Plant Molecular Biology (2019) 99:379–393
https://doi.org/10.1007/s11103-019-00824-y

GhCyP3 improves the resistance of cotton to *Verticillium dahliae* by inhibiting the E3 ubiquitin ligase activity of GhPUB17

Tao Qin[1] · Shiming Liu[1] · Zhennan Zhang[1] · Longqing Sun[1] · Xin He[1] · Keith Lindsey[2] · Longfu Zhu[1] · Xianlong Zhang[1]

Received: 6 February 2018 / Accepted: 12 January 2019 / Published online: 22 January 2019

Abstract

Key message **A U-box E3 ubiquitin ligase GhPUB17 is inhibited by GhCyP3 with antifungal activity and acts as a negative regulator involved in cotton resistance to *Verticillium dahliae*.**

Abstract E3 ubiquitin ligases, the key component enzymes of the ubiquitin–proteasome system, which contains the most diverse structural and functional members involved in the determination of target specificity and the regulation of metabolism, have been well documented in previous studies. Here, we identify GhPUB17, a U-box E3 ligase in cotton that has ubiquitination activity and is involved in the cotton immune response to *Verticillium dahliae*. The expression level of *GhPUB17* is downregulated in the *ssn* mutant with a constitutively activated immune response (Sun et al., Nat Commun 5:5372, 2014). Infection with *V. dahliae* or exogenous hormone treatment, including jasmonic acid and salicylic acid, significantly upregulated *GhPUB17* in cotton roots, which suggested a possible role for this E3 ligase in the plant immune response to pathogens. Moreover, *GhPUB17*-knockdown cotton plants are more resistant to *V. dahliae*, whereas *GhPUB17*-overexpressing plants are more susceptible to the pathogen, which indicated that GhPUB17 is a negative regulator of cotton resistance to *V. dahliae*. A yeast two-hybrid (Y2H) assay identified GhCyP3 as a protein that interacts with GhPUB17, and this finding was confirmed by further protein interaction assays. The downregulation of *GhCyP3* in cotton seedlings attenuated the plants' resistance to *V. dahliae*. In addition, GhCyP3 showed antifungal activity against *V. dahliae*, and the E3 ligase activity of GhPUB17 was repressed by GhCyP3 in vitro. These results suggest that GhPUB17 negatively regulates cotton immunity to *V. dahliae* and that the antifungal protein GhCyP3 likely interacts with and inhibits the ligase activity of GhPUB17 and plays an important role in the cotton-*Verticillium* interaction.

Keywords Cotton · Ubiquitination · Immunity · *Verticillium dahliae* · Cyclophilin

Introduction

Plants have evolved symbiotic relationships, including relationships characterized by mutual promotion and inhibition, with microorganisms in the environment. During growth and development, plants are also exposed to pathogens, such as bacteria, viruses, fungi and oomycetes (Dalio et al. 2017), and plants protect themselves against these pathogenic microorganisms by using detection mechanisms that recognize pathogen-derived molecules and then activate defence responses. To detect pathogenic microorganisms and thus protect themselves, plants have evolved an inducible innate system that allows them to minimize the influence of pathogens (Jones and Dangl 2006). Two types of defence responses are triggered by the detection of different pathogenic molecules secreted from pathogens. The first response is PAMP-triggered immunity (PTI), which is based on the recognition of conserved pathogen-associated molecular patterns (PAMPs), and this recognition leads to the production of reactive oxygen species, the activation of mitogen-activated protein kinases (MAPKs), the deposition of callose

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11103-019-00824-y) contains supplementary material, which is available to authorized users.

✉ Longfu Zhu
lfzhu@mail.hzau.edu.cn

[1] National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, Hubei, China

[2] Department of Biosciences, Durham University, South Road, Durham, UK

in the cell wall, and the synthesis of pathogenesis-related proteins (Dodds and Rathjen 2010; He et al. 2015; Thomma et al. 2011). The other response is effector-triggered immunity (ETI); as the name suggests, this response is triggered by effectors secreted from pathogens, which can suppress metabolic reactions to promote pathogen colonization in the host plants. These effectors are detected by recognition components of immune mechanisms in the host, such as nucleotide-binding leucine-rich repeat (NB-LRR) proteins, and this recognition activates the plant immune response (Boller and He 2009). The PTI process is continuous, but the ETI process is short-lived, more specific and shows increased effectiveness (Boller and He 2009; He et al. 2015).

It has been well established that protein ubiquitination is very important in many processes (Orosa et al. 2017), including the signal transduction cascade during the plant immune response (Kwon et al. 2006; Stone and Callis 2007). Ubiquitin (Ub) is a basic component of the Ub-conjugation pathway, which also involves three other enzymes or protein complexes called E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme) and E3 (Ub-protein ligase). Through a reaction catalysed by E1 enzymes, non-activated Ub molecules form a thiol-ester bond with a conserved Cys residue in the E1 enzymes, and the Ub molecules in the resulting Ub-E1 complexes then form another thiol-ester bond with a conserved Cys residues in the E2 enzymes. Subsequently, E3 ligases facilitate the formation of an isopeptide linkage between Ub and the target protein or encourages its autoubiquitination (Yee and Goring 2009). A polyubiquitin chain is subsequently formed by the addition of multiple Ub monomers via an internal Lys residue within the Ub protein. The polyubiquitin chain is then recognized by the 26S proteasome, which degrades the target and recycles the Ub monomers (Yang et al. 2006; Yee and Goring 2009). In this process, the E3 ligase plays a central role in the recruitment of specific target proteins and catalyses their ubiquitination (Trujillo and Shirasu 2010; Zhou and Zeng 2016). E3 ligases form the largest group of proteins within the Ub-enzyme cascade, indicating the wide range of potential targets in the proteome (Orosa et al. 2017). Depending on the presence of conserved domains and the mechanism of action of single-protein E3 ligases, these enzymes are classified into four main subfamilies: homologous to the E6-AP carboxyl terminus (HECT), really interesting new gene (RING), U-box and cullin-RING ligases (CRLs) (Vierstra 2009).

Recent evidence indicates that the plant U-box (PUB) E3 ligase can function as either a positive or a negative regulator of the plant immune response against diverse pathogens. The U-box domain is the representative structure of PUB genes, which usually contain other specific domains necessary for complete functional E3 ligase activity. The U-box, which is signature domain of plant U-box type E3 ligases (PUBs), mediates interaction with the Ub-conjugating enzyme (E2), and the U-box domain in combination with a variation of substrate domains, including armadillo (ARM) repeats, the Ser/Thr kinase domain, WD40 repeats, the tetratricopeptide (TPR) domain, or peptidyl-prolyl isomerase, of which ARM repeats are the most common (41 out of 64 PUBs), has been mostly shown to mediate the interaction with substrates that renders them available for ubiquitination (Trujillo 2018). The U-box gene *NtACRE74* (Avr9/Cf-9 rapidly elicited gene 74) from *Nicotiana tabacum*, which is homologous to *CMPG1* in *Petroselinum crispum* and *PUB20* and *PUB21* in *Arabidopsis thaliana*, can be rapidly induced by flagellin (Navarro et al. 2004) and acts as a positive regulator in Avr9/Cf9, AvrPto/Pto and Inf1 immune responses in tomato and potato (Gonzalez-Lamothe et al. 2006). The homologues of *PUB17* (also known as *ACRE276*) found in many plant species, including *Arabidopsis*, tobacco, potato and tomato, have been reported to be positive regulators in plant immune responses. *NtPUB17* (as well as *AtPUB17*) is required for detection of the Avr4 and Avr9 effectors from *Cladosporium fulvum* by the receptor proteins Cf4 and Cf9 in tobacco and tomato (Yang et al. 2006). Moreover, the silencing of *StPUB17* in potato and of its homologue *NbPUB17* in *Nicotiana benthamiana* enhances leaf colonization by *Phytophthora infestans* and attenuates PTI responses and programmed cell death (PCD), which is activated by flg22 and Cf4/Avr4 but is not required for all PTI- and PCD-associated responses (He et al. 2015). In *N. benthamiana* and tobacco, PUB17 acts as a target of the BTB domain E3 ligase POB1 for proteasome degradation. Plants with low levels of *POB1* expression show accelerated HR-PCD, whereas those with an increased number of *POB1* transcripts show attenuated HR-PCD. Furthermore, mutated versions of POB1 exhibit reduced interactions with PUB17 and fail to suppress HR-PCD, revealing a regulatory mechanism for PUB17 in plant immune pathways (Orosa et al. 2017). Taken together, the results show that homologues of PUB17 act as positive regulators in the Cf4- and Cf9-mediated immune response across the *Solanaceae* and *Brassicaceae*, which implies that the PUB17 homologue in cotton might have a similar function in the response to *V. dahliae*.

