HyPRP1 performs a role in negatively regulating cotton resistance to \textit{V. dahliae} via the thickening of cell walls and ROS accumulation

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Abstract

**Background:** Developing tolerant cultivars by incorporating resistant genes is regarded as a potential strategy for controlling Verticillium wilt that causes severe losses in the yield and fiber quality of cotton.

**Results:** Here, we identified the gene \textit{GbHyPRP1} in \textit{Gossypium barbadense}, which encodes a protein containing both proline-rich repetitive and Pollen Ole e I domains. \textit{GbHyPRP1} is located in the cell wall. The transcription of this gene mainly occurs in cotton roots and stems, and is drastically down-regulated upon infection with \textit{Verticillium dahliae}. Silencing HyPRP1 dramatically enhanced cotton resistance to \textit{V. dahliae}. Over-expression of \textit{HyPRP1} significantly compromised the resistance of transgenic \textit{Arabidopsis} plants to \textit{V. dahliae}. The \textit{GbHyPRP1} promoter region contained several putative phytohormone-responsive elements, of which SA was associated with gene down-regulation. We compared the mRNA expression patterns of \textit{HyPRP1}-silenced plants and the control at the global level by RNA-Seq. A total of 1735 unique genes exhibited significant differential expression. Of these, 79 DEGs involved in cell wall biogenesis and 43 DEGs associated with the production of ROS were identified. Further, we observed a dramatic thickening of interfascicular fibers and vessel walls and an increase in lignin in the \textit{HyPRP1}-silenced cotton plants compared with the control after inoculation with \textit{V. dahliae}. Additionally, silencing of \textit{HyPRP1} markedly enhanced ROS accumulation in the root tips of cotton inoculated with \textit{V. dahliae}.

**Conclusions:** Taken together, our results suggest that \textit{HyPRP1} performs a role in the negative regulation of cotton resistance to \textit{V. dahliae} via the thickening of cell walls and ROS accumulation.

**Keywords:** Cotton, Hybrid proline-rich protein (HyPRP), \textit{Verticillium dahliae}, Cell wall protein, Reactive oxygen species (ROS), Virus-induced gene silencing (VIGS)

Background

The plant cell wall contains a large set of structural proteins that are involved in defense response. Cell wall proteins are unusually rich in one or two amino acids and contain highly repetitive sequence domains. Currently, much is known about their sequence information, but there is little direct evidence of the functions of these proteins [1]. Hybrid proline-rich proteins (HyPRPs) form a subgroup of putative plant cell wall glycoproteins enriched in proline. HyPRPs are composed of three different domains: a hydrophobic signal peptide, a repetitive proline-rich domain in the N-terminus, and a hydrophobic C-terminal domain, not specifically rich in proline or glycine but containing cysteine. Based on the abundance and specific distribution of cysteine in this C-terminal domain, the HyPRPs are subdivided into A and B groups. In group A, four or six cysteine residues are present in a specific pattern (-CXXC-C-C-C-C-), while group B contains eight cysteine residues (termed the eight-cysteine motif, 8CM) in a specific order (-C-C-CC-CXC-C-C-) in the C-terminal domain [2]. Multiple studies have indicated that...
group B HyPRPs play various functional roles in specific developmental stages and in response to biotic and abiotic stresses. For example, CaHyPRP1 performs distinct dual roles as a negative regulator of basal defense and in the positive regulation of cell death in Capsicum annuum against Xanthomonas campestris [3]. GmHyPRP is involved in triggering the soybean resistance response to Phakopsora pachyrhizi [4]. The HyPRP gene EARLI1 (Early Arabidopsis Aluminum Induced 1) is induced in Arabidopsis by low temperature and salt stress [5]. GhHyPRP4 has been reported to take part in the cold stress response of Gossypium hirsutum [6]. Overexpression of CcHyPRP from Cajanus cajan increased resistance to multiple abiotic stresses in yeast and Arabidopsis [7]. However, the roles of group A HyPRPs in plant development and defense against pathogen attacks remain unclear, in contrast to the relatively better characterized proteins of group B. As of now, only one group A protein, PvPRP1, has been researched extensively. PvPRP1 can be down-regulated by Colletotrichum lindemuthianum and up-regulated by wounding in the hypocotyls of French bean (Phaseolus vulgaris) [8]. The down-regulated expression of PvPRP1 in response to fungal infection is due to mRNA destabilization through the binding of PRP-BP (PvPRP1 mRNA binding protein) to a 27-nucleotide U-rich domain in the 3′ untranslated region of PvPRP1 mRNA [9].

Verticillium dahliae Kleb is a soil-borne pathogenic fungus capable of causing vascular wilt disease in cotton (Gossypium spp.). In most cotton-growing areas, Verticillium wilt has become the most important disease of Gossypium hirsutum [10]. Unfortunately, currently available fungicides are not effective in protecting cotton from this vascular disease infection. Therefore, developing tolerant cotton cultivars by incorporating genes from resistant germplasm is now regarded as the most effective strategy for controlling this disease. Genetic dissection of Verticillium wilt resistance at the molecular level, as mediated by the relevant genes, will enhance our ability to utilize the existing germplasm to reduce cotton yield losses [11].

In recent years, high-throughput technology has been used to systematically monitor expression profiles and screen a wide spectrum of differentially expressed genes/proteins in cotton inoculated with V. dahliae with the goal of ultimately using genetic engineering to breed resistant cultivars. A total of 188 differentially expressed proteins were identified in the roots of Gossypium barbadense upon infection with V. dahliae based on comparative proteomics analysis [12]. Moreover, 3442 unigenes related to defense responses against V. dahliae were identified in G. barbadense cv. 7124 using RNA-Seq [13]. In addition, a total of 3027 Vetricillium-resistance unigenes were identified from a full-length cDNA library of G. barbadense cv Pima90–53 [14]. These comprehensive gene and protein expression data provide helpful molecular information. However, deeper insights into understanding the defense mechanisms of cotton in response to V. dahliae are needed.

Previously, we obtained a substantial number of transcript sequences from G. barbadense related to defense responses against V. dahliae [14]. Of these, a cDNA clone that encodes a group A HyPRP protein, designated as GhHyPRP1, whose expression was significantly down-regulated in cotton after V. dahliae inoculation. In the present study, GhHyPRP1 and its homologous genes were cloned from other G. hirsutum cultivars. The transcriptional expression of HyPRP1 was investigated in different tissues and in response to treatment with different hormones and V. dahliae. A potential role of HyPRP1 in negatively regulating plant resistance to V. dahliae was examined by overexpression in Arabidopsis and by virus-induced gene silencing (VIGS) in cotton. We applied transcriptomic analysis to systematize the molecular mechanisms underlying the HyPRP1-mediated cotton defensive response to V. dahliae. The important function of HyPRP1 involved in cotton resistance to V. dahliae via the thickening of the cell wall and ROS accumulation was proved.

Results

GhHyPRP1 is a cell wall protein down-regulated by V. dahliae challenge

Previously, we isolated a full-length cDNA clone from a full-length cDNA library of G. barbadense Pima90–53 challenged with V. dahliae [14]. The cDNA clone has a 5′ untranslated region (5′ UTR) of 41 bp, 3′ UTR of 186 bp with a polyA tail and an open reading frame (ORF) of 945 bp that potentially encodes a 314-amino acid protein. The protein consists of a 26-residue signal peptide (SP) sequence with an initial ATG codon, a 165-residue proline-rich domain (PRD) (containing a basic histidine-rich domain) at the N-terminus and a 123-residue hydrophobic C-terminus Pollen Ole e 1 domain (cysteine-containing domain) (Fig. 1a). Based on the sequence features and a homology search, the protein belongs to group A HyPRPs, designated as GbHyPRP1 (GenBank accession number KP162172). Gb HyPRP1 has a predicted molecular weight of ~33.59 kDa with a theoretical pI of 9.97. The alignment results showed that GbHyPRP1 from G. barbadense shared a high sequence identity with the other GhHyPRP1 proteins from six cultivars of G. hirsutum (Additional file 1: Figure S1).

