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Overexpression of *GhPFN2* enhances protection against *Verticillium dahliae* invasion in cotton

Wenyan Wang[1,2,3], Yongduo Sun[1,2,3], Libo Han[1,2], Lei Su[1,2], Guixian Xia[1,2*] & Haiyun Wang[1,2*]

[1]*Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China;*
[2]*State Key Laboratory of Plant Genomics, Beijing 100101, China;*
[3]*University of Chinese Academy of Sciences, Beijing 100049, China*

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Growing evidence indicates that actin cytoskeleton is involved in plant innate immune responses, but the functional mechanism remains largely unknown. Here, we investigated the behavior of a cotton profilin gene (*GhPFN2*) in response to *Verticillium dahliae* invasion, and evaluated its contribution to plant defense against this soil-borne fungal pathogen. *GhPFN2* expression was up-regulated when cotton root was inoculated with *V. dahliae*, and the actin architecture was reorganized in the infected root cells, with a clear increase in the density of filamentous actin and the extent of actin bundling. Compared to the wild type, *GhPFN2*-overexpressing cotton plants showed enhanced protection against *V. dahliae* infection and the actin cytoskeleton organization in root epidermal cells was clearly altered, which phenocopied that of the wild-type (WT) root cells challenged with *V. dahliae*. These results provide a solid line of evidence showing that actin cytoskeleton reorganization involving GhPFN2 is important for defense against *V. dahliae* infection.

actin cytoskeleton, profilin, *Verticillium dahliae*, cotton

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INTRODUCTION

The actin cytoskeleton plays crucial roles in diverse cellular processes during plant growth and development (Staiger and Blanchoin, 2006; Zhao et al., 2015). Accumulating evidence demonstrates that dynamic reorganization of actin structure is also an important cellular process in plant innate immunity (Lipka and Panstruga, 2005; Day et al., 2011). Tobacco BY-2 cells with disrupted actin architecture could be infected by the non-pathogenic *Erysiphe pisi*, and disruption of actin organization reduced the tolerance of barley plants to *Magnaporthe grisea* (Kobayashi and Hakuno, 2003; Jarosch et al., 2005). The actin cytoskeleton responds to pathogen invasion by increasing the density of filamentous actin (F-actin) arrays around the infection sites (Takemoto and Hardham, 2004; Hardham et al., 2007; Underwood and Somerville, 2008), and rapid increases in the F-actin density and actin bundling are associated with various processes in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). The actin cytoskeleton along with a rapid increase in F-actin abundance is therefore considered to be a new but conserved component of PTI (Henty-Ridilla et al., 2013).

The dynamic rearrangement of actin cytoskeleton depends on the participation of actin-binding proteins (ABPs). These proteins can be classified into several subgroups, including

*Corresponding authors (Guixian Xia, email: xiagx@im.ac.cn; Haiyun Wang, email: wanghy@im.ac.cn)

polymerization/elongation proteins, bundling/branching proteins, severing/depolymerization proteins, and capping proteins (Porter and Day, 2016). The function of several ABPs was found to be related to plant defense. For example, a capping protein in *Arabidopsis* was shown to modulate actin dynamics in plant innate immune response (Li et al., 2015), and alterations in the expression levels of the actin depolymerizing factor (ADF) could change the disease tolerance in various host plants (Miklis et al., 2007; Tian et al., 2009; Fu et al., 2014; Inada et al., 2016). Profilin, a key regulator of dynamic actin organization, was also involved in plant-pathogen interaction (Schütz et al., 2006; Liao et al., 2009). Profilin proteins have been detected at the *Phytophthora infestans* infection site of cultured cells of *Petroselinum crispum* (Schütz et al., 2006); accumulation of profilin proteins was increased after the plants were treated with the glycoprotein elicitor CSB I of the rice blast pathogen *Magnaporthe grisea* or with the methyl jasmonate (Liao et al., 2009; Gharechahi et al., 2013), and the expression levels of *profilin* genes were responsive to the fungi in wheat and pepper (Song et al., 2012; Wang et al., 2015).