Here, we identified the cotton homologue of PUB17, denoted GhPUB17, from the lesion mimic mutant *ssn*, which is defective in the cotton P450 gene, *SILENCE-INDUCED STEM NECROSIS* (*SSN*) (Sun et al. 2014). The knockdown of *GhPUB17* in both *Gossypium barbadense* and *G. hirsutum* by VIGS or RNAi suppressed *V. dahliae* colonization, whereas transgenic lines that overexpressed this gene were more susceptible to the fungus. Furthermore, we found a cyclophilin protein, GhCyP3, that interacts with GhPUB17. The knockdown of GhCyP3 increased the susceptibility of the seedlings to *V. dahliae*. GhCyP3 also exhibited antifungal activity in vitro, and GhCyP3 inhibited the E3 ligase activity of GhPUB17. We therefore propose

that the antifungal GhCyP3 inhibits the E3 ligase activity of GhPUB17 and is therefore involved in the resistance of cotton to *V. dahliae*.

Results

GhPUB17 is a homologue of PUB17 and is involved in the response to *V. dahliae*

Our previous study revealed that the cotton P450 gene *SSN* is downregulated in *G. barbadense* upon inoculation with *V. dahliae*. The knockdown of *SSN* causes a lesion mimic phenotype and constitutively activates systemic cell death (Sun et al. 2014). An analysis of the differentially expressed genes between *ssn* and wild-type (WT) plants revealed an EST homologous to *AtPUB17* in *Arabidopsis*, and this gene was designated *GhPUB17*. The open reading frame of *GhPUB17* contains 2136 nucleotides and putatively encodes a protein with a length 712 amino acids that is also homologous to NtPUB17 in tobacco, StPUB17 in potato and SlPUB17 in tomato (Fig. 1a and S1a). All the homologous proteins were found to share two conserved domains, including a U-box domain and an ARM repeat domain (Fig. S1a). In addition, *GhPUB17* was constitutively expressed in all cotton tissues tested (Fig. S1b) and was significantly

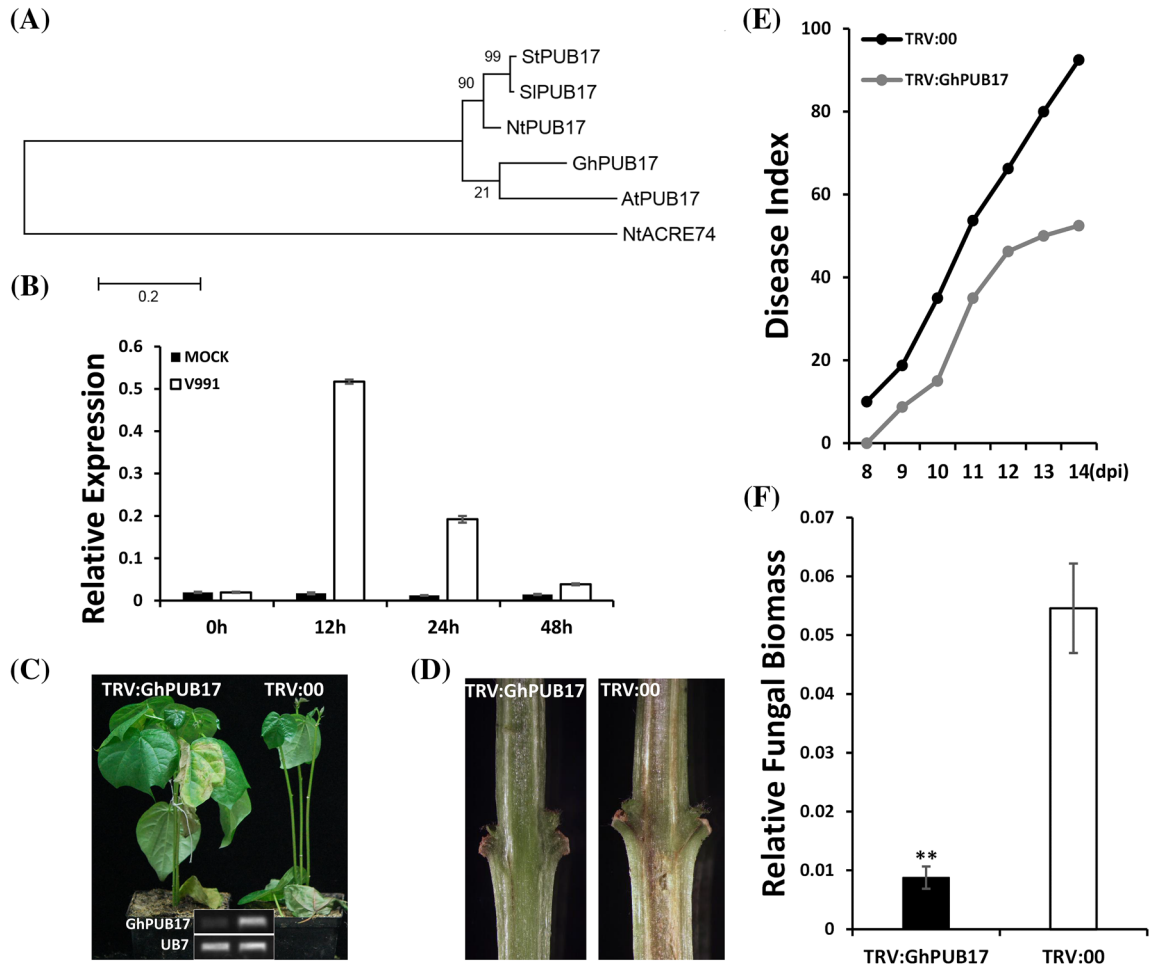

Fig. 1 GhPUB17 is involved in disease resistance. **a** Phylogenetic relationships among *GhPUB17* and its homologues in different plant species. The phylogeny was generated using the neighbour joining method with tobacco ACRE74 as the outgroup. **b** *GhPUB17* was significantly induced at several time points after *V. dahliae* inoculation. **c** The knockdown of *GhPUB17* by virus-induced gene silencing (VIGS) improves the resistance of *Gossypium barbadense* cv. 7124 to *V. dahliae*. Photographs from both control and *GhPUB17*-silenced lines were taken 12 days after *V. dahliae* inoculation. **d** Stalks dissected from control and GhPUB17-silenced plants displayed differences in the level of necrosis 12 days after *V. dahliae* inoculation. **e** Disease indexes of both lines from 8 to 14 days after *V. dahliae* inoculation. **f** Relative fungal biomass in both lines. Total DNA of stems from both lines was extracted 12 days post-inoculation and used as a template for the RT-qPCR-based detection of fungal biomass. The cotton endogenous gene *UB7* and the specific endogenous gene ITS (internal transcribed spacer) from *V. dahliae* were used as controls in the RT-qPCR analysis. The values and errors are presented as the means and standard deviations, respectively, of triplicate samples ($n \geq 15$ plants, $^{**}P < 0.01$, t-test)

induced by inoculation with *V. dahliae* strain V991 (Fig. 1b). Additionally, the exogenous application of phytohormones, including SA and JA, induced the expression of *GhPUB17* (Fig. S1c, d), suggesting a potential role for this cotton gene in response to infection with *V. dahliae*.

To explore the function of GhPUB17 in cotton immunity to *V. dahliae*, a VIGS vector was constructed with the specific domain in *GhPUB17*, as shown in Fig. S2 (Liu et al. 2002). Leaf samples were collected 14 days after the infiltration of cotyledons of *G. barbadense cv* '7124' with GV3101 infiltrated, and the expression level was determined by RT-PCR. The seedlings with decreased transcript levels were infected with 'V991', and seedlings treated with TRV:00 were used as the control. Compared with the TRV:00 plants, the knockdown of *GhPUB17* made the plants more resistant to *V. dahliae*, as determined through an analysis of the dissected hypocotyls, which showed decreased defoliation and vascular bundles with a lighter colour (Fig. 1c, d). The calculated disease indexes supported this conclusion (Fig. 1e). An analysis of the relative fungal biomass in the stems by quantitative PCR (qPCR) revealed lower levels of *V. dahliae* in the *GhPUB17*-knockdown seedlings than in the control seedlings (Fig. 1f). All these results indicate that the downregulation of *GhPUB17* decreases the colonization of cotton vascular bundles by *V. dahliae* and that GhPUB17 acts as a negative regulator in resistance of cotton to *V. dahliae*.