A GFP gene fused to the C-terminal end of GbHyPRP1 under the control of the constitutive CaMV 35S promoter was successfully transformed into tobacco epidermal cells and transiently expressed. The control GFPs appeared to be distributed throughout the whole cell,
which indicated that GFPs were localized to plasma membrane and cytoplasm. By contrast, the GFP signal indicated that the fusion HyPRP1-GFP proteins were localized to the cell periphery (Fig. 1b). In addition, GbHyPRP1 belongs to group A HyPRPs (Fig. 1a), which form a subgroup of plant cell wall glycoproteins enriched in proline. Thus, we inferred that HyPRP1 localized to the cell wall.

As shown in Fig. 1c, GbHyPRP1 was expressed in all tested parts of the cotton plants, with a significantly higher expression level in the stem compared with the leaf. In addition, the expression profiles of GbHyPRP1 in response to the highly aggressive defoliating V. dahliae strain were examined in infected G. barbadense roots and stems. The qPCR (real-time quantitative PCR) analysis showed that the expression level of GbHyPRP1 significantly decreased in either stems or roots after inoculation with V. dahliae (Fig. 1d). These data indicate that GbHyPRP1 is involved in the cotton-V. dahliae interaction.

**V. dahliae-responsive expression of HyPRP1 exhibits the same trend in G. hirsutum as in G. barbadense**

We further determined the V. dahliae-responsive expression of HyPRP1 in G. hirsutum using a resistant cv. ND601 (DI = 22.63 ± 2.28) and a susceptible cv. CCRI8 (DI = 57.59 ± 2.76) [15] inoculated with V. dahliae (Fig. 2a). The results showed that the expression of HyPRP1 dramatically decreased in both resistant and susceptible cotton stems and roots after inoculation with V. dahliae compared to the control (Fig. 2b). Moreover, the expression of HyPRP1 in the resistant cv. ND601 was significantly lower compared to the susceptible cv. CCRI8 (Fig. 2b). These results ulteriorly indicated that HyPRP1 is a negative regulator involved in the cotton-V. dahliae interaction.
Silencing of \textit{HyPRP1} enhances cotton resistance to Verticillium wilt

We further tested whether \textit{HyPRP1} was required for cotton resistance to Verticillium wilt using VIGS, which is frequently employed as a reverse genetics technique. At approximately two weeks post-infiltration, marker gene \textit{CLA1}-VIGS plants started to display the albino phenotype in the true leaves (Additional file 2: Figure S2A). At the same time, the expression of \textit{HyPRP1} in susceptible \textit{CCRI8} was not detected using semi-RT-PCR, indicating that \textit{HyPRP1} had been silenced (Additional file 2: Figure S2B). \textit{HyPRP1}-silenced plants were used for Verticillium inoculation. At 15 days post inoculation (dpi), less chlorosis and fewer wilting leaves were observed in VIGS plants compared to the control (Fig. 3a). The DI of VIGS plants (32.67 ± 1.96) was significantly lower than that of the control (81.00 ± 2.13), i.e., susceptible \textit{CCRI8} became tolerant (Fig. 3b). VIGS assays indicated that silencing of \textit{HyPRP1} significantly enhances cotton resistance to Verticillium wilt.

Over-expression of \textit{GbHyPRP1} compromises \textit{Arabidopsis} resistance to Verticillium wilt

To further determine the involvement of \textit{GbHyPRP1} in Verticillium wilt resistance, plant over-expression vectors containing the \textit{GbHyPRP1} ORF driven by a constitutive

\textbf{Fig. 2} Time-related changes in the expression of \textit{HyPRP1} in response to \textit{V. dahliae} (Vd) in \textit{G. hirsutum}. \textbf{a} ND601 showed higher tolerance to \textit{V. dahliae} (left panel) with lower disease index (right panel) compared to \textit{CCRI8} at 14 dpi. Two-week-old seedlings were dip-infected with the \textit{V. dahliae} spores. The disease indices were presented means ± SE from three biological replications with at least 15 plants per replication. \textbf{b}, The expression of \textit{HyPRP1} changed to a lower degree both in stems and roots of tolerant ND601 and susceptible \textit{CCRI8} after infection using \textit{V. dahliae} comparing to the control at the same hpi. Meanwhile, the relative expression of \textit{HyPRP1} displayed more lower both in stems and roots of tolerant ND601 comparing to the susceptible \textit{CCRI8} after infection using \textit{V. dahliae} at the same hpi. The relative gene expression was calculated using the comparative \(2^{-\Delta\Delta Ct}\) method with \textit{PP2A1} as endogenous control gene. Values are shown as the mean ± SE of three biological replicates. \textit{Sidak’s} multiple comparisons test demonstrated that there were significant differences (\(*P < 0.01, **P < 0.001\)) between Vd and control.

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35S promoter were constructed and transformed (Agrobacterium-mediated) into Arabidopsis thaliana. Three lines, L1, L2 and L3, were used for further analyses. After 15 dpi with V. dahliae, all the transgenic lines exhibited more wilting and etiolation compared to WT (wild type) (Fig. 4a). The average DI of transgenic plants, i.e., 69.5 (classified as susceptible), was significantly higher than that of the control (29.8; classified as tolerant) (Fig. 4b). These results suggest that over-expression of GbHyPRP1 compromised Arabidopsis resistance to Verticillium wilt.

GbHyPRP1 contains hormone elements in the promoter and is down-regulated by SA

The upstream region of GbHyPRP1, named pGbHyPRP1, was 1431 bp in length based on sequencing (Additional file 3: Figure S3). pGbHyPRP1 fused with the reporter gene GUS introduced into Arabidopsis plants, and GUS staining confirmed that the promoter of GbHyPRP1 functioned well with respect to the expression of the reporter gene (Fig. 5a). Promoter search using PLANTCARE indicated that several potentially inducible cis-regulatory elements corresponding to hormone, defense and pathogen elicitor responses were found in pGbHyPRP1 (Fig. 5b). Thus, we investigated the expression profile of GbHyPRP1 following treatments with plant hormones, including SA (salicylic acid), abscisic acid (ABA), jasmonic acid (JA) and ethylene (ET). The results indicated that the expression of GbHyPRP1 was strongly down-regulated by SA at 12, 24, 36 and 48 hps (hours post-spraying), whereas it was significantly up-regulated by ABA, JA and ET at various time points (Fig. 5c). To our surprise, the HyPRP1 transcript levels in the SA-treated plants were quite similar to those in the V. dahliae-inoculated cotton seedlings (Figs. 1d and 2b). These results imply that HyPRP1 may play roles in cotton resistance to V. dahliae through the SA-mediated signaling pathway.

Transcriptome analysis of VIGS cotton indicated HyPRP1 influences genes related to cell wall remodeling and ROS balance

To better understand the molecular mechanisms of HyPRP1-mediated cotton defense response to V. dahliae, we employed a combined approach of VIGS and RNA-Seq to compare the mRNA expression patterns of HyPRP1-silenced and control plants at the global level. Transcriptome libraries yielded 56,364,572 to 63,092,290 raw reads. After cleaning and quality checks, 55,741,348 to 62,384,322 high quality reads were obtained, and 79.66–80.87% of these reads were uniquely mapped to G. hirsutum L. acc. TM-1 [16], representing more than forty thousand unigenes (RPKM ≥1)(Table 1). Under V. dahliae stress, a total of 1735 unique genes exhibited significant differential expression based on VIGS compared to the control, including 816 up-regulated and 919 down-regulated genes (P-value ≤ 0.05) (Additional file 4: Data S1).