The vascular disease Verticillium wilt is caused by the soil-borne filamentous fungus *Verticillium dahliae*, and is the most devastating disease in upland cotton (Fradin and Thomma, 2006). To date, very few germplasms that are resistant to Verticillium wilt are available in cotton breeding (Cai et al., 2009). To generate cotton cultivars with resistance to Verticillium wilt by molecular breeding, many studies aimed to identify genes involved in cotton's response to *V. dahliae* invasion. A number of *V. dahliae*-responsive genes have been reported to play roles in defense against *V. dahliae* invasion in cotton (Gaspar et al., 2014; Li et al., 2014; Cheng et al., 2016; Li et al., 2016), yet no cytoskeletal gene is included in this set of genes.

In this study, we studied the defense-related function of the *GhPFN2* gene in upland cotton and found that overexpression of the gene enhanced the abundance of F-actin and actin bundles, and this was associated with increased disease tolerance of cotton plants. Our results indicate that the actin cytoskeleton architecture changes and F-actin abundance increases in response to *V. dahliae* invasion. The functional mechanism associated with changes in F-actin organization in innate immune responses is discussed.

RESULTS

Actin cytoskeleton reorganization in response to *V. dahliae* attack

Rearrangement of actin filaments in response to microbial infection has been observed in many plants (Kobayashi and Hakuno, 2003; Jarosch et al., 2005; Henty-Ridilla et al., 2013). To determine whether this occurs during the interaction of cotton plants and *V. dahliae*, actin filaments in cotton roots were stained with Alexa Fluor 488-phalloidin and imaged using a confocal microscope after dip-inoculation of cotton roots with *V. dahliae*. At 24 hours post inoculation (hpi), we observed a clear increase in both the abundance of actin filaments and the amount of actin bundles in the epidermal cells as compared with that of the un-inoculated control (Figure 1A). Statistical analysis was performed to quantify this change based on two validated parameters, the percent occupancy to measure the actin filament density and the skewness to measure the extent of actin bundling (Higaki et al., 2010). As shown in Figure 1B and C, the values for percent occupancy and skewness were higher in the infected root cells than in the control cells.

GhPFN2 expression was induced by *V. dahliae* invasion

It has been shown that profilin, one of the key modulators of the actin organization, is involved in plant-pathogen interaction (Schütz et al., 2006). This attracted our interest to see if cotton profilins contribute to the defense-responsive actin reorganization. To this end, genome sequence of *Gossypium hirsutum* was searched and 16 *profilin* genes were identified. We analyzed the expression patterns of these *profilin* genes in cotton root, which is the invasion site of *V. dahliae*. Quantitative real-time PCR (qRT-PCR) showed that *CotAD_22966/CotAD_50882*, designated *GhPFN2* previously (Wang et al., 2010), had much higher expression level than other genes (Figure 2A). As shown in Figure 2B, the expression of *GhPFN2* increased gradually in roots after *V.*

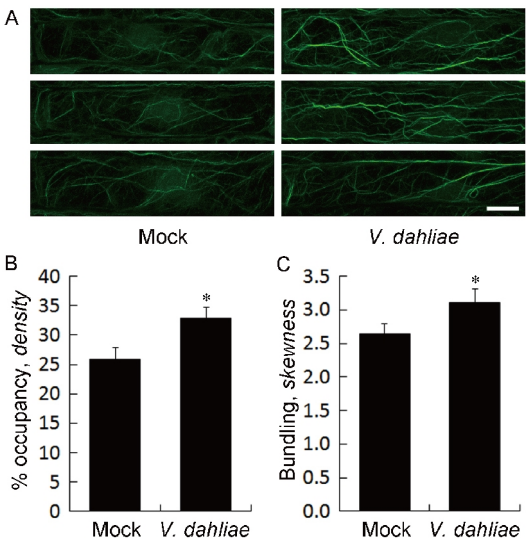

Figure 1 Actin filament restructuring response to *V. dahliae* infection in cotton root cells. A, Confocal microscopic visualization of actin structure. Representative images of root epidermal cells are displayed for each sample. Scar bar=10 μm. B, Quantitative analysis of actin density by calculation of percent occupancy in (A). C, Determination of actin filament bundling tendency (skewness) in (A). Values present means±SD. The asterisks denote statistically significant differences as determined by Student's *t*-test. *, $P<0.05$. Similar tendency was obtained from three biological replicates. About 100 root epidermal cells per line were measured for statistical analysis.