The transcript level of *GhPUB17* influences cotton resistance to *V. dahliae*

To confirm the functions of GhPUB17 in cotton, a knockdown construct for RNA interference and an overexpression construct using the 35S promoter were constructed based on the specific untranslated region (UTR) sequence and the full-length sequence of *GhPUB17*, respectively. Transgenic plants were obtained through *Agrobacterium*-mediated transformation and regeneration. The T-DNA copy numbers were evaluated by Southern blotting, and all independent lines with a single T-DNA copy were selected for analysing the expression of *GhPUB17* in cotton (Fig. S3a, b). Two RNAi lines (RNAi5 and RNAi6) with significantly reduced *GhPUB17* transcript levels and two *GhPUB17*-overexpressing lines (OE31 and OE39) with a 20-fold higher transcript level compared with that in the WT plants were selected for further analysis (Fig. S3c, d).

At the five-leaf stage, the transgenic lines were inoculated with 'V991' to analyse their susceptibility. The results showed that the downregulation of *GhPUB17* improved the resistance of the plants to *V. dahliae*, whereas the overexpression of *GhPUB17* made the plants more sensitive to the pathogen compared with the WT plants (Fig. 2a). The analysis of dissected stems and the disease indexes calculated throughout the inoculation stage revealed results that were consistent with the phenotypic findings (Fig. 2b, c). The total DNA from hypocotyls was extracted and used as a template for the qPCR analysis of the relative fungal biomass. The results showed that the pathogen DNA content varied among the different lines: less pathogen DNA was detected in the *GhPUB17*-knockdown seedlings, whereas a higher amount of pathogen DNA was found in the *GhPUB17*-overexpressing seedlings (Fig. 2d). Cotyledonary nodes from each line were collected and used for pathogen recovery assays after surface sterilization. Consistent with the results of the fungal biomass analysis, less fungus was found in the RNAi lines compared with the *GhPUB17*-overexpressing and WT lines (Fig. 2e). To evaluate the susceptibility of cotton to other fungi, cotton leaves were infected with *Botrytis cinerea*, and no significant difference was observed among these lines (Fig. S4), which suggested that GhPUB17 acts as a negative regulator in cotton resistance to *V. dahliae* and does not form part of a general response to fungal infection.

GhPUB17 interacts with GhCyP3

To identify the potential target(s) of GhPUB17, the ARM-repeat domain, which is assumed to be the substrate-interacting domain (Liao et al. 2017), was employed as the bait in a Y2H screen with the cDNA library constructed from cotton roots inoculated with strain 'V991'. This experiment was repeated twice, and one recombinant was survived on SD-4 (-Thr/-Leu/-His/-Ade/+x-α-gel) medium (Fig. 3a). After sequencing, this target was identified as a member of the cyclophilin family and designated GhCyP3 based on a sequence analysis. The full coding sequence of *GhCyP3* contains 522 nucleotides and putatively encodes a protein with a length of 174 amino acids. To confirm the interaction between GhPUB17 and GhCyP3, full-length GhPUB17 was subsequently cloned into the BD vector to confirm its hybridization with AD-GhCyP3 (Fig. 3b).

GhCyP3, as a glutathione-S-transferase (GST)-tagged fusion protein at its N terminus, was expressed in *E. coli* (BL21) and purified by affinity chromatography. The interaction of the purified protein with MBP-GhPUB17 was tested through a pulldown experiment (Fig. S5a, b), and the GST tag itself was used as a negative control. The results confirmed the interaction between these two proteins (Fig. 3c). The pulldown experiment revealed that GhCyP3 interacts with three peptides, namely, full-length MBP-GhPUB17 and two fragments of MBP-GhPUB17. The MBP-GhPUB17 protein was expressed and extracted from *E. coli*. During the extraction process, parts of the protein were degraded, and even though we performed the experiment several times, this degradation could not be avoided. Three fragments were detected in the GhPUB17 protein samples: fragment I, at 122 kDa; fragment II, at 85 kDa; and fragment III, at 75 kDa (Fig. S5b). The length of the proteins was estimated

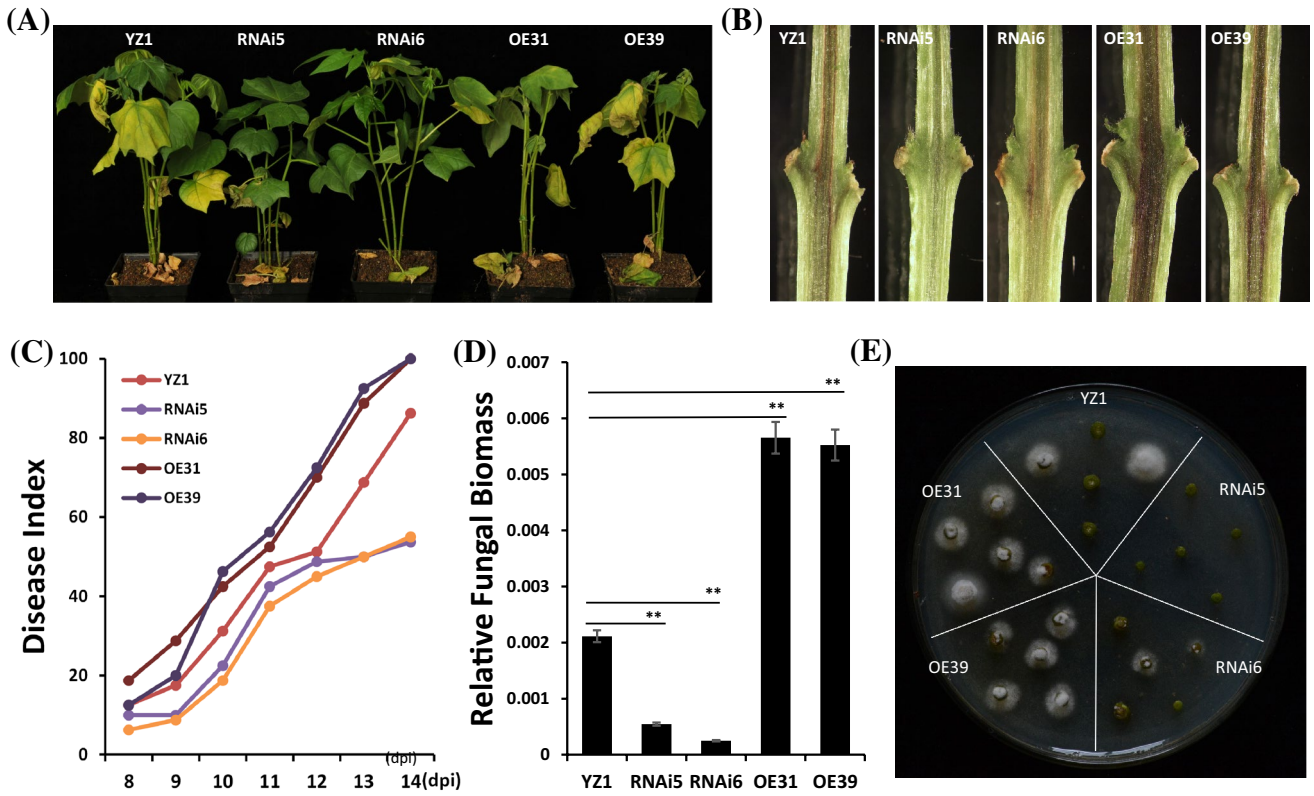

Fig. 2 The overexpression of *GhPUB17* attenuates cotton resistance to *V. dahliae*. **a** Symptoms of *GhPUB17*-knockdown plants (RNAi5 and RNAi6), *GhPUB17*-overexpressing plants (OE31 and OE39) and WT (YZ1) lines 12 days after *V. dahliae* inoculation. **b** Images of dissected stems from *GhPUB17*-knockdown plants (RNAi5 and RNAi6), *GhPUB17*-overexpressing plants (OE31 and OE39) and WT (YZ1) lines. **c** Disease index calculated from *GhPUB17*-knockdown plants (RNAi5 and RNAi6), *GhPUB17*-overexpressing plants (OE31 and OE39) and WT (YZ1) lines. **d** Relative fungal biomass in stems from *GhPUB17*-knockdown plants (RNAi5 and RNAi6), *GhPUB17*-overexpressing plants (OE31 and OE39) and WT (YZ1) lines. The values and error bars are presented as the means and standard deviations, respectively, of triplicate samples ($n \geq 15$ plants, $^{**}P < 0.01$, t-test). **e** Recovery of fungus from *GhPUB17*-knockdown plants (RNAi5 and RNAi6), *GhPUB17*-overexpressing plants (OE31 and OE39) and WT (YZ1) lines on PDA medium