Of these, we identified 79 differentially expressed genes (DEGs) that might be involved in cell wall
biogenesis in cotton according to the annotation from the cell wall genomics webserver (https://cellwall.genomics.purdue.edu/intro/index.html) (Table 2). Expansins and xyloglucan-modifying enzymes are usually up-regulated by various stresses and then become involved in cell wall remodeling [17]. Our RNA-Seq results indicated that the expression of six expansin genes (Gh_A10G1374, Gh_A05G2385, Gh_A05G3493, Gh_A13G0050, Gh_D04G1924 and Gh_D05G2650) and seven xyloglucan endotransglucosylase/hydrolase (XEH) genes (Gh_A02G1426, Gh_A11G1910, Gh_D03G0294, Gh_D11G2065, Gh_A11G0455, Gh_D05G1444 and Gh_D02G1371) were significantly up-regulated in HyPRP1 VIGS cotton seedlings inoculated with V. dahliae compared to the control. Additionally, reactive oxygen species (ROS) have been shown to be associated with cotton

Fig. 4 Over-expression of GbHyPRP1 compromised Arabidopsis resistance to Verticillium wilt. a Phenotype comparison of transgenic lines (L1, L2 and L3) and the WT inoculated with V. dahliae. Photographs were taken at 15 dpi. Expression of GbHyPRP1 was confirmed using semi-qRT-PCR. The AtActin gene was used as a control. b The disease index was measured at 15 dpi. Error bars represent the SE of three biological replicates with at least 20 plants per replication.

Fig. 5 Properties of pGbHyPRP1. a Histochemical analysis of GUS activity in Arabidopsis expressing the pGbHyPRP1-GUS chimeric gene. GUS activity was present in whole 7-day-old seedlings. b Location of cis-regulatory elements involved in hormone- and elicitor-responsive elements found in pGbHyPRP1. The conserved fungus-responsive elements ELI-box3 (−216) were present in the promoter region of WRKY in the Populus, while the ERE (elicitor responsive element) (−879) are the binding site of WRKY and required for elicitor responsiveness in the promoter of PR (pathogenesis-related protein) in the parsley [64, 65]. TC-rich repeat elements (−502) are putatively involved in plant defense and stress response. Furthermore, ABRE (ABA-responsive element) (−292), TCA-element and ERE (ethylene responsive element) (−1128) have been reported in the upstream region of many genes that show regulated expression in response to ABA, SA and Et, respectively [26–28]. c Expression profiles of GbHyPRP1 in leaves of 15-day-old Pima90–53 seedlings subjected to SA, ABA, JA and ET. Control indicates water treatment. Three plants at each time point were sampled and analysed. Values are presented as the means ± SE in three independent experiments. Asterisks represent significant differences with respect to the control as determined by Tukey’s multiple comparisons test (*P < 0.05, **P < 0.01; ***P < 0.001; ns = not significant).
resistance to fungal pathogens [18]. Here, we found 43 DEGs associated with the production and scavenging of H$_2$O$_2$ and O$_2^-$, which allows regulation of dynamic changes in ROS levels (Table 3). Thus, our results suggest that HyPRP1 as a cell wall structural protein might have a potential role in cotton resistance to V. dahliae infection through remodeling of the cotton cell wall and ROS production.

Silencing of HyPRP1 causes a drastic increase in cell wall thickness and the lignin content

To further determine the effect of silenced HyPRP1 on the cell wall, the cotton stems were histologically examined. 10-day-old seedlings with two fully expanded cotyledons were used for VIGS. After two weeks later HyPRP1-silenced plants were inoculated with V. dahliae. At 14 dpi, cross sections of the basal part of stems revealed that the thickness of interfascicular fiber walls increased obviously in the HyPRP1-silenced plants compared with the control (Fig. 6a and a’). Moreover, we examined vessel walls in ultra-thin sections using transmission electron microscopy, which revealed a dramatic reduction in thickness (Fig. 6b and b’). These results demonstrated that silencing of HyPRP1 does not affect the development of the cell wall under a pathogen-free condition, but repression of HyPRP1 expression significantly enhances cell wall thickening in response to V. dahliae. Further, lignin contents were estimated in cell wall residues. The data showed that HyPRP1-silenced plants had higher lignin content compared with the control at 0 dpi, 7 dpi and 14 dpi (Fig. 7a-c). Accordingly, the autofluorescence of lignified cell walls in these HyPRP1-silenced plants was more intense and covered a greater area compared with the control (Fig. 7d-i).

Silencing of HyPRP1 enhanced ROS accumulation in root tips of cotton infected with V. dahliae

We evaluated whether HyPRP1 silencing would result in cotton generating more ROS in response to V. dahliae infection. DAB and NBT staining showed that HyPRP1 VIGS cotton had significantly higher levels of O$_2^-$ and H$_2$O$_2$ compared to the control under V. dahliae infection (Fig. 8a and b). In particular, VIGS plants not only showed darker staining approximately one millimeter from the tip, but staining was also detected in the upper part of the root (Fig. 8a and b). DCFH-DA staining further confirmed enhanced ROS levels in HyPRP1 VIGS cotton roots compared to the control (Fig. 8c). These results suggest that HyPRP1 is involved in ROS production in cotton infected with V. dahliae.

Discussion

HyPRP1 is a negative regulator in cotton resistance to Verticillium wilt

The roles of HyPRPs in response to multiple abiotic and biotic factors such as cold, salinity, drought and pathogens have been inferred primarily from their expression profiles [3, 8, 19–22]. Remarkably, all of these HyPRP genes have been reported to be up-regulated by abiotic factors and down-regulated upon infection with pathogens, e.g., HyPRP1 in C. annuum and N. benthamiana following P. capsici infection [3] and PvPRP1 in cell cultures of P. vulgaris treated with an elicitor [8]. Similarly, in our study, transcriptional suppression of HyPRP1 in roots of both resistant and susceptible cotton was also detected after V. dahliae infection (Figs. 1d and 2b). This indicates that HyPRP1 is involved in the interaction between cotton and Verticillium. Further, based on loss- and gain- of function studies comprising VIGS and over-expression in cotton and Arabidopsis plants, respectively, we showed that HyPRP1 was negatively correlated with resistance to V. dahliae (Figs. 3 and 4). Thus, it is reasonable to speculate that HyPRP1 functions in the process of cotton resistance to Verticillium wilt as an important negative regulator.