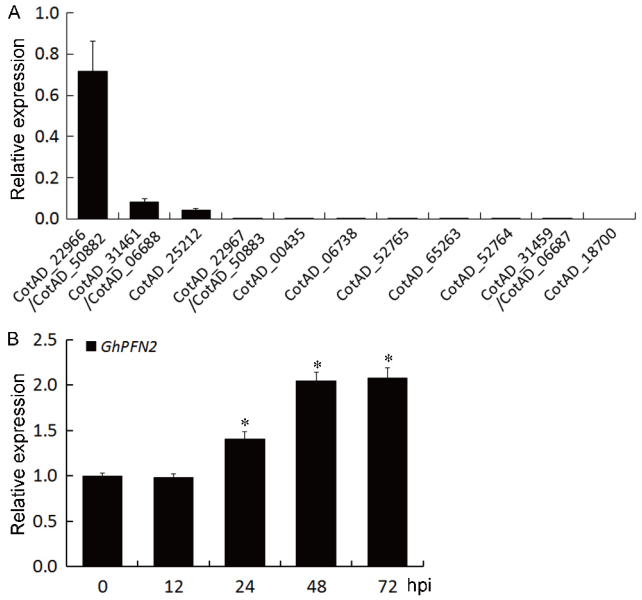

Figure 2 Expression patterns of cotton *PFNs* by qRT-PCR. A, Transcriptional expression levels of *GhPFNs* in cotton root. B, Accumulation of *GhPFN2* transcripts after inoculation of cotton plants with *V. dahliae*. Values represent means±SD. *, $P<0.05$, as determined by Student's *t*-test. Three biological repeats were performed.

dahliae infection and reached the peak around 48–72 h post inoculation, indicating that *GhPFN2* expression is associated with defense response against the pathogen.

GhPFN2-overexpressing cotton plants were more tolerant to *V. dahliae* infection

GhPFN2-overexpressing transgenic cotton plants generated in our previous study (Wang et al., 2010) were used to investigate the defense-related function of the gene. As shown in Figure 3B, the *GhPFN2* mRNA level increased approximately two folds in the root of *GhPFN2*-overexpressing seedlings (L156 and L176) compared with that in the control. The transgenic and control cotton plants were subjected to *V. dahliae* inoculation, and the disease-related phenotypes were examined. Approximately two weeks post inoculation, the leaves of control plants became severely withered, but the symptoms of *GhPFN2*-overexpressing plants appeared much weaker (Figure 3A). The disease index for the *GhPFN2*-overexpressing plants was significantly lower than that for the control (Figure 3C). We performed qRT-PCR to analyze the expression levels of two defense-related genes in wild-type (WT) and *GhPFN2*-overexpressing plants. The results showed that expression of *GhPR1*, an important gene in the salicylic acid (SA) signaling pathway, was activated significantly in the transgenic plants, whereas no significant change was detected with *GhPDF1.2* gene (Figure 3D). This result indicates that up-regulation of *GhPFN2* had a positive effect on the disease tolerance of cotton plants, which may be associated with SA-mediated defense response.

Overexpression of *GhPFN2* increased the percent occupancy and skewness of actin filaments in cotton root epidermal cells

To determine if there is a relationship between increased disease tolerance and actin organization, the actin filament arrays in root epidermal cells of GhPFN2-overexpressing cotton were imaged and compared with those in control cells. The results showed that both the actin density and the extent of actin bundling in the *GhPFN2*-overexpressing plants increased significantly (Figure 4A) as compared to that in the control. Measurements of the percent occupancy and skewness of actin filaments showed that both values were elevated in *GhPFN2*-overexpressing plants (Figure 4B and C). These changes in actin filament reorganization in *GhPFN2*-overexpressing plants phenocopied the changes in the root cells of wild-type plants challenged with *V. dahliae* (Figure 1B and C).

