymes required for fiber cell wall formation, such as soluble acid invertase, sucrose synthase, sucrose phosphate synthase, and β -1,3-glucanase, resulting in cellulose content and fiber strength decrease [36]. At the levels of crop cultivation and physiology, these studies provided evidence that phosphorus played a crucial role in fiber elongation and secondary cell wall development. However, the journey of phosphorus from soil to fiber cells involves complex processes, including uptake, transport, and utilization. Different organs, tissues, and cells exhibit significant variations in phosphorus transport and utilization efficiency. Therefore, the specific functions and regulatory mechanisms of phosphorus in governing fiber cell growth and development require further investigation.

Sphingolipids are composed of nonpolar ceramides and various glycosylated residues in plant cells [37], and are an important component of plant cell membranes [38]. They not only play a significant role in maintaining the integrity of cell membranes but also act as key

signaling molecules to participate in various physiological processes within plants, including cell division and differentiation, information transmission, and plant morphogenesis [39]. In recent years, studies on *Arabidopsis thaliana* and other crops such as cotton and rice have gradually revealed the complex functions and regulatory networks of sphingolipid in plant growth and development [40–44]. For instance, overexpression of the ceramide synthase gene *GhCS1* in cotton leads to the accumulation of ceramides containing long-chain fatty acids, inducing programmed cell death and inhibiting the elongation of fibers [44]. Regulating the levels and ratios of sphingosine (Sph) and sphingosine 1-phosphate (S1P) can affect the elongation of fiber cells [43]. Recently, a study has proved that phosphorus deficiency can cause changes in sphingolipid content in plants. For instance, under phosphorus-deficient conditions, *Arabidopsis thaliana* non-specific phospholipase C4 (NPC4) can degrade and reduce the content of glycosylinositolphosphoceramide (GIPC). *Arabidopsis* glucosylceramide synthase 1 (AtGCS1) responds to phosphorus-deficient conditions by remodeling the dynamic balance of sphingolipid to promote phosphorus utilization [19,45]. However, it is still unclear whether phosphorus affects sphingolipid changes during the growth and development of the cotton plant and the elongation of cotton fibers, as well as the molecular mechanism by which it regulates the elongation of cotton fibers by causing sphingolipid changes.

To explore the function and molecular mechanism of phosphorus regulating fiber cell growth and development, this study identified a phosphorus-responsive factor, GhPHR1, a homolog of phosphate starvation response 1 (PHR1) in upland cotton from fibers treated with various phosphorus concentrations. Furthermore, we clarified the regulatory relationship between this transcription factor and its target gene, *Gossypium hirsutum* GCS1 (*GhGCS1*), and demonstrated the role of *GhGCS1* in fiber growth and phosphorus metabolism. These results elucidate a novel mechanism by which the GhPHR1-*GhGCS1* molecular module regulates fiber cell elongation, providing new insight into the regulatory mechanism of phosphorus in cotton fiber development.

2. Materials and Methods

2.1. Plant Materials and Strains

This study used upland cotton (*Gossypium hirsutum* L.) cv. Jimian 14, supplied by the Cotton Research Institute of the Chinese Academy of Agricultural Sciences. Plants for phenotypic observation under phosphorus deficiency were grown in a growth chamber, whereas those used for harvesting were cultivated in the field at the Southwest University, Chongqing, China.

The strains used in this study included the following: *Escherichia coli* DH5 α competent cells, *Agrobacterium tumefaciens* GV3101 competent cells, and *Saccharomyces cerevisiae* NMY51 competent cells.

2.2. Sample Collection and Processing

To obtain samples of ovules and fibers at corresponding developmental stages, all flowers were marked on the day of anthesis. 25 d after, leaves, roots, stems, flowers, and cotton bolls at different developmental stages were collected. The fibers were promptly separated from the ovules using autoclave-sterilized forceps and immediately frozen in liquid nitrogen. The same procedure was followed for samples obtained from in vitro ovule cultures, wherein fibers were similarly detached from the ovules with sterilized forceps and snap-frozen in liquid nitrogen. All samples were stored at -80°C for subsequent analysis.

2.3. In Vitro Cotton Ovules Culture and Fiber Length Measurement

Cotton flowers at 2 days post-anthesis (DPA) were collected for ovule culture in vitro. After removing petals and sepals, ovaries containing ovules were retained. The ovaries

were first surface-sterilized with 75% ethanol for 1 min, followed by sterilization with 0.3% hydrogen peroxide solution under continuous shaking for 10 min. Ovules were gently excised along their longitudinal axis by using a scalpel blade. Uniformly developed ovules were selected and transferred to Beasley and Ting's (BT) medium for incubation in a 32 °C dark environment. Cultured ovules were divided into groups with incubation durations of 5, 10, or 15 d, respectively.

After 15 d of culture on BT medium, a subset of ovules was randomly selected, observed under a stereomicroscope, and photographed to document their growth status. To prepare the fiber characterization, the cultured ovules were first soaked in 30% glacial acetic acid and then heated in boiling water to disperse the individual fibers. Then we placed the ovules on a glass slide, rinsed with water 3–5 times, and finally straightened the fibers for morphological measurement.

2.4. Total RNA Extraction from Plants and qRT-PCR

Total RNA was extracted from tissues, organs, ovules, and fibers at different developmental stages using the Plant Polysaccharide and Polyphenol RNA Rapid Extraction Kit (DP441, TIANGEN, Beijing, China). The obtained RNA was either stored at −80 °C or immediately used for cDNA synthesis. cDNA was synthesized by using HiScriptIII RT SuperMix for qPCR (+gDNA wiper) cDNA synthesis Kit (R323-01, Vazyme, Nanjing, China). qRT-PCR analysis was performed using the SYBR Green Pro Taq HS premixed qPCR kit (AG11701, Accurate Biology, Changsha, China) with the following program: 40 cycles of 95 °C for 3 min, 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s. The programmed temperature of the melting curve is 65 to 95 °C. The cycle threshold (CT) of each sample was standardized using HISTONE3 (GenBank accession AF024716), and the relative folding change (FC) of gene expression was calculated based on the $2^{-\Delta\Delta CT}$ method [46]. Each sample was repeated in three groups. The specific primers of qRT-PCR are shown in Table S1.

2.5. Measurement of P Contents

When measuring phosphorus concentration, we first prepared the chromogenic solution by mixing the ammonium molybdate–sulfuric acid test solution with 10% (*w/v*) ascorbic acid solution at a ratio of 6:1 for later use. Subsequently, 0.5 g of fresh sample was weighed, thoroughly ground using a mortar and pestle, and diluted to 10 mL with extraction solution (0.3 M trichloroacetic acid). After thorough mixing, the mixture was incubated at 4 °C for 1 h. Following centrifugation, 0.5 mL of the supernatant was transferred into a 2 mL centrifuge tube, mixed with 1 mL of the chromogenic solution, and incubated at 40 °C for 20 min. The absorbance was then measured at 820 nm using a spectrophotometer.

The 60 mg/L standard phosphorus solution was diluted with the extractant to prepare 0.6, 1.2, 2.4, 3.6, 4.8, and 6 mg/L phosphorus standard series solutions, respectively. The reaction solutions of the extractant and the working solution were used as blanks to establish the phosphorus standard curve. Finally, the content of available phosphorus in plants (g Pi/kg FW) was obtained based on the OD conversion value $Y \times 0.02$.