based on the protein marker in the same SDS-PAGE gel. All tags were confirmed by mass spectrometry to verify the protein sequence. The peptides detected in the three fragments compared with the reference sequence of MBP-GhPUB17 are listed in Table S3. According to the size of each fragment and the structure of MBP-GhPUB17, fragment I was extrapolated as full-length MBP-GhPUB17 containing conserved U-box and ARM domains. In addition, fragment II was estimated to be a fragment of MBP-GhPUB17 that contains only the conserved U-box domain, whereas fragment III does not contain the U-box and ARM domains. All the fragments contained an MBP protein tag (Fig. S5d). Based on the findings that GhCyP3 was acquired by a Y2H with the GhPUB17-ARM domain as the bait and that GhCyP3 protein, which was identified in the GST pulldown assay, could interact in vitro with all fragments of GhPUB17 with or without the ARM domain, we propose that more than one binding site is involved in the interaction of these proteins.

To confirm the interaction between GhPUB17 and GhCyP3 in vivo, we constructed expression vectors for the transient transformation of *Nicotiana benthamiana* plants with *Agrobacterium tumefaciens* strain GV3101 for bimolecular fluorescence complementation (BiFC) and split luciferase complementation (SLC) assays. Because PUB17 and its homologues showed very low accumulation in *N. benthamiana* due to Ub-mediated degradation, as previously demonstrated, the proteasome inhibitor MG132 (50 μM) was infiltrated into leaves 6 h prior to imaging. The results from the BiFC assay indicated that both GhPUB17ARM and GhPUB17 interacted with GhCyP3 at the plasma membrane and in the nucleus. Inducer of CBF expression 1 (GhICE1) fused to the C-terminal portion of yellow fluorescence protein (YFP) served as the negative control in the analysis of the interaction with GhPUB17ARM&GhPUB17, and the interaction of nYFP with GhCyP3 also served as a negative control. No YFP fluorescence was observed with the other three combinations, suggesting a specific interaction between GhPUB17 and GhCyP3 (Fig. 3d, e). The interaction results were confirmed with the SLC assay, and a luciferase signal could

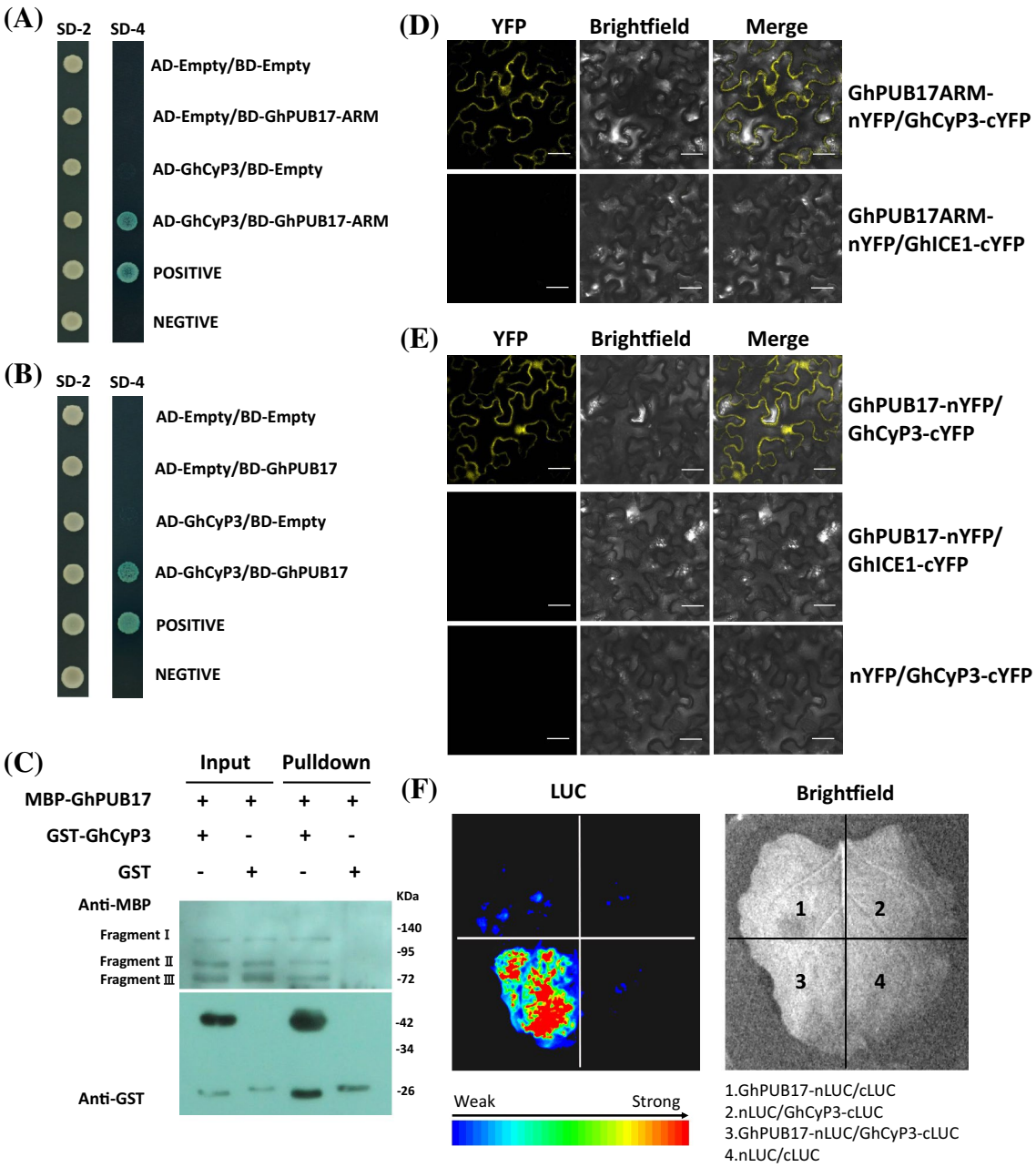

Fig. 3 GhPUB17 interacts with GhCyP3. **a** Yeast two-hybrid assays indicate that the ARM domain from GhPUB17 interacts with GhCyP3. **b** Yeast two-hybrid assays indicate that full-length GhPUB17 interacts with GhCyP3. **c** A GST pulldown experiment indicates that full-length MBP-GhPUB17 and its fragments interact with GST-GhCyP3. **d, e** GhPUB17ARM/GhPUB17 interact with GhCyP3 at the plasma membrane and in the nucleus. Bimolecular fluorescence complementation (BiFC) analysis was performed in *N. benthamiana*. GhPUB17ARM/GhPUB17 was fused to the N terminus of yellow fluorescence protein (YFP), and GhCyP3 was fused to the C terminus of YFP. Inducer of CBF expression 1 (GhICE1) was fused to the C terminus of YFP to serve as the negative control. Confocal microscopy images were obtained 3 days after infiltration. Bars, 50 μm. **f** GhPUB17 can interact with GhCyP3, as indicated by the split luciferase (LUC) complementation assay. GhPUB17 was fused to the N-terminal portion of LUC (nLUC), and GhCyP3 was fused to the C-terminal portion of LUC (cLUC). Agrobacteria carrying different plasmids, as indicated, were co-expressed in *N. benthamiana*. Three days after infiltration, images were taken using a Photek camera. The experiment was repeated twice with similar results. Six hours prior to imaging, MG132 (50 μM) was infiltrated into agrobacteria-infiltrated leaves in BiFC and SLC analyses due to the ubiquitination of PUB17 in the 26S proteasome

only be captured after the co-expression of GhPUB17-nLUC and GhCyP3-cLUC but not with the other combinations (Fig. 3f). All the results indicate that GhPUB17 interacts with GhCyP3.