Table 1 Summary of sequencing, read processing, mapping, and differential expression analysis

| Sample names & Replicates | Control-12 hpi (C12) | VIGS-12 hpi (V12) |
|---------------------------|---------------------|------------------|
|                           | C12–1               | V12–1            |
| Raw reads                 | 60,758,592          | 59,201,676       |
| Clean reads               | 60,228,094          | 58,628,694       |
| Q20 (%)                   | 96.12               | 96.13            |
| Q30 (%)                   | 92.44               | 92.46            |
| GC content (%)            | 44.62               | 45.03            |
| Total mapped              | 56,294,373 (93.47%) | 54,610,037 (93.15%) |
| Uniquely mapped           | 46,067              | 42,211           |

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| Stages of cell wall biogenesis | Gene   | Cotton ID | Arabidopsis ID | Biological process description                          | log2FC | padj     |
|--------------------------------|--------|-----------|----------------|----------------------------------------------------------|--------|----------|
| 1. Pathways of substrate generation | MPG     | Gh_D13G1445 | AT4G26850      | Mannose-1-phosphate guanylyltransferase                   | 1.3226 | 0.00074702 |
|                                 | MPG     | Gh_A04G0114 | AT4G26850      | Mannose-2-phosphate guanylyltransferase                   | 1.0154 | 0.0038664 |
|                                 | MPG     | Gh_D05G3607 | AT4G26850      | Mannose-3-phosphate guanylyltransferase                   | 0.87197 | 0.016364  |
|                                 | PAL2    | Gh_D06G0758 | AT3G53260      | Phersylanine ammonia-lyase 2                             | -4.7679 | 0.031477  |
|                                 | COMT    | Gh_D08G2702 | AT5G54160      | O-methyltransferase 1                                     | -2.1454 | 1.04E-05  |
| 2. Polysaccharide synthases and glycosyl transferases | CSLDS   | Gh_D12G1289 | AT1G02730      | Cellulose synthase-like D5                               | -1.8163 | 4.86E-06  |
|                                 | CSLDS   | Gh_A12G1169 |               |                                                         | -1.7886 | 0.00023721 |
|                                 | CSA2    | Gh_D05G2313 | AT4G39350      | Cellulose synthase A2                                     | 0.94515 | 0.0088594 |
|                                 | UGT     | Gh_A01G1073 | AT5G65550      | UDP-Glycosyltransferase superfamily protein               | -1.0906 | 0.0034716  |
|                                 | UGT     | Gh_A08G0702 | AT5G12890      |                                                         | 1.3521  | 0.0044964  |
|                                 | UGT2    | Gh_D02G0230 | AT1G05530      | UDP-glucosyl transferase 75B2                             | 1.7995  | 0.04585   |
|                                 | UGT     | Gh_A12G0455 | AT3G21780      | UDP-glucosyl transferase 71B6                             | 1.1842  | 0.026133  |
|                                 | GT35    | Gh_A13G0714 | AT3G29320      | Glycosyl transferase, family 35                          | -1.4741 | 0.0049557  |
| 3. Secretion and targeting pathways | DJC24   | Gh_A11G1350 | AT4G12780      | DNA J protein C24                                         | 0.93104 | 0.032233  |
|                                 | DJC24   | Gh_D03G0085 | AT2G17880      |                                                         | 0.83759 | 0.036168  |
|                                 | DJC75   | Gh_D05G2948 | AT4G09350      | DNA J protein C75                                         | 1.28    | 0.00014472 |
|                                 | DJC75   | Gh_A05G2646 |               |                                                         | 1.3309  | 0.00072978 |
|                                 | DNJ     | Gh_D11G2787 | AT1G56300      | Chaperone DnaJ-domain superfamily protein                 | -2.2389 | 1.13E-09  |
|                                 | DNJ     | Gh_D12G1290 |               |                                                         | 0.84609 | 0.048095  |
|                                 | DNJ     | Gh_A11G2469 |               |                                                         | -2.4022 | 2.03E-10  |
|                                 | DNJ     | Gh_A12G1170 |               |                                                         | 1.2513  | 0.00021456 |
| 4. Assembly, architecture, and growth | CML12   | Gh_Sca058336G01 | AT2G41100 | Calmodulin-like 12                                       | 1.1651  | 0.00085002 |
|                                 | CML12   | Gh_A05G1998 |               |                                                         | 0.97104 | 0.03541   |
|                                 | CML12   | Gh_D07G0377 |               |                                                         | 0.9888  | 0.0122    |
|                                 | CML30   | Gh_A11G3105 | AT2G15680      | Calmodulin-like 30                                        | 0.90289 | 0.019411  |
|                                 | CaBP    | Gh_D01G1024 | AT1G73630      | Calcium-binding EF-hand family protein                   | 1.1131  | 0.033647  |
|                                 | CaBP    | Gh_D05G2265 | AT1G21550      |                                                         | 1.1609  | 0.00045229 |
|                                 | CaBP    | Gh_A05G2022 |               |                                                         | 1.0194  | 0.02553   |
|                                 | EXP5    | Gh_A10G1374 | AT3G29030      | Expansin A5                                               | 1.0678  | 0.015918  |
|                                 | EXP8    | Gh_A05G2385 | AT2G40610      | Expansin A8                                               | 1.1376  | 0.00071198 |
|                                 | EXP8    | Gh_A13G0050 |               |                                                         | 1.2031  | 0.00047444 |
|                                 | EXP8    | Gh_D04G1924 |               |                                                         | 1.0126  | 0.020419  |
|                                 | EXP8    | Gh_D05G2650 |               |                                                         | 1.0878  | 0.0013803 |
|                                 | EXP11   | Gh_A05G1576 | AT1G20190      | Expansin A11                                              | -1.5462 | 0.00073074 |
|                                 | XTH6    | Gh_A02G1426 | AT5G65730      | Xyloglucan endotransglycosylase/hydrolase 6               | 1.0514  | 0.0026916  |
|                                 | XTH6    | Gh_A11G1910 |               |                                                         | 1.7179  | 0.0045263  |
|                                 | XTH6    | Gh_D03G0294 |               |                                                         | 1.3393  | 0.00049521 |
|                                 | XTH6    | Gh_D11G2065 |               |                                                         | 1.3826  | 4.78E-05  |
|                                 | XTH7    | Gh_A11G0455 | AT4G37800      | Xyloglucan endotransglycosylase/hydrolase 7               | 1.0837  | 0.025074  |
|                                 | XTH16   | Gh_D05G1444 | AT3G23730      | Xyloglucan endotransglycosylase/hydrolase 16              | 1.0522  | 0.022367  |
|                                 | XTH28   | Gh_D02G1371 | AT1G14720      | Xyloglucan endotransglycosylase/hydrolase                 | 0.96782 | 0.021935  |
HyPRP1 is potentially down-regulated by SA in cotton resistance against *V. dahliae*

Upon pathogen attack, plants can rapidly initiate an immune response that is regulated by specific phytohormones, which vary greatly in composition, quantity and timing [23, 24]. Through analysis of differential gene expression and transcription profiling of cotton inoculated with *V. dahliae*, SA-, ABA-, JA- and ET-mediated signaling pathways have been proven to contribute to *V. dahliae* resistance [14, 25]. ABRE (ABA-responsive element)

### Table 2 DEGs involving in cell wall biogenesis according to the annotation from the cell wall genomics webserver (Continued)