2.6. Subcellular Localization

The full-length ORF of *GhPHR1* was cloned into pCambia2300 of the CaMV 35S promoter to construct a plant expression vector containing the *GmPHR1*-eGFP fusion gene and obtain an over-expression vector of *GmPHR1*. The constructed 35S::GmPHR1-eGFP vector and nuclear marker NLS-mCherry were transformed into *Agrobacterium tumefaciens* strain GV3101 and injected into tobacco leaves for transient expression, respectively. The fluorescence signal was analyzed by confocal scanning microscopy (Leica TCS SP8; Leica Microsystems GmbH, Wetzlar, Germany) 2–3 d after injection. Three independent biological replicates were conducted on the experiment. The amplification primers are shown in Table S2.

2.7. Yeast One-Hybrid Assay

First, clone *GhPHR1* into the pGADT7-AD vector to construct pGADT7-GhPHR1. The about 200 bp *GhGCS1* promoter fragment containing the P1BS element sequence was cloned into the pAbAi vector to construct pAbAi-GhGCS1P as bait. The pAbAi-GhGCS1P vector was linearized by BstBI endonuclease and co-transformed with pGADT7-GhPHR1 and pGADT7-AD (negative control) to co-transform the yeast receptor NMY51, respectively. Carried DNA was placed in a PCR instrument at 95 °C for 5 min, pre-cooled on ice with PEG/LiAC conversion solution (PT1183-ZH, PYEAST, Wuhan, China). First, add 5 µL of the carried DNA to a 2 mL centrifuge tube, then add 1–3 µg of the corresponding plasmid, and finally add 100 µL NMY51. Add 500 µL of the pre-cooled conversion solution, gently flip, and incubate at 30 °C for 30 min. Add 20 µL filtered DMSO, and incubate at 42 °C for 15 min. Centrifuge at 4000 rpm for 5 min at the end and spread it onto SD/-Leu solid medium plates. Incubate in a 30 °C incubator for 2–3 d. The monoclonal clones were picked and activated and then cultured on SD-Leu selection medium containing the antibiotic basidiocin (ABA) (250 µg/mL) at 30 °C for 4 d.

2.8. LUC Activity Analysis

The *GhGCS1* promoter fragment (2500 bp) was ligated onto the pGreen0800 II-LUC vector to obtain the pGreen0800II-GhGCS1-LUC vector. It was transformed into *Agrobacterium tumefaciens* strain GV3101 (PSoupP19) along with the aforementioned 35S::GhPHR1-GFP vector and 35S::GFP vector (negative control), and then instantaneously transformed into tobacco leaves for co-expression. Two days after inoculation, the leaves and their surfaces were treated with D-luciferin. The LUC signal was detected using a plant imaging system in 10 min. In addition, referring to the method of Yan et al. [47], the total protein in the leaves was extracted using a plant total protein extraction kit, and the luciferase activity of the total protein solution was detected by an automatic microplate reader. The activity of the promoter was calculated using the value of firefly luciferase/renilla luciferase.

2.9. Hairy Root Transformation Experiment

Construct the overexpression vectors of *GhPHR1* according to the above method. We cloned the CDS reverse complementary sequence of *GhPHR1* and obtained the RNA-interference (RNAi) vector. Then, cotton hypocotyls were infected with *Agrobacterium rhizogenes* strain K599 containing the above vectors. About two weeks later, roots grew from the incision. Gene-specific primers were used to perform PCR experiments on the transgenic hairy roots (Table S2) to confirm the overexpression of functional gene hairy roots, and GUS staining was used to prove the acquisition of antisense-inhibited functional gene hairy roots. It was proved by qRT-PCR that the expression of functional genes in hairy roots was increased or decreased. Prove that transgenic hairy roots were obtained.

2.10. Plasmid/Vector Construction and Plant Transformation

To construct plant expression vectors, the pBI121 vector was modified. The recombinant vector pBI121-GN contained the fusion genes CaMV 35S promoter::GUS::nos terminator and nos promoter::NPTII::nos terminator. The cloned *GhGCS1* gene fragment was inserted into the pBI121-GN vector under the control of the CaMV 35S promoter, successfully generating the *GhGCS1* overexpression vector, designated as pBI121-GN-OGCS1. Additionally, a reverse complementary fragment of a partial *GhGCS1* sequence was inserted into the pBI121-GN vector to create an antisense *GhGCS1* fragment, yielding the antisense suppression vector named pBI121-GN-AGCS1. Both successfully constructed plasmids were introduced into the *Agrobacterium tumefaciens* strain LBA4404 [41].

Cotton plant transformation was subsequently performed following a previously described method. Cotton seeds were sterilized and germinated on MS (Murashige and Skoog) medium in the dark at 28 °C for 4–5 days. The LBA4404 strain harboring the respective plasmid was cultured in YEB medium (containing 100 mg/L kanamycin and 125 mg/L streptomycin) at 28 °C for 20 h. Bacterial cells were collected by centrifugation and resuspended in MS medium supplemented with 30 g/L glucose and 100 µM acetosyringone to an OD600 of 0.8–1.0 for subsequent use. The germinated cotton hypocotyls were cut into 1 cm segments, infected with the *Agrobacterium* suspension for 20–30 min, placed on sterile filter paper, and transferred to co-cultivation medium. After growth in the dark at 25 °C for 2 days, the explants were transferred to selection medium for further growth. Approximately four months later, kanamycin-resistant calli formed embryogenic calli on the selection medium. Following histochemical GUS staining, embryogenic calli that were both kanamycin-resistant and GUS-positive were cultured in MSB liquid medium. These cultures were then inoculated onto elongation medium. The elongated somatic embryos were transferred to Schenk and Hildebrandt medium containing 0.5% (*w/v*) activated charcoal. The transgenic plants obtained were identified by histochemical GUS staining of leaf tissues. Subsequently, GUS-positive plants were transplanted into the greenhouse for cultivation [48].

2.11. Statistical Analyses

We used MEGA11 (version 11.0.13 [49]) for phylogenetic tree construction, DNAMAN (Informer Technologies Inc., Los Angeles, CA, USA) for multiple sequence alignment, and TBtools (version TBtools-I [50]) for visualization analysis. The software used for statistical data analysis is GraphPad Prism (version 9.5.0; Boston, MA, USA). Values are means ± SD. Statistical data analysis was performed by the one-tailed Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, no significant.