Identification of proteins interacting with GhPFN2 by yeast-two hybrid assay

In order to understand the molecular mechanism by which GhPFN2 acts in the defense response, yeast two hybrid assay was conducted to identify proteins that interact with GhPFN2. A cDNA library of cotton roots infected with *V. dahliae* was constructed in AD vector pGADT7 and used as prey, and the open reading frame (ORF) of *GhPFN2* was inserted into BD vector pGBKT7 and used as bait. A total of nine proteins that interacted with GhPFN2 were identified, including four mitochondrial outer membrane proteins, two proline-rich proteins, one DEAD-box ATP-dependent RNA helicase, one BEL1-like homeodomain protein and one polyphenol oxidase (Table 1). Among these proteins, the proline-rich protein has been reported to interact with profilin, which is important for the regulation of dynamic actin reorganization (Gibbon et al., 1998; Aparicio-Fabre et al., 2006; Boukhelifa et al., 2006). Interestingly, about one half of the identified proteins are the mitochondrial outer membrane proteins. It was shown that yeast mitochondrial outer membrane proteins possess ATP-sensitive actin binding activity, and function in mitochondria-actin interactions (Boldogh et al., 1998). It is intriguing for us to undergo further studies for the functional relations of these proteins with profilin.

DISCUSSION

During development and in response to environmental stimuli, the actin cytoskeleton undergoes active dynamic changes in plant cells. Many studies show that such dynamic changes of actin organization occur during interactions between pathogens and plant hosts. The actin cytoskeleton appears to be one of the cellular targets of the invading microbes. For instance, effectors released by the pathogen *Pseudomonas*