GhPUB17 functions as an E3 Ub ligase, and this activity can be repressed by GhCyP3

To test whether GhPUB17 possesses E3 Ub ligase activity, the fused protein MBP-GhPUB17 was used for in vitro ubiquitination assays. In the presence of all components, including E1, E2, MBP-GhPUB17 and Ub, ubiquitination activity was observed in immunoblots probed with monoclonal antibodies to Ub. No ubiquitination was observed in the absence of any of the above-mentioned components. The Val residue within different U-box domains is highly conversed and very important for E3 ligase activity, as demonstrated in a study of AtPUB17 and NtACRE276 (Yang et al. 2006). In vitro ubiquitination assays revealed that E3 ligase activity was completely abolished in GhPUB17 protein with a Val-to-Ile point mutation at residue 311 (V311I), suggesting the specific E3 Ub ligase activity of GhPUB17 (Fig. 4a).

To explore the interaction between GhCyP3 and GhPUB17, the fused protein GST-GhCyP3 was used as an additional additive reagent in the ubiquitination assays of GhPUB17 E3 ligase activity. Each reaction contained the same components (at the same quantities) as those used in the above-described ubiquitination assays and a specific amount of GST-GhCyP3 (0.05, 0.5, 5, 50, 100, or 200 μg) in a total volume of 50 μl. The results showed that a higher quantity of GhCyP3 exerted a stronger repressive effect on the E3 Ub ligase activity of GhPUB17 (Fig. 4b). Furthermore, the ubiquitination of GST-GhCyP3 by GhPUB17 was not detectable with GST antibody. These results demonstrated the E3 Ub ligase activity of GhPUB17 and revealed that this activity was suppressed by GhCyP3 in vitro. In addition, no GST-GhCyP3 ubiquitinated by GhPUB17 could be detected with GST antibody in each lane, which suggested that GhCyP3 might not be the substrate responsible for the Ub ligase activity of GhPUB17 (Fig. 4b). The stability of recombinant MBP-GhPUB17 and GST-GhCyP3 after co-incubation for 3 or 6 h at 25 °C was assessed by immunoblotting. No significant dispersion zone was observed at 3 and 6 h compared with the control at 0 h, confirming the stability of these proteins (Fig. 4c). In addition, the transcript levels of GhCyP3 and GhPUB17 in cotton roots were evaluated by RT-qPCR, and the results showed that the GhCyP3 transcript level was approximately 48.7-fold higher than that of GhPUB17 (Fig. 4d), which suggested that the protein concentrations of GhPUB17 and GhCyP3 used in the in vitro activity assay should be biologically significant.

GhCyP3 acts as a positive regulator in the resistance of cotton to *V. dahliae*

The cyclophilin family is a large gene family that might have biological functions in a range of metabolic processes, including plant immunity. To explore the potential function of GhCyP3 in cotton resistance to *V. dahliae*, the GhCyP3-VIGS vector was constructed, and the empty vector TRV:00 was used as a control in plant infection assays. Different constructs of *Agrobacterium* GV3101 were infiltrated into the GhPUB17-RNAi6, GhPUB17-OE31, and WT (YZ1) lines, and 14 days after VIGS, the expression level of *GhCyP3* was assessed by RT-PCR. *GhCyP3*-knockdown plants were inoculated with *V. dahliae* strain V991. Disease symptoms were observed 1 week after inoculation, and the diseased leaves were analysed to calculate the disease index.

The results revealed that the *GhCyP3*-silenced plants with the WT background showed increased susceptibility to *V. dahliae* compared with the WT plants (Fig. 5a), and similar results were also observed in the GhPUB17-RNAi6/GhPUB17-OE31 plants. More serious defoliation was detected in the GhPUB17-OE31 plants in which *GhCyP3* was knocked down, which indicated that the overexpression of GhPUB17 in plants in which GhCyP3 is suppressed increases the susceptibility of the plants to *V. dahliae*. Additionally, the knockdown of *GhCyP3* impaired the resistance of GhPUB17-RNAi6 to *V. dahliae* (Fig. 5a). The stems dissected from *GhCyP3*-silenced plants presented a more severe level of necrosis than those from the control plants, and this finding was obtained from all three tested lines (Fig. 5b). In addition, symptoms of *Verticillium* wilt were observed earlier and showed increased severity in the GhPUB17-OE31 plants after the knockdown of *GhCyP3*. At 8 days post-inoculation (dpi), the disease index of the GhPUB17-OE31 seedlings was higher than 20, whereas those of the WT and GhPUB17-RNAi6 plants were less than 10 and close to zero, respectively (Fig. 5c-e). The results from both the pathogen recovery growth assay on PDA medium and the detection of the proportion of pathogen DNA in the total DNA of plants demonstrated that GhCyP3 repressed the colonization of *V. dahliae* in cotton seedlings (Fig. 5f, g), and thus, GhCyP3 acts as a positive regulator of cotton resistance to *V. dahliae*.

GhCyP3 protein shows antifungal activity against *V. dahliae* in vitro

The cyclophilin (CyP) family has various metabolic functions. In fact, several CyP proteins, such as C-CyP in cabbage (Lee et al. 2007), CaCyP1 in pepper (Kong et al. 2001) and StCyP in tomato (Godoy et al. 2000), have been reported to have antifungal activity. A sequencing and phylogenetic analysis revealed that GhCyP3 is highly homologous to these CyP proteins (Fig. 6a, b). To explore