3. Results

3.1. Phosphorus Deficiency and Excess Inhibited Fiber and Ovule Growth

To investigate the effects of phosphorus on the growth and development of cotton fibers and ovules, we cultured cotton ovules (with attached fibers) in the medium with various phosphorus concentrations in the ovule culture system *in vitro*. After growing for 10 d, growth was observed and statistically analyzed. The results showed that the growth of both ovules and fibers was severely inhibited in the medium without phosphorus and was suppressed both in the medium with a low concentration of phosphorus (0.25 mmol L⁻¹) and in the medium with a high concentration of phosphorus (1.0 mmol L⁻¹) (Figure 1A). Based on the fiber length, phosphorus concentrations of 0.5 mmol L⁻¹ and 0.75 mmol L⁻¹ were the optimal concentrations for fiber growth (Figure 1A,B). In contrast, based on the ovule size, 0.25 mmol L⁻¹ was the optimal phosphorus concentration for ovule growth, while concentrations of 0.5 mmol L⁻¹ and 0.75 mmol L⁻¹ exhibited inhibitory effects on ovule growth (Figure 1A,C,D). These results indicated that phosphorus concentration influenced the growth of fibers and ovules. Furthermore, the optimal phosphorus concentration for ovule growth differed from that for fiber growth. Compared to ovule growth, fiber cell growth has a higher demand for phosphorus. Under phosphorus deficiency, the ovules utilize their own phosphorus to complete ovule development as much as possible, while fiber growth is halted. These findings also suggest that phosphorus is precisely regulated in the development of ovules and fiber cells.

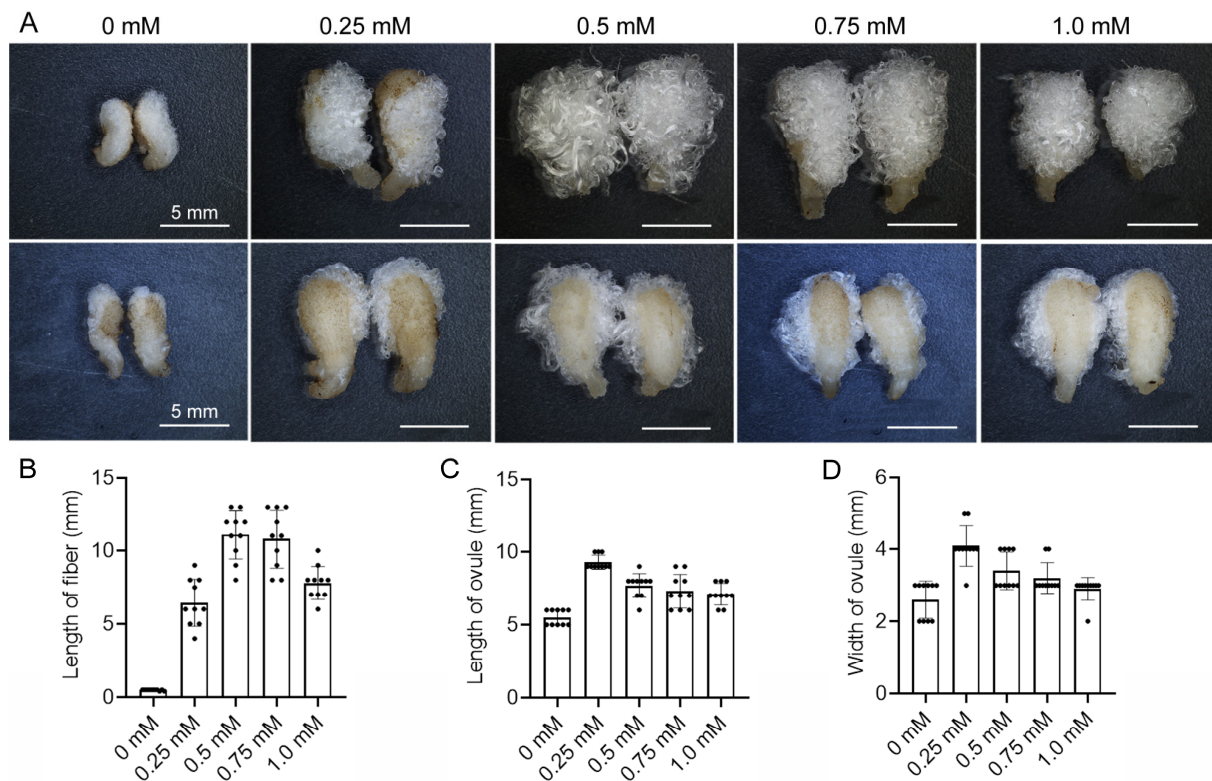


Figure 1. The effects of various concentrations of phosphorus on the growth of fibers and ovules. (A) The fibers (upper panel) and ovules (lower panel) after 10 d cultured in vitro. (B) The length of fiber cells from various phosphorus concentrations. (C) The length of ovules from various phosphorus concentrations. (D) The width of ovules from various phosphorus concentrations. SD represents the standard deviation from 10 cultured ovules.

3.2. Identification of GhPHR1

To explore the regulatory mechanism of phosphorus homeostasis in fibers and ovules, and given that phosphate starvation response 1 (PHR1) plays a key role in containing phosphorus homeostasis, we screened for PHR1 homologs in upland cotton (*Gossypium hirsutum* L.). There are four annotated *GhPHRs* in the upland cotton genome (Figure 2A). All four genes are highly expressed in the stage of fiber cell differentiation and initiation, especially the expression levels of *Gohir.D11G256400* and *Gohir.A11G246400* are far higher in −1 DPA and 1 DPA ovule than in the other samples (Figure 2A). Under different phosphorus treatments, the expression of *GhPHR1* (*Gohir.D11G256400*) was significantly upregulated in fibers and ovules under phosphorus-deficient conditions (Figure 2C), suggesting *GhPHR1* might play a role in phosphorus homeostasis of ovules and fiber cells. We further cloned the *GhPHR1* gene from cotton fiber cells. The resulting fragment is 1347 bp in length, containing an open reading frame (ORF) encoding 448 amino acid residues. The predicted molecular weight is 49.4 kDa, and the isoelectric point is 5.76. Multiple sequence alignment using *GhPHR1* and *PHR1* proteins from other species showed that *GhPHR1* possesses characteristic conserved domains of MYB family members, the MYB_SHAQKYF domain (225–279 AA) and the MYB_CC_LHEQLE domain (312–356 AA) (Figure 2B), indicating *GhPHR1* is a MYB family member and a homolog of *PHR1* in upland cotton.

Phylogenetic analysis indicated that *GhPHR1* is closely related to *PHR1* from *Gossypium barbadense* and *Glycine max*, but more distantly related to *PHR1* from *Oryza sativa* (Figure 2D). These results confirm that the cloned sequence is the *GhPHR1* gene, laying a foundation for further research.