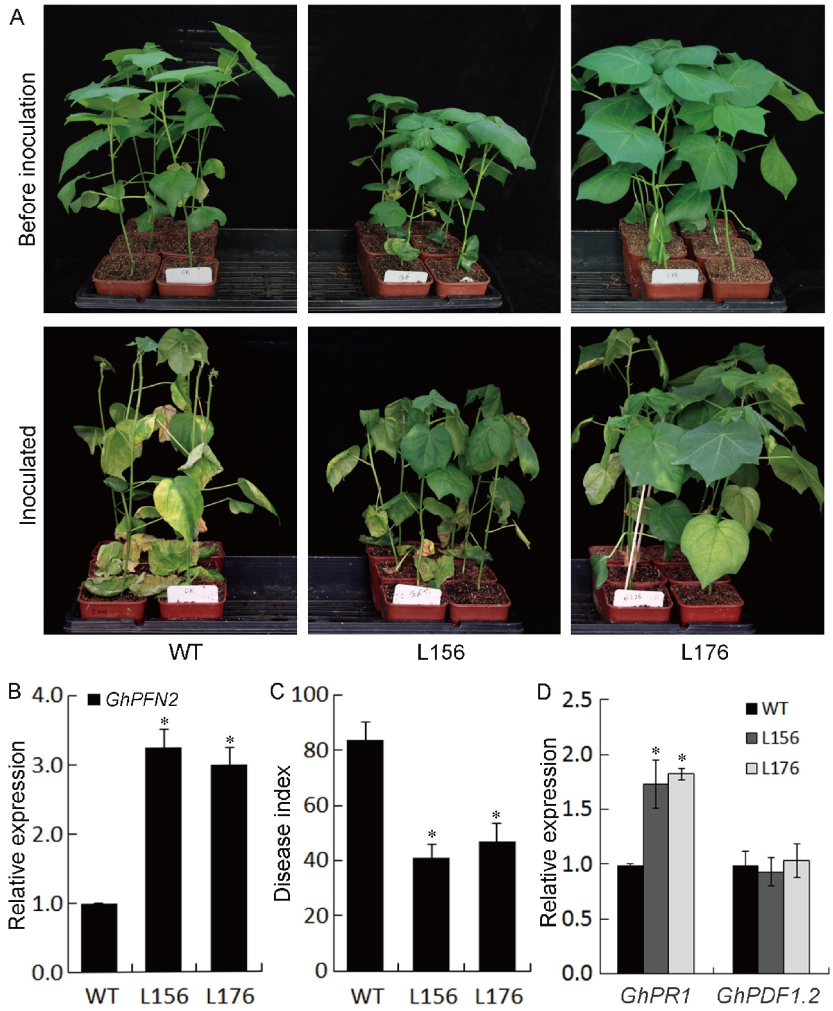

Figure 3 Increased tolerance of *GhPFN2*-overexpressing cotton plants to *V. dahliae* infection. A, Disease symptoms of control and *GhPFN2*-overexpressing (L156 and L176) plants infected by *V. dahliae*. Pictures were taken before inoculation (upper panel) and two weeks after *V. dahliae* infection (lower panel). B, Expression levels of *GhPFN2* in WT and transgenic plants (L156 and L176). Values represent means±SD. *, $P<0.05$. Three biological repeats were performed. C, Disease index of the WT and *GhPFN2*-overexpressing (L156 and L176) plants. Values present means±SE ($n=20$, from $n=3$ biological repeats). *, $P<0.05$. D, Expression profile of *GhPR1* and *GhPDF1.2* genes in WT and *GhPFN2*-overexpressing (L156 and L176) plants. Values represent means±SD. *, $P<0.05$. Similar result was obtained from three biological replicates.

syringae can interact with the host actin cytoskeleton and reduce the content of actin filaments, thereby inhibiting endocytosis (Kang et al., 2014); and the VD toxin released from *V. dahliae* caused dose-dependent destruction of the actin cytoskeleton in *Arabidopsis* suspension cells (Yuan et al., 2006). It has been shown that plant actin cytoskeleton undergoes rapid remodeling of the architecture in response to pathogen attack, such as increasing the density and bundling of actin filaments (Takemoto and Hardham, 2004; Henty-Ridilla et al., 2013). In the present study, we observed that the actin cytoskeleton in cotton root cells was reorganized after challenging with the filamentous fungus *V. dahliae*, and the density of F-actin and the extent of actin bundling were increased in the infected cells. Our results add a new line of evidence showing that reorganization of the F-actin structure and increase of F-actin abundance is an important event during the defense process in higher plants.

The actin cytoskeleton plays crucial roles in intracellular trafficking by providing tracks for material transportation (Takemoto and Hardham, 2004; Hardham et al., 2007; Underwood and Somerville, 2008). A possible mechanism for the reorganization of the actin cytoskeleton in response to pathogen invasion may be attributed to its function in cellular trafficking. Indeed, a number of studies revealed the association between actin cytoskeleton-mediated cellular transportation and pathogen invasion. It was shown that antimicrobial compounds delivery to the site of infection and callose deposition at the plant cell wall require actin cytoskeleton (Day et al., 2011). The specific targeting of defense-related proteins to the plasma membrane is also dependent on the actin cytoskeleton (Underwood and Somerville, 2008). The accumulation of resistance protein RPW8.2 requires the actin cytoskeleton to reach the pathogen penetration sites (Wang et al., 2009). Based on the literature