| Stages of cell wall biogenesis | Gene   | Cotton ID | Arabidopsis ID | Biological process description | log2FC | padj  |
|-------------------------------|--------|-----------|----------------|-------------------------------|--------|-------|
| 28                            | GH17   | Gh_A01G0299 | AT2G39640      | Glycosyl hydrolase family 17 protein | -2.7529 | 0.0037067 |
|                               | GH17   | Gh_A05G0191 |                |                                | -1.5162 | 0.0061888 |
|                               | GH17   | Gh_Sca016465G01 |            |                                | -1.5804 | 0.023458  |
|                               | GHB13  | Gh_D03G0779 | AT4G02290      | Glycosyl hydrolase 9B13        | -1.7925 | 0.0030996 |
|                               | GH32   | Gh_A06G0779 | AT3G13790      | Glycosyl hydrolases family 32 protein | -1.6456 | 0.011594  |
|                               | BG3    | Gh_D06G2277 | AT3G57240      | Beta-1,3-glucanase 3           | -2.4743 | 0.041623  |
|                               | PLL    | Gh_A03G0087 | AT5G47500      | Pectin lyase-like superfamily protein | -3.5536 | 1.03E-10  |
|                               | PLL    | Gh_A10G1707 | AT1G65570      |                                | -4.0659 | 0.0010399 |
|                               | PLL    | Gh_D03G1564 | AT4G13710      |                                | -2.7246 | 7.22E-10  |
|                               | PLL    | Gh_D05G3049 |                |                                | -1.5099 | 0.011417  |
|                               | PLL    | Gh_D12G2158 |                |                                | -2.0499 | 0.0090874 |
|                               | GRP    | Gh_D10G1727 | AT3G06780      | Glycine-rich protein           | 1.0437  | 0.036293  |
|                               | HRGP   | Gh_A06G1050 | AT3G25690      | Hydroxyproline-rich glycoprotein family protein | -1.3346 | 0.027913  |
|                               | HRGP   | Gh_D11G0769 | AT3G02120      |                                | -1.742  | 0.00058494 |
|                               | GER3   | Gh_A05G3949 | AT5G20630      | Germin 3                       | -1.8095 | 0.012068  |
|                               | TPX1   | Gh_D05G1251 | AT1G65980      | Thioredoxin-dependent peroxidase 1 | 1.0563  | 0.029966  |
| 5. Differentiation and secondary wall formation | PRX    | Gh_A07G0275 | AT2G24800      | Peroxidase superfamily protein | -1.5209 | 0.0086829 |
|                               | PRXR1  | Gh_A05G0507 | AT2G24800      |                                | -2.0256 | 0.037339  |
|                               | PRX52  | Gh_A09G2334 | AT5G05340      |                                | 1.4635  | 0.0033052 |
|                               | PRX53  | Gh_D08G2420 | AT5G06720      |                                | -3.5232 | 0.0049155 |
| 6. Signaling and response mechanisms | PR5    | Gh_A01G1376 | AT4G38670      | Pathogenesis-related thaumatin superfamily protein | 1.3177  | 0.0017125 |
|                               | PR5    | Gh_A03G0347 | AT2G28790      |                                | 1.6781  | 0.031724  |
|                               | PR5    | Gh_D12G0310 | AT4G38670      |                                | 1.3177  | 0.0017125 |
|                               | LTPG   | Gh_A08G0720 | AT1G55260      | Lipid-transfer protein         | 0.81073 | 0.046398 |
|                               | LTPG   | Gh_A08G1543 | AT2G45180      |                                | 1.9495  | 0.022371 |
|                               | LTPG   | Gh_D08G1844 |                |                                | 1.2797  | 0.011976  |
|                               | LTPG   | Gh_A07G0235 |                |                                | 0.88365 | 0.016833  |
|                               | LYM2   | Gh_A12G0303 | AT2G17120      | Lysm domain GPI-anchored protein 2 precursor | -1.5665 | 0.040813 |
|                               | LYM2   | Gh_D12G0361 |                |                                | -1.4063 | 0.0022 |
|                               | SKS4   | Gh_A06G1309 | AT4G22010      | SKUS similar 4                 | -1.3884 | 0.046854  |
|                               | SKS4   | Gh_D06G1637 |                |                                | -1.8953 | 0.0019284 |
|                               | PMEI   | Gh_A08G1555 | AT5G20740      | Plant invertase/pectin methylesterase inhibitor superfamily protein | 1.3284  | 0.00058494 |
|                               | PMEI   | Gh_D03G1026 | AT5G62360      |                                | -2.289  | 3.90E-13  |
|                               | PMEI   | Gh_D05G0356 |                |                                | 1.1199  | 0.0018667 |
|                               | PMEI   | Gh_D08G1863 |                |                                | 1.2925  | 0.00051083 |
|                               | CIF1   | Gh_D10G1801 | AT1G47960      | Cell wall / vacuolar inhibitor of fructosidase 1 | 1.1969  | 0.00078794 |
|                               | CIF1   | Gh_A10G1552 |                |                                | 0.85616 | 0.047869  |
| Cotton ID | log2FC | padj   | Arabidopsis ID | Biological function                                    |
|---------|--------|--------|----------------|--------------------------------------------------------|
| Gh_D05G1251 | 1.0563 | 0.029966 | AT1G65980      | Thioredoxin-dependent peroxidase 1                     |
| Gh_D10G1930 | −2.4149 | 6.56E-07 | AT3G06730      | Thioredoxin z                                         |
| Gh_A10G1673 | −2.3963 | 0.0013815 | AT3G06730      | Thioredoxin z                                         |
| Gh_A05G3692 | −1.1174 | 0.006171 | AT1G76760      | Thioredoxin Y1                                        |
| Gh_D11G1225 | 1.4097 | 2.15E-05 | AT2G30540      | Thioredoxin superfamily protein                       |
| Gh_D09G1113 | −1.432 | 8.29E-05 | AT2G31840      | Thioredoxin superfamily protein                       |
| Gh_D08G2582 | 1.5176 | 0.0029746 | AT4G33040      | Thioredoxin superfamily protein                       |
| Gh_D05G2291 | 0.96122 | 0.0058703 | AT4G03520      | Thioredoxin superfamily protein                       |
| Gh_D05G0426 | 1.1171 | 0.012918 | AT4G33040      | Thioredoxin superfamily protein                       |
| Gh_A12G0064 | 1.0842 | 0.03541 | AT2G30540      | Thioredoxin superfamily protein                       |
| Gh_A11G1072 | 1.4971 | 8.01E-06 | AT2G30540      | Thioredoxin superfamily protein                       |
| Gh_A09G1107 | −1.1404 | 0.0022929 | AT2G31840      | Thioredoxin superfamily protein                       |
| Gh_A05G2047 | 1.0221 | 0.0026406 | AT4G03520      | Thioredoxin superfamily protein                       |
| Gh_A05G0320 | 2.1073 | 0.0079978 | AT4G33040      | Thioredoxin superfamily protein                       |
| Gh_A01G0925 | 0.93891 | 0.0080796 | AT3G51030      | Thioredoxin H-type 1                                  |
| Gh_D12G1273 | −0.85546 | 0.036125 | AT2G47470      | Thioredoxin family protein                            |
| Gh_D07G2378 | −2.2432 | 0.0089634 | AT4G29720      | Polyamine oxidase 5                                   |
| Gh_A08G1751 | 1.1958 | 0.0029528 | AT5G21105      | Plant L-ascorbate oxidase                             |
| Gh_A11G0771 | 0.86508 | 0.024855 | AT1G01820      | Peroxin 11c                                           |
| Gh_D07G0417 | 0.83262 | 0.032604 | AT3G47430      | Peroxin 11B                                          |
| Gh_A07G0355 | 1.0949 | 0.0017285 | AT3G47430      | Peroxin 11B                                          |
| Gh_A09G2334 | 1.4635 | 0.0033052 | AT5G05340      | Peroxidase superfamily protein                       |
| Gh_A07G0275 | −1.5209 | 0.0006829 | AT2G24800      | Peroxidase superfamily protein                       |
| Gh_A05G0507 | −2.0256 | 0.037339 | AT2G24800      | Peroxidase superfamily protein                       |
| Gh_D08G2420 | −3.5232 | 0.0049155 | AT5G06720      | Peroxidase 2                                         |
| Gh_D01G1856 | −1.675 | 0.01222 | AT1G77510      | PDI-like 1–2                                         |
| Gh_A05G3724 | −1.0492 | 0.012948 | AT1G21750      | PDI-like 1–1                                         |
| Gh_A04G0409 | 0.88963 | 0.01631 | AT1G17180      | Glutathione S-transferase TAU 25                     |
| Gh_A04G0830 | 0.77974 | 0.047869 | AT1G59700      | Glutathione S-transferase TAU 16                     |
| Gh_D13G0032 | −2.0914 | 0.045894 | AT5G01420      | Glutaredoxin family protein                           |
| Gh_D12G0079 | 1.7875 | 0.010834 | AT2G47880      | Glutaredoxin family protein                           |
| Gh_D11G0244 | 1.1813 | 0.00090549 | AT1G64500      | Glutaredoxin family protein                           |
| Gh_A11G0230 | 1.2723 | 0.0011017 | AT1G64500      | Glutaredoxin family protein                           |
| Gh_A09G1302 | −1.7113 | 0.047546 | AT5G03870      | Glutaredoxin family protein                           |
| Gh_A05G2978 | −2.0779 | 7.21E-07 | AT5G40760      | Glucose-6-phosphate dehydrogenase 6                  |
| Gh_D07G0457 | 1.2396 | 0.036439 | AT5G51100      | Fe superoxide dismutase 2                            |
| Gh_A07G0392 | −1.4372 | 0.0035132 | AT5G51100      | Fe superoxide dismutase 2                            |
| Gh_D11G1719 | −0.94414 | 0.01944 | AT1G65930      | Cytosolic NADP+ – dependent isocitrate dehydrogenase |
| Gh_A05G0722 | −2.2649 | 0.0088139 | AT2G28190      | Copper/zinc superoxide dismutase 2                   |
| Gh_D06G0021 | 1.3352 | 0.023812 | AT4G35090      | Catalase 2                                            |
| Gh_A13G0827 | 1.1257 | 0.0022698 | AT1G08570      | Atypical CYS HIS rich thioredoxin 4                  |
| Gh_D10G2577 | 1.2429 | 0.034842 | AT5G65110      | Acyl-coa oxidase 2                                   |
(-292), TCA-element and ERE (ethylene responsive element) (-1128) have been reported in the upstream region of many genes that showed regulated expression in response to ABA, SA and ET, respectively [26–28]. In this paper, these potential cis-regulatory elements were also found in pGbHyPRP1 (Fig. 5b). Furthermore, the expression profiles of GbHyPRP1 following treatment with these phytohormones were examined. The expression of GbHyPRP1 was significantly down-regulated by SA but up-regulated by ABA, JA and ET (Fig. 5c). Activation of complicated and concerted phytohormone signaling networks is an important regulatory mechanism of immunity employed by plants. In many cases, these hormones interact antagonistically or synergistically with each other [29]. Generally, pathogens that require a living host (biotrophs) are implicated in SA-mediated defense responses, whereas pathogens that kill the host and feed on the contents (necrotrophs) are associated with JA/ET-mediated defenses [23, 30]. Interestingly, however, V. dahliae is a hemibiotrophic phytopathogenic fungus. Thus, we infer that HyPRP1 taking part in cotton resistance to V. dahliae is probably regulated by a complex phytohormone signaling network. The HyPRP1 transcript levels showed quite similar down-regulation in SA-treated and V. dahliae-inoculated cotton seedlings (Figs. 1d, 2b and 5c). Additionally, V. dahliae infection significantly increased SA levels in G. thurberi [31], G. hirsutum and G. barbadense seedlings (our unpublished data). Thus, we speculate that HyPRP1 may be mainly and negatively regulated by SA signaling in cotton resistance against V. dahliae.