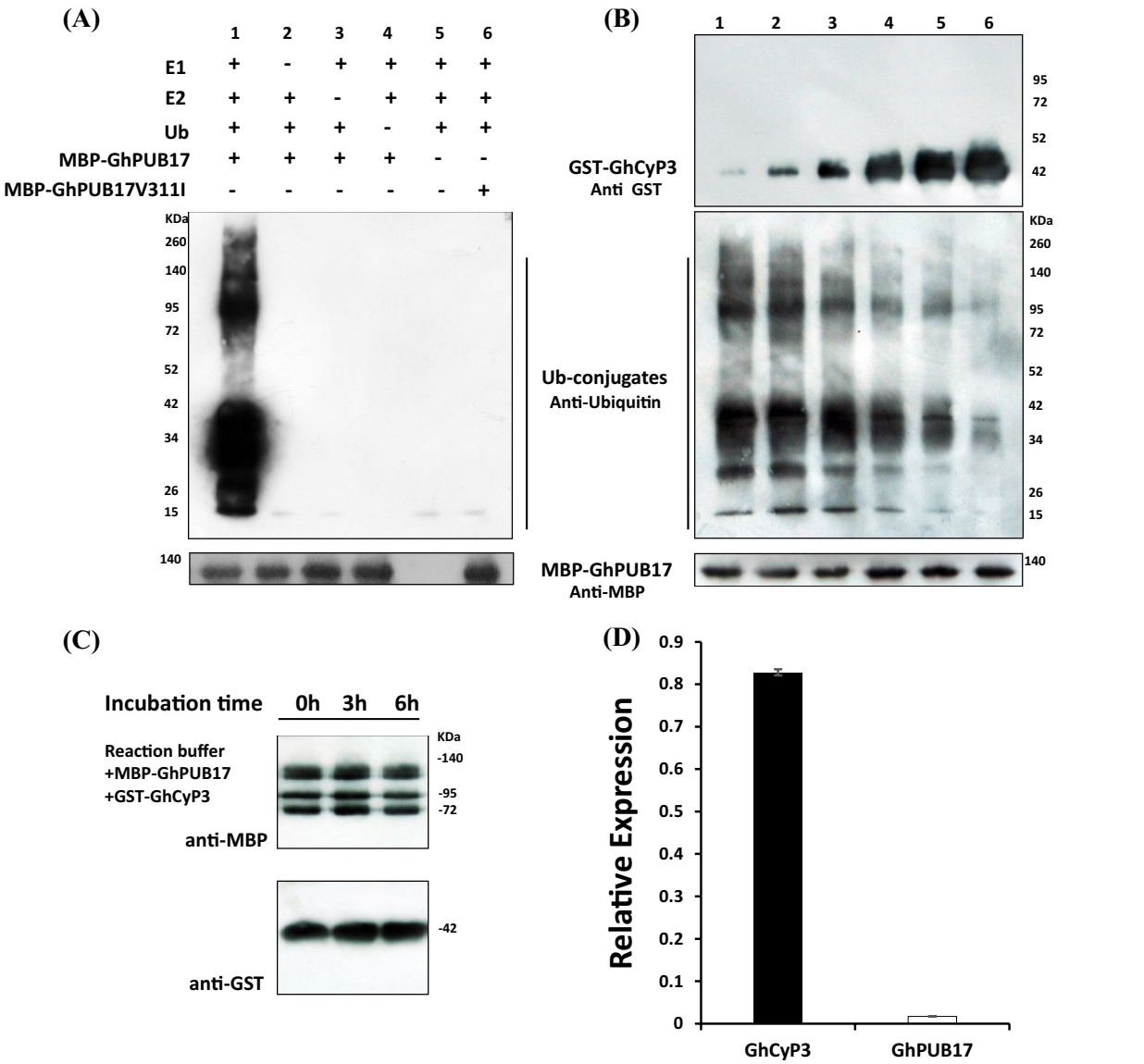

Fig. 4 GhPUB17 has E3 Ub ligase activity and is inhibited by GhCyP3 in vitro. **a** E3 Ub ligase activity assay of GhPUB17. Purified MBP-GhPUB17 fusion protein was incubated in the presence or absence of E1, E2, and Ub. The reactions were analysed by immunoblotting using an anti-Ub antibody. The E3 ligase activity of MBP-GhPUB17 was only detected in the presence of all components (lane 1). A mutated version of GhPUB17 (the conversed U-box domain with a Val-to-Ile substitution mutation at residue 311, MBP-GhPUB17) was used as a controls in the E3 activity assay, and no Ub conjugates were detected with the Val-to-Ile mutation at residue 311 (lane 6). The other lanes (sequential deletion of E1, E2, Ub and MBP-GhPUB17) yielded negative results. **b** GST-GhCyP3 quantitatively repressed the E3 ligase activity of MBP-GhPUB17 in vitro. Different amounts of GST-GhCyP3 were used in the ubiquitination system as the variable factor in this experiment. In lanes 1 to 6, the amounts of GST-GhCyP3 were 0.05, 0.5, 5, 50, 100 and 200 μg, respectively, in a total reaction volume of 50 μl. These results indicate that GhCyP3 cannot be ubiquitinated by GhPUB17. The quantity of PUB17 in each reaction was immunoprecipitated with MBP antibody as the loading control. **c** Stability of the recombinant proteins. The recombinant proteins MBP-GhPUB17 and GST-GhCyP3 were co-incubated at 25 °C for 3 and 6 h in E3 ligase reaction buffer, and the stability of the recombinant proteins was assessed by immunoblotting with anti-GST and anti-MBP antibodies, respectively. **d** Transcript level of *GhPUB17* and *GhCyP3* in cotton roots; the cotton gene *GhUb7* was used as a control

whether GhCyP3 has similar antifungal activity, GhCyP3 fused with a HIS tag at the N terminus was expressed by a prokaryotic expression system (*BL21*). The expression of GST fused to HIS was used as a control.

Recombinant proteins were extracted from IPTG-induced *E. coli* (*BL21*), and their concentrations were measured by the Bradford method and adjusted using a lyophilizer to a final concentration of 300 μg/ml. The stability of

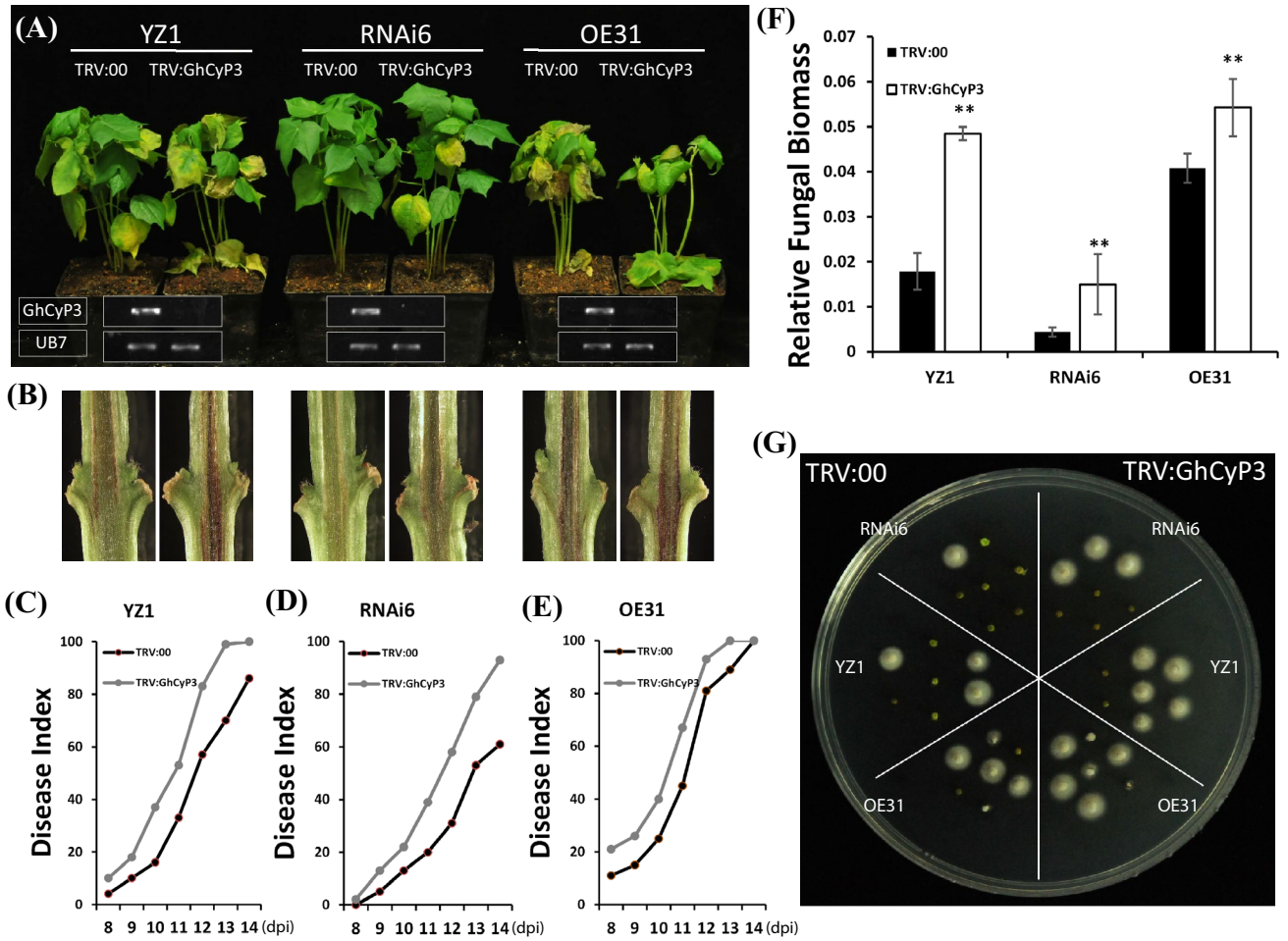

Fig. 5 The knockdown of GhCyP3 impairs cotton resistance to *V. dahliae*. **a** Symptoms of TRV:00 and TRV:GhCyP3 in YZ1, RNAi6 and OE31 lines 12 days after *V. dahliae* inoculation. The expression level of GhCyP3 in the roots of the different lines is shown below each image. **b** Stems dissected from the control and GhCyP3-silenced plants displayed differences in the level of necrosis 12 days after *V. dahliae* inoculation. **c, d** and **e** Disease indexes of *V. dahliae* in the TRV:00 and TRV:GhCyP3 lines at 7 days; these values were calculated from the GhCyP3-knockdown and control plants. **f** Relative fungal biomass in stems of the different lines. The values and error bars are presented as the means and standard deviations, respectively, of triplicate samples ($n \geq 15$ plants, $^{**}P < 0.01$, t-test). **g** Recovery of fungus from different lines with *GhCyP3* knockdown on PDA medium