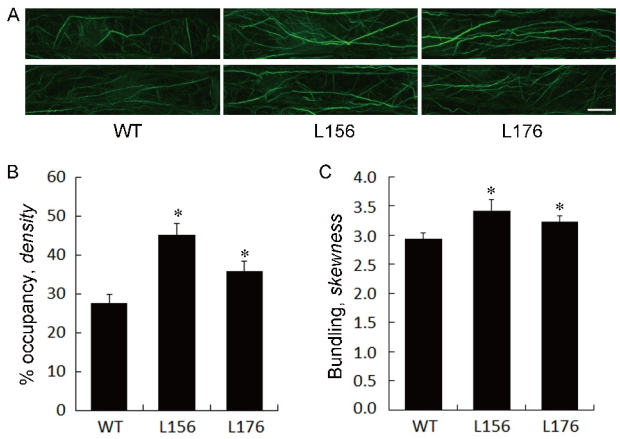

Figure 4 Reorganization of actin filament arrays in *GhPFN2*-overexpressing cotton root cells. A, Confocal microscopy visualization of actin filament arrays in root cells. Representative images of root epidermal cells are displayed for each sample. Scar bar=10 μm. B and C, Determination of actin filament percent occupancy (density) and bundling (skewness). Values present means±SD. The asterisks denote statistically significant differences as determined by Student's *t*-test. *, $P<0.05$. Similar tendency was obtained from three biological replicates. About 100 root epidermal cells per line were measured for statistical analysis.

Table 1 Putative GhPFN2-interacting proteins

Clone No.	Protein description	Accession No.
5	*Gossypium arboreum* mitochondrial outer membrane protein porin of 34 kD-like	XP_017606824
29	*Gossypium arboreum* mitochondrial outer membrane protein porin of 34 kD	XP_017607996
9	*Gossypium arboreum* mitochondrial outer membrane protein porin of 36 kD-like	XP_017602978
13	*Gossypium arboreum* mitochondrial outer membrane protein porin of 36 kD-like	XP_017602978
14	*Gossypium hirsutum* 36.4 kD proline-rich protein-like	XP_016696423
28	*Gossypium hirsutum* 36.4 kD proline-rich protein-like	XP_016725665
7	*Gossypium raimondii* DEAD-box ATP-dependent RNA helicase 56-like	XP_012477379
22	*Gossypium arboreum* BEL1-like homeodomain protein 4	XP_017634650
35	*Gossypium raimondii* polyphenol oxidase, chloroplastic-like	XP_012484697

information, we assume that the increased F-actin density and actin bundles in *GhPFN2*-overexpressing plants may favor the trafficking of defense-related components in the cells, which consequently leads to enhanced tolerance to *V. dahliae* infection.

The dynamic reorganization of actin arrays depends on the activities of various ABPs. Some of these act to promote actin polymerization or bundling of actin filaments, such as profilin or fimbrin, whereas others function to induce depolymerization of actin filaments, such as ADF (Porter and Day, 2016). As a key modulator of actin organization, profilin could accelerate both polymerization and depolymerization at the barbed end of actin filaments (Yarmola and Bubb, 2006). In addition, it has been shown that profilin is also required for formation of actin cables/bundles, possibly through interacting with other ABPs such as formin (Evangelista et al., 2002; Wang and Riechmann, 2008; Pujol et al., 2009). In a previous study, we found that GhPFN2 could enhance the actin bundling activity of formin *in vitro* (Wang et al., 2010). Hence, we speculate that the presence of more abundant actin bundles in *GhPFN2*-overexpressing root cells could be a result of interaction between cellular formin and overproduced GhPFN2 proteins, and GhPFN2-formin interaction may also contribute to the actin bundle formation in wild-type root cells challenged with *V. dahliae*.

Finally, it was reported that suppression of all members of subclass I *ADFs* (*ADF1-4* RNAi) led to accumulation of *PR1* mRNA in uninfected leaf cells in *Arabidopsis* (Inada et al., 2016). Likewise, we detected induced expression of *PR1* genes in *GhPFN2*-overexpressing cotton plants. We speculate that the defense-related actin reorganization involving up-regulation of *GhPFN2* may be linked to SA-mediated defense signaling. Further study is required to verify this assumption.

MATERIALS AND METHODS

Plant materials

Seedlings of upland cotton varieties MY (from the Institute of Cotton Research, Shanxi Academy of Agricultural Sciences) of greencolored cotton (*Gossypium hirsutum* L.) were either grown in soil in a greenhouse or under hydroponic conditions in an artificial climate simulator under 16 h light/8 h dark conditions at 28°C, and irrigated with Murashige and Skoog (MS) nutrient solution weekly.