**HyPRP1 participates in complex interactions within the cell wall polymer network in cotton infected with V. dahliae**

Cell wall proteins are essential constituents of plant cell walls and are involved in modification of the cell wall structure [32]. Arabinogalactan protein 31 (AGP31), a remarkable plant cell wall protein, comprises an SP, a short AGP domain, an His-stretch, a PRD and a PAC (PRP-AGP containing Cys) domain [33–35]. Arabidopsis AGP31 is able to bind methylesterified polygalacturonic acid, possibly through its His-stretch, and to interact with itself in vitro through its PAC domain [34]. Similarly, cotton HyPRP1 also contains an SP, a basic histidine-rich domain embedded within the PRD and a cysteine-containing domain embedded within the Pollen Ole e I domain (Fig. 1a and Additional file 1: Figure S1). The cell wall, a physical barrier that pathogens need to breach to colonize the host plant, is typically reinforced with the phenolic polymer lignin [36, 37]. Lignin is believed to play a critical role in the resistance of cotton to V. dahliae [13]. By comparative transcriptome analysis of HyPRP1-silenced cotton plants and the control inoculated with V. dahliae, 79 DEGs potentially involved in cell wall biogenesis were identified (Table 2). Of these genes, Gh_D13G1445 was described as a Mannose-1-phosphate guanylyltransferase catalyzing the production of GDP-mannose. Arabidopsis cyt1 (encodes mannose-1-phosphate guanylyltransferase) mutant cause changes in the cell wall composition, such as dramatic decrease in cellulose content [38]. UDP-glycosyltransferases (UGTs) can influence the resistance of plants to infection by pathogenic microorganisms through regulating the
glycosylation of phenylpropanoid and phenylpropanoid-derived compounds, which are essential for the synthesis of lignin [39]. The xyloglucan endotransglucosylase/hydrolases (XTHs), specifically hydrolyzing xyloglucan as a substrate, are considered to be involved in the construction and restructuring of xyloglucan cross-link in plant cell wall [40]. Calmodulin, a highly conserved Ca$^{2+}$-binding protein, acts as an intermediary connecting Ca$^{2+}$ signals involved in plant defence reactions [41]. Most of DEGs mentioned above have higher transcript levels in HyPRP1-silenced plants, suggesting that HyPRP1 is a negative regulator of cell wall-related genes. Further, we observed a dramatic thickening of interfascicular fiber walls and vessel walls (Fig. 6) and an increase in lignin (Fig. 7) in the HyPRP1-silenced cotton plants compared with the control after inoculation with V. dahliae. On the other hand, some DEGs were down-regulated, which seems to be a negative impact on cell-wall thickening and lignin accumulation. For example, phenylalanine ammonia-lyase (PAL) is the first committed enzyme in the phenylpropanoid biosynthesis, which engenders a variety of precursors of important secondary metabolites, mainly including flavonol glycosides and lignin. In our study, PAL2 (Gh_D06G0758) was shown to be significantly downregulated. We inferred that PAL transcription was feedback regulated by particular biosynthetic intermediates [42], which means that the expression of PAL may not be always positive correlation with lignin accumulation. Although the roles of many cell wall proteins have been studied broadly, the knowledge on the interaction between components is lacking. Thus, we speculated that HyPRP1 participates in complex interactions within the cell wall polymer network in cotton infected with V. dahliae.

**HyPRP1 contributes to Veticillum defense by enhancing ROS accumulation**

ROS have been studied extensively for their roles in interactions between plants and foliar pathogens. Little is known about ROS synthesis and function in defense reactions of the root, but ROS are consistently observed to accumulate in a plant after the perception of pathogens [18]. In the Verticillium-cotton interaction, the generation of H$_2$O$_2$ was observed in the roots of cotton infected with V. dahliae [43]. Moreover, transgenic tomato plants expressing the Ve resistance gene accumulated...
H₂O₂ upon *V. dahliae* infection [44]. Likewise, transgenic cotton plants expressing a fungal endochitinase gene were more resistant and accumulated ROS faster than the control following pathogen inoculation [45]. These results indicate that cotton plants infected with *V. dahliae* are accompanied by increased ROS accumulation. In our study, silencing of *HyPRP1* markedly enhanced ROS accumulation in the root tips (Fig. 8). Therefore, we reasonably inferred that cotton negatively modulates *HyPRP1* transcription to generate ROS used for defense against *Verticillum*. Further studies are needed to elucidate the roles of ROS in cotton resistance to *Verticillum*. However, we suggest that ROS perform three possible functions: (i) ROS are primary immune signaling molecules [46]; (ii) ROS mediate cell wall modifications [47]; and (iii) ROS are important modulators that play a role in defense-related protein post-translational modifications [48].