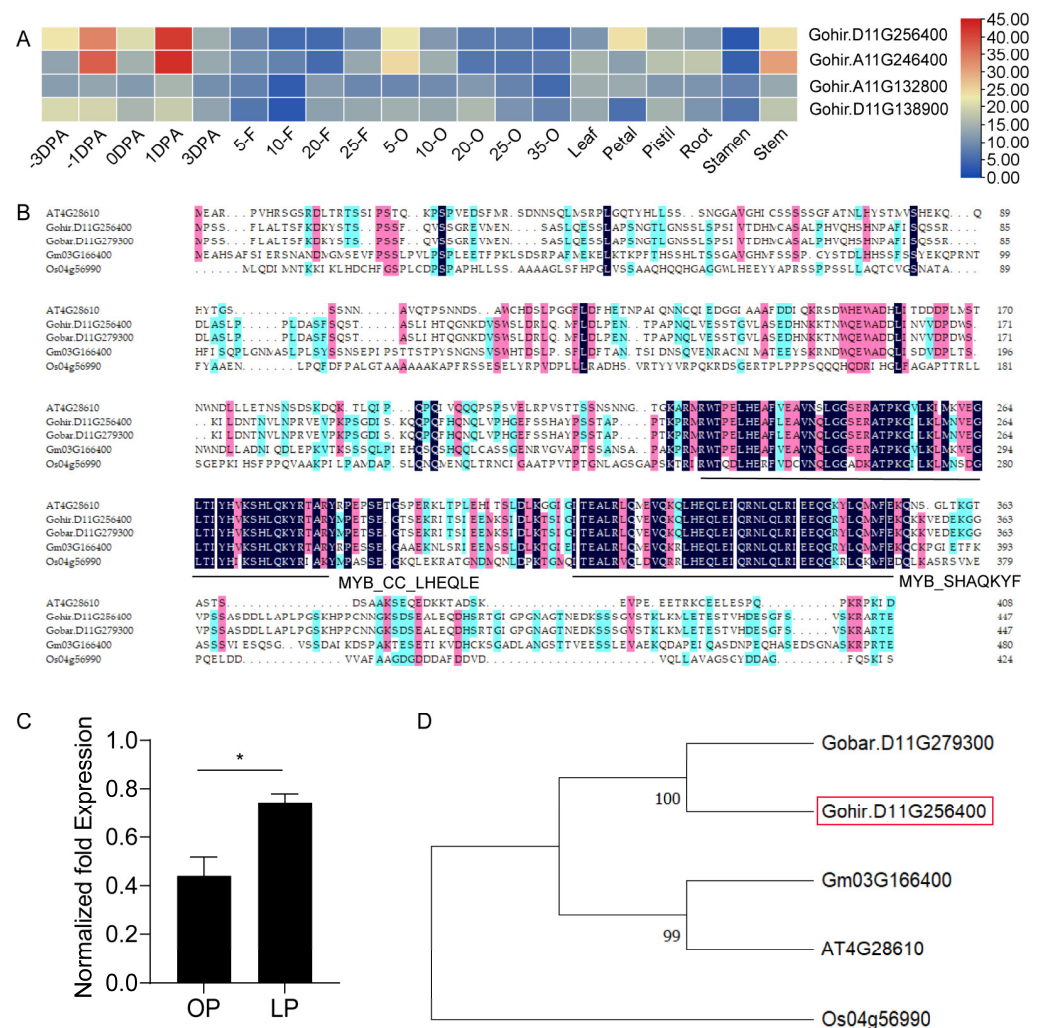


Figure 2. Sequence analysis of *GhPHR1*. (A) The expression profiles of four annotated *GhPHR1* in the upland cotton genome. (B) Multiple sequence alignment of *GhPHR1* with PHR1 from other species. (C) Expression of *GhPHR1* in ovules and fibers under phosphorus deficiency conditions. (D) Phylogenetic tree of *GhPHR1* and PHR1 from other species. Color coding is as follows: a dark background indicates residues identical in all five sequences; pink indicates identity in four sequences; blue indicates identity in three sequences. —3 DPA and —1 DPA: the ovules at 3 days and 1 day before anthesis; 5-F~25-F: fiber cells at 5 DPA~25 DPA; 5-O~35-O: ovules at 5 DPA~35 DPA. MYB_CC_LHEQLE and MYB_SHAQKYF are characteristic conserved domains of members of the MYB family. OP: P-sufficient condition (0.5 mM KH_2PO_4). LP: P-deficient condition (0 mM KH_2PO_4). Gobar.D11G279300: *Gossypium barbadense* PHR1. Gohir.D11G256400: *Gossypium hirsutum* PHR1. Gm03G166400: *Glycine max* PHR1. At4G28610: *Arabidopsis thaliana* PHR1. Os04G56990: *Oryza sativa* PHR1. The red box indicates the location of the target protein, *GhPHR1*. Data analysis was performed using a *t*-test. *, $p < 0.05$.

3.3. Subcellular Localization and Expression Analysis of *GhPHR1*

To investigate the subcellular localization of *GhPHR1*, we constructed a plant expression vector of *GhPHR1::eGFP* driven by the CaMV 35S promoter. We performed transient expression in tobacco leaf cells and observed the eGFP fluorescence signals using a confocal laser scanning microscope. The result revealed that the *GhPHR1*-eGFP fluorescence signal overlapped with the signal of the nuclear marker protein(NLS::mCherry) (Figure 3A), indicating that *GhPHR1* localized in nuclear. Gene expression patterns can provide important clues to elucidating gene function. To clarify the expression characteristics of the *GhPHR1* gene, we investigated the expression pattern of *GhPHR1* in various organs and tissues, as

well as in fibers and ovules at various developmental stages. The results showed that the expression level of *GhPHR1* was lower at 6–14 DPA than at 16–24 DPA (Figure 3C) during fiber development, while its expression remained relatively stable in the ovules at different stages (Figure 3D). *GhPHR1* was expressed in various floral tissues (Figure 3B). Given that PHR1 is a phosphorus starvation-responsive transcription factor, its lower expression during the rapid elongation stage of fiber cells (6–14 DPA) suggested that this stage might be associated with relatively high phosphorus content.

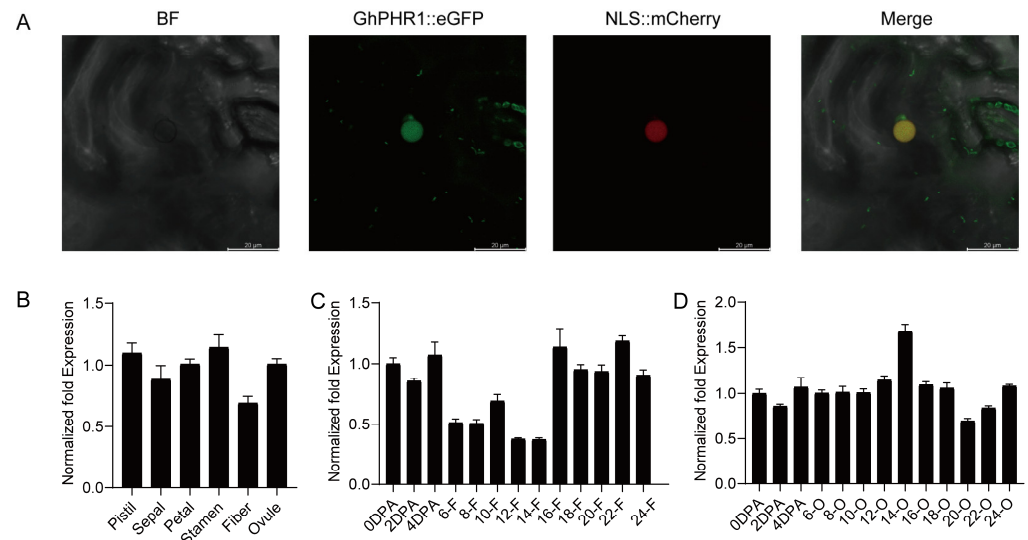


Figure 3. Subcellular localization and expression pattern of *GhPHR1*. (A) Subcellular localization of *GhPHR1*. (B) The expression of *GhPHR1* in different tissues of the floral organ. (C) The expression of *GhPHR1* at different developmental stages of the fiber cell. (D) The expression of *GhPHR1* at different developmental stages of the ovules. BF: bright field. 0 DPA~4 DPA: the fibers and ovules at 0 DPA~4 DPA; 6-F~24-F: fiber cells at 6 DPA~24 DPA; 6-O~24-O: ovules at 6 DPA~24 DPA.