recombinant His-GhCyP3 was determined by immunoblotting with anti-HIS, and the results confirmed its stability for the subsequent assays (Fig. 6c). A concentration gradient consisting of 1, 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 of the final concentration was obtained. The diluted proteins were subsequently mixed with isopycnic V991 spore solution (10^3/ml), and the mixtures were separately inoculated in PDA medium and Czapek medium for cultivation. Three days later, the colonies containing less GhCyP3 protein showed improved growth compared with those containing more GhCyP3 (Fig. 6d), and the effective concentration of GhCyP3 was estimated to equal 9.375 μg/ml (1:32 dilution). The spore concentration in Czapek culture 3 days after inoculation displayed similar trends as the results obtained on PDA medium, i.e., increases in the concentration of GhCyP3 decreased the spore number (Fig. 6e), and the effective concentration of GhCyP3 was estimated as 4.6875 μg/ml (1:64 dilution). To summarize, the effective concentration of GhCyP3 that is able to repress the development of *V. dahliae* spores (10^3/ml) was found to range from 4.6875 to 9.375 μg/ml. The data from the samples treated with HIS-GST in the same culture medium revealed no significant differences between the PDA and Czapek media and this experiment was repeated three times with similar results.

Discussion

Pathogens and plant hosts have coevolved over millions of years and generated complex mechanisms to regulate infection and defence. During infection, constitutive structural molecules or effectors secreted from pathogens are delivered

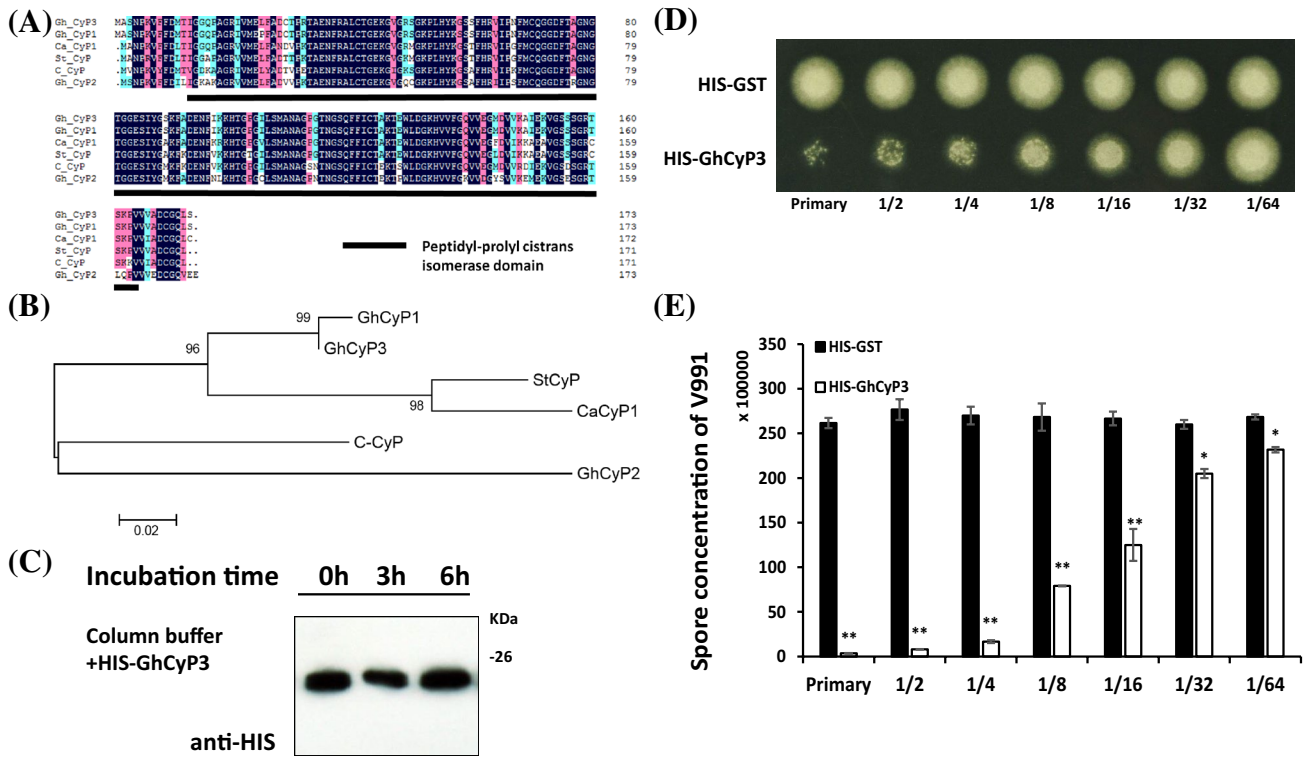

Fig. 6 GhCyP3 inhibits the proliferation of *V. dahliae*. **a** Alignment of homologues of CyPA amino acid sequences from cotton (GhCyP1, 2, 3), potato (StCyP), Chinese cabbage (C-CyP) and pepper (CaCyP1); GhCyP2 was used as the outgroup. The coloured boxes indicate the conserved amino acids. The peptidyl-prolyl cis–trans isomerase domain is marked by the black line. **b** Phylogenetic relationships among GhCyP3 and its homologues with antifungal activity. **c** The recombinant protein HIS-GhCyP3 was incubated at 25 °C for 3 and 6 h in column buffer, and its stability was assessed by immunoblotting with anti-HIS. **d** GhCyP3 protein inhibits the growth of V991 on a plate with PDA medium, and a higher content of GhCyP3 was associated with stronger antifungal activity. Primary protein concentration: 300 μg/ml. **e** Statistical analysis of the numbers of V991 spores in Czapek culture with different contents of GhCyP3. Primary protein concentration: 300 μg/ml. The standard deviations were calculated from the results of three independent experiments (n = 3, $^{**}P < 0.01$, $^{*}P < 0.05$, t-test). GST was recombined into the HIS vector and used as the negative control

to plants to repress their immunity and thus allow colonization. These pathogen-derived molecules are also recognized by plant hosts and activate host immune responses, including PTI and ETI (Sang et al. 2018). Plants can also produce antifungal metabolites or peptides to inhibit pathogen growth and colonization (Sang et al. 2018). A series of physiological reactions during host immune responses are generated by signalling pathways activated by hormones, metabolites, and enzymes (Zipfel and Oldroyd 2017). All the steps and components in these pathways are precisely managed by enzymes that are regulated by other systems, such as the ubiquitin 26S proteasome system (UPS), a major pathway that regulates protein degradation and modification (Du et al. 2009; Zhou et al. 2017). E3 ligases constitute the key component of the UPS and determine the diversity of the protein substrates of the UPS. Based on the classification of E3 ligases based on structural features, the PUB E3 ligase is a major subfamily that contains the conserved U-box domain (Yang et al. 2006), and E3 ligases were recently found to play roles in various physiological and metabolic processes, including plant immunity (Trujillo and Shirasu 2010; You et al. 2016).

The PUB gene family members usually contain a U-box domain that transfers activated Ub to substrates, whereas the ARM domain binds the substrates to allow their recognition (Trujillo 2018). PUB17 is a member of the PUB family and functions as an E3 ligase to regulate plant immunity, and the mutation of conversed Val sites within the U-box domain of PUB17 in tobacco abolishes its E3 ubiquitination activity (Yang et al. 2006). A previous study found that homologues of PUB17 play a positive role in the management of plant immunity by enhancing the immune responses of plants to specific pathogens or their effectors through the Cf-9- and Cf-4-mediated HR-PCD pathway across *Solanaceae* and *Brassicaceae* (He et al. 2015; Orosa et al. 2017; Yang et al. 2006). Effectors such as Avr4 and Avr9 can activate the immune responses managed by Cf-4 and Cf-9, and PUB17 plays a crucial role in both pathways. During the potato immune response to *P. infestans*, PUB17 is localized in the nucleus, which is consistent with its role in *N. benthamiana*

HR-PCD. Interestingly, in this study, we found that the PUB17 homologue GhPUB17 acts as a negative regulator in cotton resistance to *V. dahliae* (Figs. 1, 2). No homologues of Cf-9 and Cf-4 were found in the cotton genome, indicating the potential existence of a different immune pathway in cotton. In addition, *V. dahliae* is a fungal pathogen that infects cotton starting at the roots and thus blocks vascular bundles through colonization in the host (Shi et al. 2012), and this process differs from that of other pathogens, such as *Pst* DC3000 and *P. infestans*. Although our study provides more evidence indicating that GhPUB17 plays a negative role in cotton resistance to *V. dahliae*, more questions, e.g., the specific substrate(s) of GhPUB17 and the downstream signalling pathway of GhPUB17 in cotton defence against *V. dahliae*, remain to be explored.