**Conclusions**

Based on our research and existing developments on how plants resist *Verticillum* wilt, we propose a model of *HyPRP1*-mediated cotton defense against *V. dahliae*. Upon *V. dahliae* attack, recognition by cotton plants results in the activation of immune responses, including the production of a specific combination of the signals...
such as SA and JA, which have been proved to be involved in the plant-V. dahliae interaction [14, 25]. The expression of HyPRP1 is most likely and mainly down-regulated by SA signaling. A significant reduction in HyPRP1 may affect the cell wall polymer network, including an increase in the thickness of the cell wall and the content of lignin required to prevent V. dahliae infection. Alternatively, down-regulation of HyPRP1 obviously enhances the accumulation of ROS, which could mediate the establishment of a cotton defensive response to V. dahliae.

Methods
Plant materials and growth conditions
The cotton seeds G. barbadense cv. Pima90–53, G. hirsutum cv. CCR18 and ND601 were preserved at the North China Key Laboratory for Crop Germplasm Resources of Education Ministry, Hebei Agricultural University, Baoding, China. For transcriptional analysis of HyPRP1 in different tissues and response to V. dahliae by qPCR, two-week-old cotton seedlings were cultivated on Murashige and Skoog (MS) medium and were inoculated with V. dahliae as described by Zhang et al. [14]. For VIGS and the hormone treatment experiment, seeds were sterilized in 20% (V/V) commercial bleach (the final concentration of sodium hypochlorite was approximately 1%) for 20 min followed by washing four times with distilled water. Seeds were soaked in distilled water for 2 days and then germinated on wet towels for another 2 days at 25°C. Germinant seeds were transferred to pots containing commercially sterilized soil (a mixture of soil, peat, and composted pine bark) and covered with a plastic dome in a growth room at 25°C under a 14-h light/10-h dark cycle. The A. thaliana accession Columbia was grown in commercially sterilized soil at 22°C, 70% relative humidity, and ~150 μE m⁻² s⁻¹ under a 9-h photoperiod.

V. dahliae cultivation
A highly aggressive defoliating fungus, V. dahliae strain Linxi2–1, was isolated from a symptomatc upland cotton plants growing in agricultural fields near Linxi, Hebei Province, China, and preserved in North China Key Laboratory for Crop Germplasm Resources of Education Ministry, Hebei Agricultural University. V. dahliae was cultivated on potato dextrose agar (PDA) plates for 10 d and then inoculated into Czapek’s broth on a shaker at 150 rpm for 1 week at 25°C in the dark. Spores were harvested by filtration through folded Fisherbrand® lens paper and were re-suspended in sterile distilled water to a specific density.

Cloning of HyPRP1 and the GbHyPRP1 promoter region
GbHyPRP1 was identified from a full-length cDNA library of G. barbadense Pima90–53 [14]. The full-length cDNA of HyPRP1 from other upland cotton cultivars was obtained by homology-based cloning. The genomic walking method was performed to amplify its 5′ flanking (promoter) region using the Genome Walking Kit (Takara, Dalian, China) according to the manufacturer’s instructions. Nested sequence-specific primers designed on the basis of the known GbHyPRP1 gene sequence and shorter arbitrary degenerate primers were chemically synthesized or provided by the kit. The HyPRP1 protein sequences were identified using NCBI Web BLAST (http://www.ncbi.nlm.nih.gov/) and were aligned using the Clustal W program (http://www.clustal.org/). The promoter sequence was analyzed using the software programs PlantCARE and PLACE to define putative cis-elements or binding sites for transcription factors [49, 50].

qPCR and semi-RT-qPCR analysis
Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Each treatment, imposed on three pooled root, stem or leaf samples, was repeated at least three times in all experiments. RNA was quantified using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). Subsequently, first-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). qPCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and SYBR® Green reagent (TaKaRa, Dalian, China) as the reporter dye. Data were collected using CFX Manager™ software (Bio-Rad, Hercules, CA, USA). Target gene relative expression was normalized using PP2A1 (catalytic subunit of protein phosphatase 2A) [51]. For semi-RT-qPCR, the reactions were run with a denaturation step of 95°C for 5 min, followed by 25 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gels.

Subcellular localization analysis of transiently expressed fusion proteins
For subcellular localization studies, the GbHyPRP1 ORF was amplified using PCR. The resulting product was inserted into the vector pDONR™207. Subsequently, the fragment was recombined into the destination vector pK7WGFl2 [52] using L/R-Clonase. The construct was verified by sequencing and was transferred to the Agrobacterium tumefaciens GV3101 strain using the freeze/thaw method [53]. Transient transformation of tobacco leaf epidermal cells was performed as described in [54].
Localization of fluorescent proteins was monitored 3 days after infiltration using a confocal laser scanning microscope (FluoView FV1000; Olympus). A pCAMBIA derivative (pCamE) carrying a cauliflower mosaic virus 35S-driven GFP was used as the control [55].

**Hormone treatments**

Cotyledon seedlings at the 2-cotyledon stage were sprayed with 100 μM SA, ABA, JA or ET and were covered with plastic bags to maintain 100% humidity. Cotyledon tissues were collected from hormone-treated plants at 6, 12, 24, 36 and 48 hps, immediately frozen in liquid nitrogen and then stored at −80 °C until RNA extraction. The control seedlings were sprayed with distilled water.

**Generation of transgenic Arabidopsis lines**

The coding sequence of GbHyPRP1 was amplified from G. barbadense Pima90–53 cDNA and cloned into a pBI121 vector. A genomic GbHyPRP1 upstream fragment was amplified and cloned into the pBI121 vector containing beta-glucuronidase (GUS) gene coding sequences, where the 35S promoter region was excised by digestion using PstI and BamHI restriction enzymes and was replaced with the sequence of the GbHyPRP1 upstream fragment. The recombinant plasmid was transformed into A. thaliana Columbia wild type (WT) plants through Agrobacterium tumefaciens strain G V3101-mediated plant transformation using the floral dip method [56]. Primary transformants were selected for survival on ½ MS medium with 50 μg ml⁻¹ kanamycin. Homozygous plants were isolated on media using gene-specific primers. Semi-RT-qPCR was then performed on cDNA from overexpression lines to confirm the status of transcription using the full-length GbHyPRP1 primers. Expression was normalized to the expression of actin. T3 transgenic A. thaliana plants carrying the GbHyPRP1 promoter were used in histochemical assays for GUS staining as described by [57].

**VIGS assays in cotton**

pTRV1 and pTRV2 from the Yule Liu research group at Tsinghua University (China) were used for the VIGS assays [58]. HyPRP1 fragments (364 bp) were amplified (Additional file 5: Table S1) and inserted into the pTRV2 vector to generate the derivative pTRV-HyPRP1, which were transformed into A. tumefaciens strain GV3101. An Agrobacterium tumefaciens strain G V3101-mediated plant transformation using the floral dip method [56]. Primary transformants were selected for survival on ½ MS medium with 50 μg ml⁻¹ kanamycin. Homozygous plants were isolated on media using gene-specific primers. Semi-RT-qPCR was then performed on cDNA from overexpression lines to confirm the status of transcription using the full-length GbHyPRP1 primers. Expression was normalized to the expression of actin. T3 transgenic A. thaliana plants carrying the GbHyPRP1 promoter were used in histochemical assays for GUS staining as described by [57].