3.4. *GhGCS1* Is a Target Gene of *GhPHR1*

PHR1 is a central transcription factor responding to phosphorus stress in plants. It binds to the PIBS (GNATATNC) cis-regulatory element and regulates target gene expression. To elucidate the relationship between *GhPHR1* and sphingolipid synthase genes in upland cotton, we analyzed the promoter sequences of several sphingolipid synthase genes in upland cotton. The results revealed that the promoter of the glucosylceramide synthase (*GhGCS1*) gene contains the PIBS element (GAATATGC), while other sphingolipid synthase genes, such as *GhKDSR1* (3-Ketosphingina nine reductase 1), *GhSBH1* (Sphingoid base hydroxylase 1), *GhSLD1* (Sphingoid LCB desaturase 1), and *GhORM1* (Orosomucoid protein 1), lack the PIBS element in their promoter sequences (Figure S1). *GhGCS1* is a key rate-limiting enzyme in sphingolipid synthesis, catalyzing the combination of glucose and ceramide to produce glucosylceramide. The result suggested that *GhGCS1* might be a target gene of *GhPHR1*.

To clarify the binding and regulatory characteristics of *GhPHR1* with the promoter of the *GhGCS1* gene, the promoter of *GhGCS1* was cloned into the pGreen0800II-LUC reporter vector, and a constitutive effector vector was constructed in which the CaMV35S promoter drives *GhPHR1* (Figure 4A). These vectors were co-expressed in tobacco leaves, and the fluorescence signal was investigated. The results demonstrated that the LUC intensity at the sites co-transformed with pGreen0800II-*GhGCS1*-LUC and *GhPHR1*::eGFP was significantly higher than that of the control group (Figure 4B). Moreover, we extracted the total protein from the leaves and detected the luciferase activity of the total protein solution by using an automatic microplate reader. The luciferase activity is far higher in the co-expressing *GhPHR1* and *GhGCS1p* sample than in the *GhGCS1p* sample (Figure 4C).

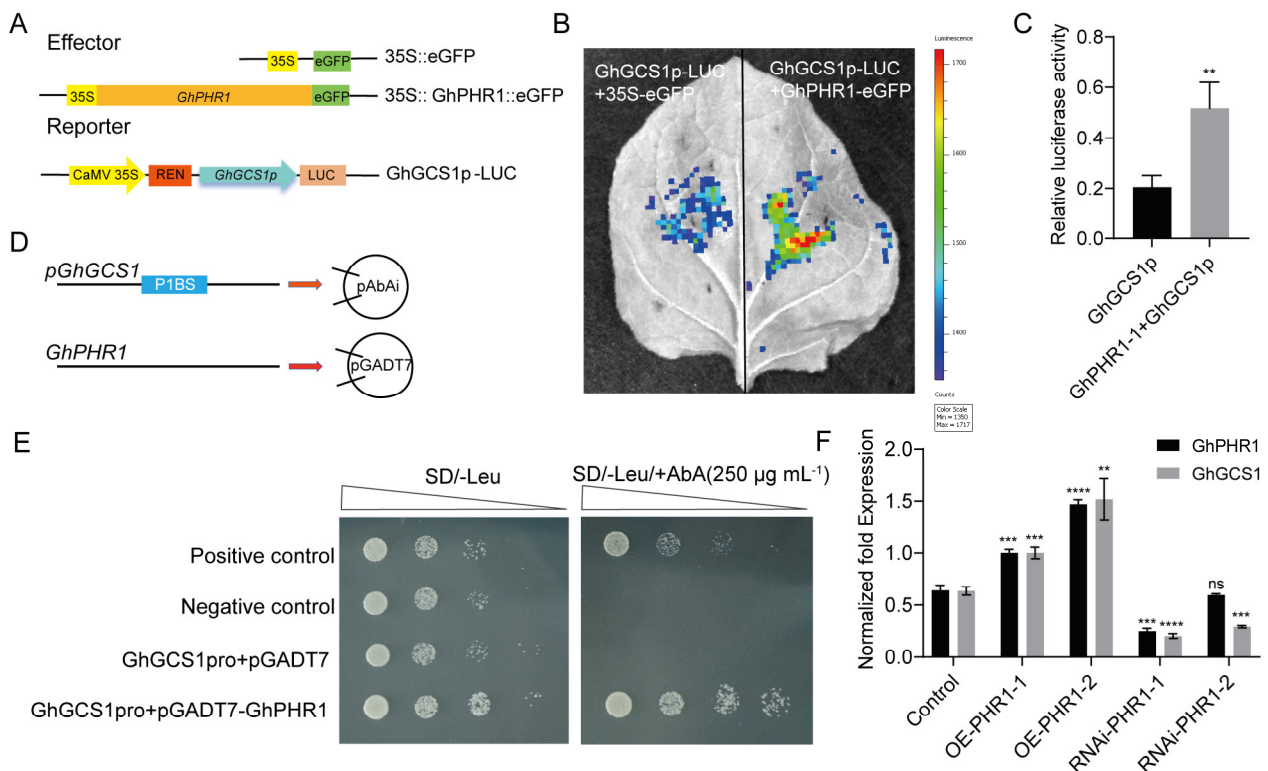


Figure 4. GhPHR1 directly binds to the *GhGCS1* promoter and positively regulates its expression. (A) The diagram of effector and reporter vectors in a dual-luciferase assay. (B) Dual-luciferase assay. (C) Relative luciferase activity in leaves. (D) The diagram of pAbAi-pGhGCS1 and pGADT7-GhPHR1 vectors. (E) Y1H assay. (F) The expression levels of *GhPHR1* and *GhGCS1* in transgenic roots. Positive control: pAbAi-p53 + pGADT7-53, Negative control: pAbAi-p53 + pGADT7. SD represents the standard deviation from three replications. Data analysis was performed by a *t*-test. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, no significant.

Subsequently, we conducted yeast one-hybrid assays and dual-luciferase experiments. First, a promoter fragment of *GhGCS1* containing the GAATATGC element was cloned into the pAbAi-bait vector, and the ORF of the *GhPHR1* gene was constructed into the pGADT7 vector (Figure 4D). The two resulting vectors were co-transferred into yeast competent cells and allowed to grow in media containing different concentrations of AbA. The results showed that yeast colonies co-transformed with pGADT7-GhPHR1 and pAbAi-GhGCS1 were able to grow in the medium with $250 \text{ ng} \cdot \text{mL}^{-1}$ AbA, similar to the positive control (Figure 4E). This indicates that GhPHR1 can directly bind to the promoter of the *GhGCS1* gene, confirming that *GhGCS1* is a target gene of the GhPHR1 transcription factor.