Pathogen cultivation and inoculation

The *V. dahliae* strain V991, a highly aggressive defoliating isolate, was used in this study. Fungal colonies were grown on potato dextrose agar (PDA) plates for 1 week at 28°C. Spores were inoculated into Czapek medium (1 g KH_2PO_4, 2 g $NaNO_3$, 1 g $MgSO_4 \cdot 7H_2O$, 1 g KCl, 2 mg $FeSO_4 \cdot 7H_2O$, and 30 g Sucrose per L) and harvested at the 5th day. The spores were adjusted to a concentration of 1×10^6 colony-forming units (CFU) mL^{-1} with sterile distilled water for infection. The roots of cotton seedlings grown under hydroponic conditions for seven days were dipped into the spore suspension for 30 min, then harvested at 0, 12, 24, 48, and 72 hpi for RNA extraction. To infect soil-grown plants, 5 μL of spore suspension was injected into the hypocotyl,

1 cm under the cotyledons. Disease index was calculated according to the method described by Wang et al. (Wang et al., 2004).

RNA extraction and qRT-PCR

Total RNA from roots was extracted using the Plant Total RNA Purification Kit (GM biolab, Taichung City). 1 μg of total RNA was used to synthesize cDNA by reverse transcription according to the protocol of TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen, Beijing). For the qRT-PCR assays, SYBR Green Realtime PCR Master mix (Toyobo, Japan) and a real-time PCR detection system (CFX96 Touch, BIO-RAD, USA) were used. The cotton *Histone3* gene was used as the internal control. The expression levels of genes were calculated by using the $2^{-\Delta\Delta C_t}$ or $2^{-\Delta C_t}$ method. All reactions were repeated three times. The primers used in qRT-PCR are listed in Table S1 in Supporting Information.

yeast two-hybrid (Y2H) assay

For the yeast two-hybrid screening, the ORF of *GhPFN2* was cloned into the BD vector pGBKT7 to construct BD-GhPFN2 as bait. The cDNA library of cotton roots infected by *V. dahliae* was inserted into the AD vector pGADT7-DEST to produce the prey library. According to the instructions of the manufacturer of the Matchmaker Gold Yeast Two-Hybrid System (Clontech, USA), the bait culture and the prey library were combined and incubated for 24 h, then plated onto SD/-Leu/-Trp DO (DDO) medium supplemented with X-α-gal and Aureobasidin A and grown at 30°C for 72 h. The blue clones were confirmed by transferring to the SD/-Leu/-Trp/-Ade/-His DO (QDO) medium supplemented with X-α-gal and Aureobasidin A. The blue clones were considered as positive interactors, and inserts in the colonies were analyzed by colony PCR and sequenced.

Labeling of actin filaments in cotton root epidermal cells

Cotton roots grown under hydroponic conditions were harvested, washed with PBS buffer, and then subjected to Alexa Fluor 488-phalloidin staining in PBS buffer (0.1 mol L^{-1} PIPES, pH 6.9, 0.05% (V/V) Triton X-100, 1 mmol L^{-1} $MgCl_2$, 3 mmol L^{-1} DTT, 0.3 mmol L^{-1} PMSF, 5 mmol L^{-1} EGTA, and 0.25% glutaraldehyde) to image the actin filament arrays as described previously (Van Gestel et al., 2001). The *Z*-series stacked images were collected by confocal microscopy (Leica TCS SP8; Leica Microsystems, Germany). The excitation wavelengths/emission filters were 488 nm/500−550 nm.

Evaluation of actin filament arrays

Analysis of actin filament arrays was conducted primarily based on the method described previously by Higaki et al. (Higaki et al., 2010). For the skewness value, the background noise in the *Z*-series stacks of all optical sections was eliminated by subtracting background (set to 50 pixels) and filtering with Gaussian blur (set to 1.0), and the skeletonization was then assessed using the ThinLine procedure (a JAVA plug-in procedure; Higaki et al., 2010). The actin filament pixels were collected into a single image using maximum intensity projections, and the skewness values were calculated. For the percent occupancy, pixels of actin filaments were measured after subtracting the background noise by setting a minimal threshold (set to 50 pixels).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Table S1 Primers used in this study

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