Two independent sets from both control and HyPRP1-silenced plants were sampled to generate two biological replicas. For each sample, the first true leaves from at least six plants were collected at 12 hpi and pooled to minimize plant-to-plant variation. Then, total RNA was extracted using TRIzol® reagent following the manufacturer’s instructions, after which genomic DNA was removed using DNase I (Invitrogen, Carlsbad, CA, USA). RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), and concentration and purity were calculated using a Qubit® RNA Assay Kit (Life Technologies, CA, USA). Twenty-four-day-old cotton seedlings were subjected to V. dahliae inoculation by root dipping in a spore suspension (10⁷ spores ml⁻¹) for 2 min and were then returned to their original pots. Four-week-old Arabidopsis plants were infected with V. dahliae by soil drenching using a 10 ml conidial suspension (10⁶ spores ml⁻¹) per pot (80 ml). Control plants were inoculated with distilled water in the same way. Cotton and Arabidopsis symptoms and disease index (DI) were scored at 15 dpi. The DI was calculated based on five disease grades as described previously [60]. Thirty-five plants were used per treatment, and each treatment was repeated three times. Plant resistance to V. dahliae was determined based on the DI, where >35 = susceptible, 20 to 35 = tolerant, and 10 to 20 = resistant (National Standards of the People’s Republic of China GBT 22101.5-2009, Technical Specification for Evaluating Resistance of Cotton to Disease and Insect Pests - Part 5: Verticillium wilt).

**Primer**

All primers used in this paper are listed in Additional file 5: Table S1.

**RNA-Seq library construction and analysis of transcriptome sequencing data**

Two independent sets from both control and HyPRP1-silenced plants were sampled to generate two biological replicas. For each sample, the first true leaves from at least six plants were collected at 12 hpi and pooled to minimize plant-to-plant variation. Then, total RNA was extracted using TRIzol® reagent following the manufacturer’s instructions, after which genomic DNA was removed using DNase I (Invitrogen, Carlsbad, CA, USA). RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), and concentration and purity were calculated using a Qubit® RNA Assay Kit (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Transcriptome sequencing was performed using an Illumina HiSeq™ 2000 sequencer (Illumina, San Diego, CA, USA).

Sequence tag preprocessing was performed according to a previously described protocol [61]. Paired-end clean reads were aligned to the reference genome of G. hirsutum L. acc. TM-1 (https://www.cottongen.org/) using Bowtie v2.0.6 and TopHat v2.0.9. Differential expression analysis of HyPRP1-silenced and unsilenced (mock) groups (two biological replicates per group) was performed using the DESeq R package (1.10.1). The
resulting \(P\)-values were adjusted using Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted \(P < 0.05\) according to DESeq were assigned as differentially expressed.

**Examination of cell walls**

Stems were cut into pieces (1 mm\(^3\)) and fixed in 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide, and then embedded in Spurr’s resin. Semi-thin sections (1 \(\mu\)m), prepared using a Leica UC6 (Leica, Illinois, USA), were hot-stained with a 1% toluidine blue water solution. Images were captured using a digital camera (Digital sight DS-L1, Nikon, Japan) attached to a Olympus BX51 microscope (Tokyo, Japan). Ultra-thin sections (70 nm thick), prepared using a Leica Ultracut R ultramicrotome (Leica, Illinois, USA), were stained with uranyl acetate and lead citrate and examined under a JEM-1230 electron microscope (JEOL Ltd., Tokyo, Japan). Approximately 60 cells were measured per sample to determine the thickness of the cell walls.

**Lignin extraction and quantification**

Stem samples were collected from both HyPRP1-silenced and control plants at 0, 7 and 14 dpi. Three samples were collected per plant at different time intervals; the samples were crushed immediately in liquid nitrogen and freeze-dried. The extraction and quantification of lignin in cell walls were performed according to the method of Schenk et al., 2014 [62].

**Identification of lignin deposition in cell walls using histochemistry and autofluorescence**

Standard transverse free-hand sections were collected from the stems of cotton seedlings (5 cm from the stem base). Lignin autofluorescence was imaged using a BX51 microscope with both UV excitation and brightfield modes.

**Biological analysis of ROS accumulation**

Since ROS include various forms of reduced and chemically reactive molecules such as hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and the superoxide oxygen anion (\(\text{O}_2^-\)), we used NBT and DAB dyes, as well as the ROS-reactive fluorescent probe DCFH-DA, to detect the accumulation of ROS. Yellow, water-soluble NBT can be reduced by \(\text{O}_2^-\) to blue, water-insoluble formazan. DAB polymerizes at (\(\text{H}_2\text{O}_2\)) and the superoxide oxygen anion (\(\text{O}_2^-\)) of peroxidase activity into a reddish brown polymer to blue, water-insoluble formazan. DAB polymerizes at (\(\text{H}_2\text{O}_2\)) and the superoxide oxygen anion (\(\text{O}_2^-\))

**Statistical analysis**

All experiments were repeated at least three times for each determination. Statistical analysis of data was conducted with the software of GraphPad Prism\textsuperscript{\textregistered} 6 (Graph Pad, San Diego, CA, USA). The \(P\)-value less than 0.05 was considered to be statistically significant.

**Additional files**

**Additional file 1:** Figure S1. Alignment of the amino acid sequences of Sea Island cotton Pima90–53 HyPRP1 with those of six other upland cotton cultivars including TM-1, Coker312, ND601, CCR8, JImian20, and NongDaMian7. The alignment results showed that HyPRP1 shares a significant degree of sequence identity in cotton. (TIF 2613 kb)

**Additional file 2:** Figure S2. A DNA fragment upstream of the \(G_b\text{HyPRP1}\) coding sequence was isolated and then designated as \(pG_b\text{HyPRP1}\), which is 1431 bp in length and contains 41 nucleotides of the 5’-terminal region of the \(G_b\text{HyPRP1}\) cDNA. The first base of the cDNA was designated as the putative transcription start site (+1). (TIF 9955 kb)

**Additional file 3:** Figure S3. At approximately two weeks post \(Agr\text{obacterium}\) infiltration, the leaves of CLA1-VIGS plants started to displayed the albino phenotype on the true leaves (A). At the same time, the silencing of HYPP1 gene expression in VIGS and control plants was confirmed by semi-RT-qPCR analysis (B). (TIF 9955 kb)

**Additional file 4:** Data S1. DEGs in V12 (VIGS at 12 hpi) versus C12 (Control at 12 hpi) using \(\text{Padj}\) (adjusted \(P\)) value < 0.05 as criteria. (XLSX 106 kb)

**Additional file 5:** Table S1. Primers used in this study. (DOCX 15 kb)

**Abbreviations**

ABA: Abscisic acid; CLA1: Cloroplastos alterados 1; DEGs: Differentially expressed genes; DI: Disease index; ET: Ethylene; GUS: beta-glucuronidase; HyPRP: Hybrid proline-rich protein; JA: Jasmonic acid; ORF: Open reading frame; PDA: Potato dextrose agar; ROS: Reactive oxygen species; SA: Salicylic acid; VIGS: Virus-induced gene silencing; WT: Wild type

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Availability of data and materials

All sequence reads analyzed during the current study have been deposited in the NCBI Genbank datasets (https://www.ncbi.nlm.nih.gov/nuccore) under the SRA accession PRJNA490422. Other data generated or analyzed during the current study are included in this published article and its supplemental data files and available from the corresponding author on reasonable request.

Authors’ contributions

YJ and MZY designed the experiments. YJ carried out the study and wrote the manuscript. ZY, WXF, WWQ and WGM performed the experiments. LZK, WH, WLO and ZGY carried out the data analysis. All authors discussed the results and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval and consent to participate results and reviewed the manuscript. All authors read and approved the final manuscript.

Consent for publication

Ethics approval and consent to participate

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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