Next, in order to further clarify the regulatory effect of GhPHR1 on *GhGCS1*, we constructed the overexpression vector and RNA-interference (RNAi) vector of *GhPHR1* and obtained the corresponding transgenic complex through the hairy root transformation experiment. Additionally, we detected the expression of the *GhGCS1* gene in the transgenic hairy roots. The results indicated that *GhGCS1* expression was elevated in *GhPHR1*-overexpressing hairy roots but reduced in *GhPHR1*-RNAi hairy roots (Figure 4F), further demonstrating that GhPHR1 positively regulates the expression of *GhGCS1*. These results indicated that GhPHR1 directly binds to the *GhGCS1* promoter and positively regulates its expression.

3.5. Upregulating *GhGCS1* Gene Expression Promoted Phosphorus Uptake in Plants

To clarify the function of the *GhGCS1* gene in the uptake and utilization of phosphorus in cotton plants, we used the transgenic cotton seedlings of up- and downregulating

GhGCS1 in this assay. The transgenic cotton plants and wild-type plants were treated with phosphorus deficiency, and their phenotypes were observed. The results showed that under phosphorus deficiency conditions, the growth of the overexpression line was better than that of the wild-type (Figure 5A,B). Moreover, the height of the shoot and root length were significantly higher than those of the wild-type, and the fresh weight and phosphorus content were also higher than those of the wild-type (Figure 5C–E). The growth of the RNAi transgenic lines was inferior to that of the wild-type, and the height of the shoot and the root length were significantly lower than those of the wild-type. The fresh weight and phosphorus content were also lower than those of the wild-type. These results demonstrate that increasing *GhGCS1* gene expression can promote the uptake and transport of phosphorus in cotton plants.

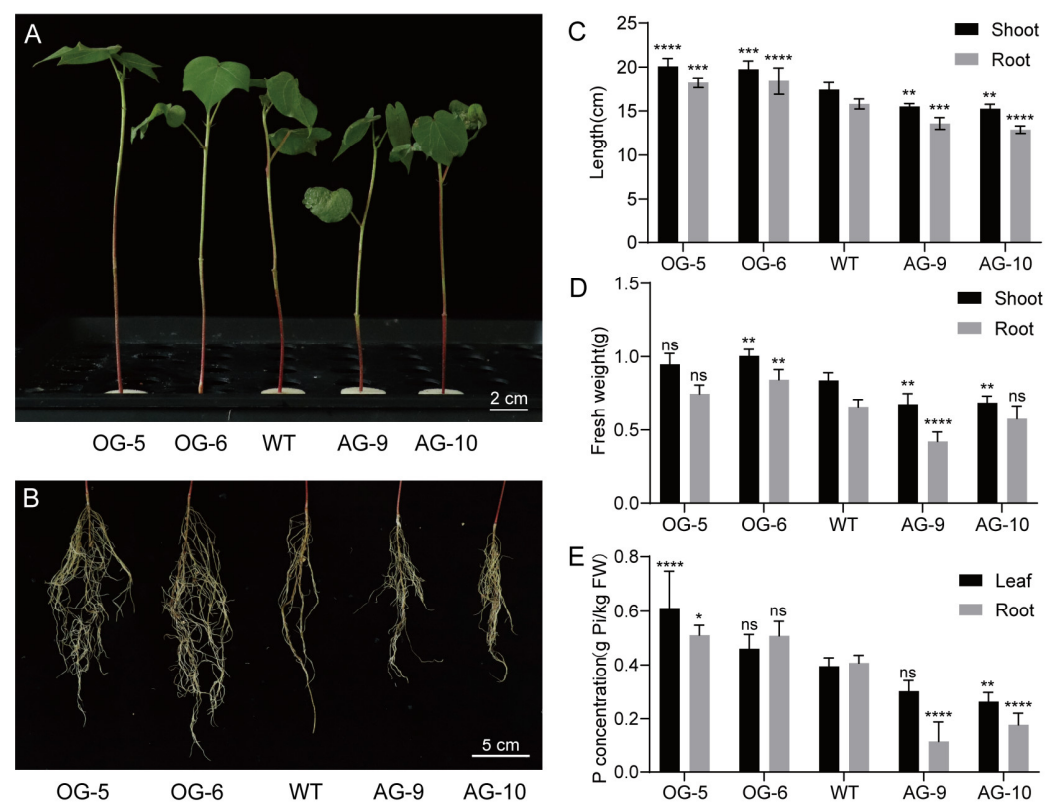


Figure 5. Up- or downregulating *GhGCS1* expression affects plant phosphorus uptake under P-deficient conditions. (A) Phenotypes of the aboveground parts of transgenic plants and the wild-type. (B) Root phenotypes of transgenic plants and the wild-type. (C) Shoot and root length. (D) Fresh weight. (E) Phosphorus content. OG-5, OG-6: overexpressing transgenic lines of *GhGCS1*. AG-9, AG-10: RNA interference (RNAi)-mediated down-regulation transgenic lines of *GhGCS1*. WT: wild-type Jimian 14. SD represents the standard deviation from three biological replications. Data analysis was performed by a *t*-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, no significant.

3.6. *GhGCS1* Promotes Phosphorus Uptake, Transport, and Utilization in Fibers

To investigate the role of *GhGCS1* in phosphorus metabolism during the growth and development of cotton fibers and ovules, we analyzed the expression of *GhGCS1* in cotton fibers and ovules grown in ovule culture systems under different phosphorus concentrations. The results indicated that under phosphorus deficiency conditions, the expression level of *GhGCS1* was significantly upregulated both in ovules and fibers (Figure 6A), and the increase was greater in ovules. It indicates that *GCS1* is involved in phosphorus metabolism in cotton fibers and ovules.

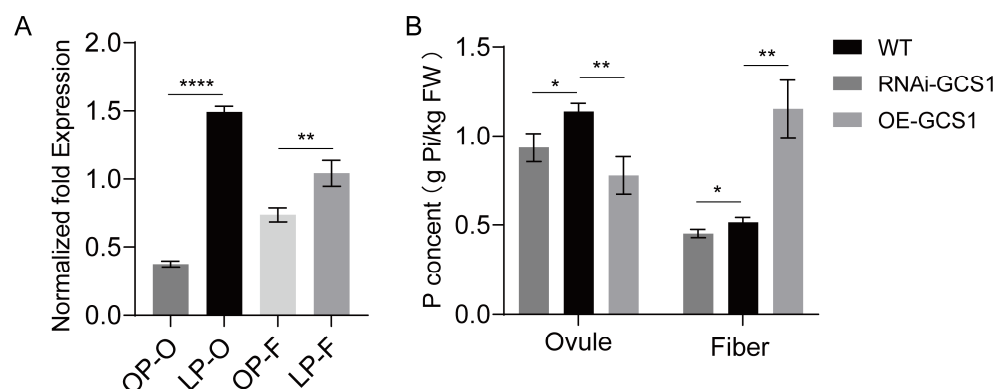


Figure 6. *GhGCS1* promotes phosphorus uptake, transport, and utilization in fibers. (A) The expression of *GhGCS1* in cotton fibers and ovules cultured in ovule culture systems with different phosphorus concentrations. (B) The phosphorus content in the fibers and ovules of transgenic cotton. OP-O: ovule in P-sufficient condition (0.5 mM KH_2PO_4). LP-O: ovule in P-deficient condition (0 mM KH_2PO_4). OP-F: fiber in P-sufficient condition (0.5 mM KH_2PO_4). LP-F: fiber in P-deficient condition (0 mM KH_2PO_4). WT: wild-type Jimian14. RNAi-GCS1: the RNAi transgenic lines of *GhGCS1*. OE-GCS1: the overexpression line of *GhGCS1*. SD represents the standard deviation from three biological replications. Data analysis was performed using a *t*-test. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

To clarify the effect of up- or downregulating the *GhGCS1* gene on the phosphorus content of transgenic ovule and fiber cell, we then measured the phosphorus content in the fibers and ovules of transgenic cotton. The results indicated that the phosphorus content in the fibers of the overexpression lines was much higher than that of the wild-type. On the contrary, the phosphorus content in the fibers of the RNAi transgenic lines was significantly lower than that of the wild-type (Figure 6B). Interestingly, the phosphorus content in the ovules of both the overexpression line and the RNAi transgenic lines was significantly lower than that of the wild type, with the reduction in phosphorus content being more significant in the overexpression line. Based on the above results, we speculate that the decrease in phosphorus content in the ovules of the RNAi transgenic line is caused by the reduced expression of *GhGCS1*, which is not conducive to the uptake of phosphorus by the plants.

This result further indicates that the overexpression of *GhGCS1* promotes not only phosphorus uptake, transport, and utilization in cotton but also facilitates phosphorus allocation to developing fibers.

3.7. *GhGCS1* Played a Role in Cotton Fiber and Ovule Growth

The GhPHR1 transcription factor responds to changes in phosphorus levels and regulates the expression of the *GhGCS1* gene in cotton ovule and fiber. It is hypothesized that altering the expression of *GhGCS1* may affect the growth of the fiber and ovule. To address this issue, we examined fiber length, as well as seed and embryo weight in transgenic cotton lines overexpressing or suppressing *GhGCS1* expression. The results showed that overexpression of *GhGCS1* increased the length of mature fibers, whereas suppression of this gene shortened fiber cell length (Figure 7A). Overexpression of *GhGCS1* also increased the size of seeds and embryos, while suppression of the gene reduced seed and embryo size (Figure 7B,C,E,F).

Since the lint percentage is the ratio of fiber weight to the total weight of fiber and seed, overexpression of *GhGCS1* had little effect on lint percentage, likely due to a significant increase in seed weight (seed index). In contrast, suppression of *GhGCS1* expression led to a reduction in seed weight and a decrease in lint percentage, indicating a greater decline in fiber weight (Figure 7D). These results demonstrate that the *GhGCS1* gene plays an important role in regulating the growth and development of fibers and ovules.

Furthermore, *GhGCS1* could promote phosphorus uptake and transport in cotton fibers, promote fiber cell elongation, and increase seed size and weight.

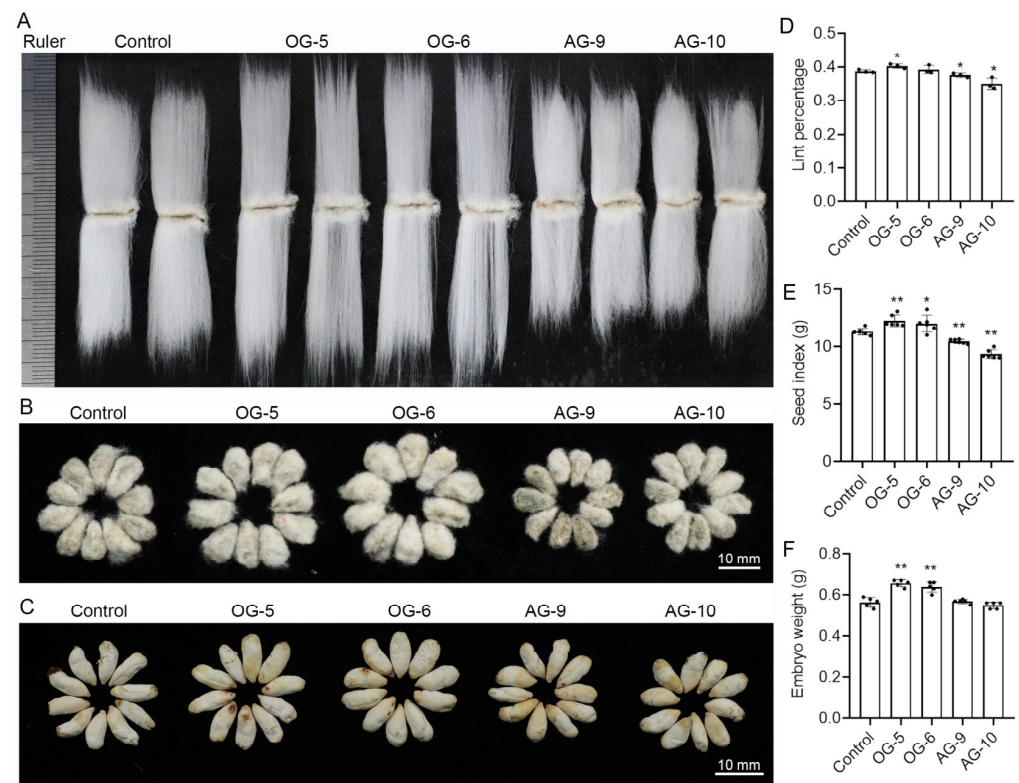


Figure 7. The effects of up- or downregulating *GhGCS1* expression on the growth of fibers and ovules. (A) The length of mature fiber from control plants and transgenic plants. (B) The size of the seed from control plants and transgenic plants. The unit of the ruler is mm. (C) The size of the embryo in control plants and transgenic plants. (D) The lint percentage of control plants and transgenic plants. (E) The seed index of control plants and transgenic plants. (F) The weight of the mature embryo from control plants and transgenic plants. Control: The transgenic cotton plant was generated by the empty vector. SD represents the standard deviation from three or six biological replications. Data analysis was performed using the method of the *t*-test. *, $p < 0.05$; **, $p < 0.01$.

4. Discussion

4.1. Phosphorus Is Important for the Growth of Ovule and Fiber While They Displayed Different Sensitivity to Phosphorus

Phosphorus, as one of the vital macronutrients for plant growth and development, functions as both an “energy hub” and a “structural cornerstone” for plant survival and growth. It participates in ATP synthesis to supply energy and serves as a key component of nucleic acids and phospholipids, thereby supporting fundamental cellular architecture [18]. Cotton fiber cells are not only the primary economic product of cotton but also an ideal model for studying plant cell growth and development [5]. However, the specific functions and mechanisms through which phosphorus directly influences fiber development remain unclear.

Under phosphorus-deficient conditions, both ovule growth and fiber elongation are severely suppressed, with fiber cell growth being relatively more inhibited and almost completely halted (Figure 1). At a low phosphorus level (0.25 mmol L^{-1}), fiber cell growth recovers to some extent, whereas ovule growth returns to normal. When phosphorus concentration reaches 0.5 mmol L^{-1} , fiber growth reaches its optimal value, but ovule growth begins to show some degree of inhibition. This indicates that fiber cells have a higher phosphorus demand than ovules and are more sensitive to phosphorus availability.

Under normal growth conditions, the phosphorus content in the ovules of wild-type cotton is higher than that in the fibers. However, in *GhGCS1*-overexpressing lines, phosphorus content in the fibers significantly increases, and that in the ovules significantly decreases (Figure 6B). This suggests that overexpression of *GhGCS1* can enhance phosphorus allocation from ovules to fibers, thereby promoting the growth of fibers. In contrast, the phosphorus content in both tissues of the antisense lines was significantly decreased, indicating that reducing the expression of the *GhGCS1* gene is not conducive to the uptake of phosphorus by cotton plants, weakens the transport effect of phosphorus from ovules to fibers, and thereby is not conducive to the elongation of fibers.

Under phosphorus-deficient conditions, cotton prioritizes ovule development to ensure reproductive success while restricting the growth of fiber cells, which are less critical for propagation. This differential phosphorus demand between ovules and fiber cells also suggests complex regulatory mechanisms governing phosphorus transport and utilization between them. Given that fiber cells originate from the outer epidermal layer of the ovule and given that lint percentage is defined as the ratio of fiber weight to the total weight of fibers and seeds, a critical agronomic trait in cotton production, it is hypothesized that elucidating phosphorus transport and utilization between cotton fibers and ovules holds significant theoretical value and practical application potential for improving lint percentage.

4.2. The *GhPHR1*-*GhGCS1* Molecular Module Is a Nutritional Regulatory Mechanism That Regulates Fiber Cell Growth

The MYB family is one of the most important classes of transcription factor families in plants. Phosphate starvation response 1 (PHR1), a member of the MYB transcription factor family, is a central transcription factor mediating plant responses to phosphate starvation [51,52]. It is well known that inorganic phosphate is essential for normal plant growth and development. However, in most natural environments and agricultural ecosystems, plants frequently face phosphorus deficiency due to low availability of inorganic phosphate [17,53–55]. Under phosphate-deficient conditions, PHR1 could bind to the P1BS element in the promoter of the Pi starvation-induced (PSI) gene, initiating downstream phosphate response pathways [51,56,57]. In this study, we detected a significant upregulation of the PHR1 homolog *GhPHR1* under phosphorus deficient condition, indicating that *GhPHR1* is a phosphate starvation-responsive transcription factor (Figure 2B). PHR1 proteins can bind to the P1BS cis-regulatory element in the promoters of target genes, thereby regulating their expression. Previous studies in *Arabidopsis* have demonstrated that PHR1 can regulate the expression of sphingolipid synthase genes, thereby modulating phosphate metabolism pathways [7]. By screening the promoter sequences of cotton sphingolipid synthase genes, we identified that the promoter of the *GhGCS1* gene contains a P1BS sequence (Figure S1). Through yeast one-hybrid and dual-luciferase assays, we further demonstrated that *GhPHR1* could directly bind to *GhGCS1* promoter and activate its expression (Figure 4), which is consistent with that from *Arabidopsis* [45].

Sphingolipids are essential both as key components of membranes and as bioactive molecules. Our previous studies revealed the role and mechanism of the glucosylceramide synthase and its product, glucosylceramide (GluCer), in cotton fiber growth [41,42,58]. *GhGCS1* is preferentially expressed during the fiber elongation stage and localizes to the endoplasmic reticulum. Its overexpression promoted GluCer synthesis and fiber elongation, consistent with the effects of exogenous application of GluCer (containing very long-chain unsaturated fatty acids) in ovule culture. Conversely, suppression of *GhGCS1* expression reduced both GluCer synthesis and fiber elongation, similar to the effect of the GluCer synthesis inhibitor PDMP. We further demonstrated that *GhGCS1* regulates the synthesis and signaling of the important plant hormone brassinosteroid during the promotion of fiber elongation [41]. Given the diverse regulatory functions of sphingolipids in plant growth and

development, and considering that nutrition and hormones are two closely interconnected and indispensable aspects during organism development, our study establishes a molecular regulatory network between sphingolipid and phosphate metabolism. In transgenic plants overexpressing *GhGCS1*, the contents of both GluCer and phosphorus increased, whereas in plants with suppressed *GhGCS1* expression, the levels of GluCer and phosphorus decreased (Figure 5). Collectively, these results suggest that *GhGCS1* and its product GluCer play important roles in both plant nutrient metabolism and hormone metabolism, potentially coordinating plant hormone and nutrient balance to regulate growth and development by perceiving environmental factors (sphingolipids are key functional components of membranes). Further in-depth research on the coordination mechanisms of these two aspects will help to elucidate the regulatory mechanisms of plant growth and development, and related studies are currently underway.

5. Conclusions

Cotton is a crucial economic crop globally and a major source of natural textile materials. Fiber quality is sensitive to phosphate fertilization, yet the specific role and regulatory mechanism of phosphorus in fiber cell growth remain poorly understood. This study investigated the effects of different phosphorus concentrations on cotton ovule and fiber development. The results demonstrate that phosphorus plays a critical role in fiber cell elongation and ovule growth, with differential sensitivity to phosphorus between ovules and fiber cells. We identified a phosphorus starvation-responsive transcription factor, GhPHR1, which localizes to the nucleus and is expressed in various tissues and organs of cotton, as well as at different developmental stages of fiber and ovules, with relatively lower expression during the fiber cell elongation stage. GhPHR1 directly binds to the promoter of *GhGCS1* and activates its expression. Overexpression of *GhGCS1* promotes sphingolipid synthesis in fiber cells, enhances phosphorus uptake and transport in cotton, increases phosphorus content in fiber cells, and enhances fiber elongation. Conversely, suppression of *GhGCS1* expression inhibits sphingolipid synthesis, inhibits phosphorus uptake and transport in cotton, reduces phosphorus content in fiber cells, and restrains fiber elongation. These findings demonstrate the importance of the GhPHR1-*GhGCS1* molecular module in regulating fiber elongation and elucidate the phosphorus nutrition regulatory mechanism underlying fiber elongation. This study provides insights into the intrinsic relationship between sphingolipid homeostasis and mineral nutrition during plant growth and development, offering a theoretical foundation for improving cotton fiber yield (e.g., increasing lint percentage) and quality (e.g., enhancing fiber length). Further study may focus on the coordination mechanisms of phosphorus, sphingolipid, and phytohormone, which will elucidate the regulatory mechanisms of plant growth under specific environmental conditions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16030324/s1>. Figure S1: Analysis of the promoter sequence of sphingolipid synthase gene in upland cotton; Table S1: Primers used for qRT-PCR; Table S2: Primers used for PCR.

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Abbreviations

The following abbreviations are used in this manuscript:

OP	P-sufficient condition (0.5 mM KH ₂ PO ₄)
LP	P-deficient condition (0 mM KH ₂ PO ₄)
OE	Overexpression
RNAi	RNA interference
WT	Wild type
Y1h	Yeast one-hybrid
OD	Optical density
OEF	Open reading frame
DPA	Day post-anthesis
cDNA	Complementary DNA
gDNA	Genomic DNA
qRT-PCR	Quantitative real-time PCR
min	Minute
d	Day
s	Second
h	Hour
GhGCS1	<i>Gossypium hirsutum</i> GCS1
GhPHR1	<i>Gossypium hirsutum</i> PHR1
GhGCS1p	<i>Gossypium hirsutum</i> GCS1 promoter

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