The activity of E3 ligase is hypothesized to be regulated as part of the trade-off between plant growth and defence. POB1, an E3 ligase, is a negative regulator of plant immunity that could affect Cf-9/AVR9-mediated HR-PCD in tobacco and also destabilizes PUB17 in the nucleoplasm. This finding indicates that the level of E3 ligases could be regulated through degradation by other components (Orosa et al. 2017). Here, we found that GhCyP3 can repress the E3 ligase activity of GhPUB17, indicating that the regulation of GhPUB17 in cotton could involve the regulation of enzyme activity rather than the protein level (Fig. 4b). The GST pulldown assays revealed that GhCyP3 can interact with different fragments of GhPUB17, regardless of the presence of the ARM domain (Fig. 3c). This result was also confirmed by BIFC analysis (Fig. 3d), which suggested the existence of more than one binding site in the interaction between GhPUB17 and GhCyP3 and that this interaction could be due to the activity of the PPIase domain within GhCyP3.

The CyP family, which has been well studied in animals and plants, is involved in a wide range of metabolic pathways, including immunity (Kan et al. 2002; Mainali et al. 2014; Piechota-Polanczyk et al. 2013). For example, the CyP protein is the receptor for the effector cyclosporine A (CsA), a 11-amino-acid peptide that acts as an inhibitor of the immune response in animals (Piechota-Polanczyk et al. 2013). In plants, CyPs are highly expressed in metabolically active tissues and are involved in the regulation of development and abiotic/biotic stress responses (Kullertz et al. 1999; Oh et al. 2006). GhCyP1, which shares significant identity with GhCyP3, is reportedly involved in abiotic and biotic stresses. The overexpression of *GhCyP1* in tobacco increased the tolerance of the transgenic plants to salt stress and *Pseudomonas syringae pv. tabaci* (*Pst*) infection through the maintenance of membrane protein stability (Zhu et al. 2011). CaCyP1 in pepper is involved in the response to abiotic stress and is also induced by pathogenic bacteria and plant hormones (Kong et al. 2001). Similarly, StCyP in tomato is involved in the response to abiotic stress, hormones, mechanical damage and pathogens (Godoy et al. 2000). In our study, GhCyP3 was found to be involved in cotton resistance to *V. dahliae*. The knockdown of *GhCyP3* by VIGS increases the susceptibility of the plants to *V. dahliae*.

The results from various interaction assays, including Y2H, GST pulldown, BiFC and SLC, confirmed the interaction between GhPUB17 and GhCyP3 (Fig. 3). The findings revealed an inhibitory effect of GhCyP3 on the E3 Ub ligase activity of GhPUB17 (Fig. 4), but the mechanism underlying the inhibitory effect of GhCyP3 remains unclear. Nearly all CyP proteins contain a conserved domain with peptidyl-prolyl cis–trans isomerase (PPIase) activity, which is helpful for expediting specific pathways (Göthel and Marahiel 1999). Moreover, the activity of PPIase can make these proteins act as molecular chaperones that play a key role in protein refolding and synthesis and might be involved in pre-mRNA splicing (Horowitz et al. 2002). The homologue AtROC1 in *Arabidopsis* has been demonstrated to be involved in immune pathways by catalysing the *cis–trans* isomerization of the RIN4 peptide (Li et al. 2014b), which provides a possible link between GhPUB17 and GhCyP3 and implies that the spatial structure of GhPUB17 might be changed by the PPIase domain of GhCyP3. CyPA proteins are secreted around areas of inflammation in animals (Andreas et al. 1997). GhCyP3 was found to exhibit antifungal activity against *V. dahliae* in vitro in this study and was found at a high transcript level in cotton roots, which is the site of *V. dahliae* infection. However, this protein contains only a cyclophilin-like domain (CLD, consisting of approximately 109 amino acids) without any secretion signal, which indicates the potential existence of an intracellular passive defence activity to protect cotton against colonization of *V. dahliae*.

Materials and methods

Plant material and growth conditions

The cotton plants used in this study, namely, *Gossypium barbadense* cv. 7124, *Gossypium hirsutum* cv. YZ1, transgenic lines derived from YZ1 (RNAi5, RNAi6, OE31, OE39), and *Nicotiana benthamiana* seedlings, were grown in soil-filled pots or Hoagland's solution under greenhouse conditions with a 14-h light/10-h dark cycle and a day/night temperature of 28/20 °C.

Isolation and characterization of GhPUB17 and GhCyP3

As determined by RNA-Seq, the expression level of *GhPUB17* was downregulated in the *ssn* mutant compared

with the WT plants (Sun et al. 2014). The full-length sequence of *GhPUB17* was obtained by PCR using specific primers. The gene sequence of *GhCyP3* was acquired from a Y2H screen of a cDNA library constructed of RNAs extracted from the roots of YZ1 seedlings 12 h after inoculation with *V. dahliae* (Cao and Yan 2013); in this screen, the GhPUB17-ARM domain was used as the bait. The homologous protein sequences of these two genes were acquired from GenBank (http://www.ncbi.nlm.nih.gov/genbank).

Vector construction

Full-length and specific UTR sequences of *GhPUB17* were used to construct vectors for the overexpression of *GhPUB17* (in the pK2GW7.0 plasmid), for RNAi (in the pHellsgate4 plasmid), for VIGS (in the TRV:00 plasmid), for MBP-tag fusion (in the pMal-c4x plasmid), for Y2H (in the BD-pGBKT7 plasmid), and for GFP-tag fusion (in the pMDC43 plasmid). The GhCyP3 sequence was used to construct vectors for VIGS (in the TRV:00 plasmid), HIS-tag fusion (in the pET-28a-LR plasmid), for GST-tag fusion (in the pGEX4T-1 plasmid) and for GFP-tag fusion (in the pMDC43 plasmid), as listed in Tables S1 and 2. Both full sequences were generated for vector construction in the pBiFC-2in1-NN (V256) plasmid for BiFC analysis and in pCAMBIA-GW-nLUC and pCAMBIA-GW-cLUC for the SLC assay.

Virus-induced gene silencing

Non-conserved domains within the *GhPUB17* gene were used to generate the TRV:GhPUB17 construct, and the GhCyP3 sequence was used to generate the TRV:GhCyP3 construct. The TRV vectors and *Agrobacterium tumefaciens* for VIGS were prepared as previously described (Gao et al. 2013). *Agrobacterium* with TRV vectors was injected into cotyledons of cotton seedlings, and 14 days later, leaves from the injected plants were collected for RNA extraction and RT-qPCR detection of the gene expression level.

Pathogen inoculation

The protocols used for the preparation of *V. dahliae* strain V991 spores and cotton seedlings and pathogen infection were previously described (Gao et al. 2013). Strain V991 was cultured at 25 °C in Czapek solution, and the spores were collected, diluted to a concentration of 3×10^5/L or 1.5×10^6/L and inoculated into *G. hirsutum* or *G. barbadense* plants, respectively. Specifically, the roots of cotton plants were dipped into the spore suspension solution for 1 min, and the plants were then allowed to grow in soil. Approximately 1 week later, disease symptoms were observed and recorded.

Plant disease symptom phenotyping, photography, and data processing

Photographs were taken once symptoms of morbidity appeared, approximately 7 days post-infection with *V. dahliae* strain V991. Data for calculation of the disease index were gathered during the disease stages until the seedlings were close to death, as described by Xu et al. (2014). Once obvious wilt symptoms appeared after infection, the stems of the seedlings were collected. Cotyledonary nodes were used in the fungal recovery growth assay, the browning of vascular bundles was visualized in the dissected stems, and the relative fungal biomass was determined as previously described (Xu et al. 2014). Approximately 1 to 2 cm above the cotyledonary node, the stems were sterilized by mercury dichloride (1 g in 1 L water), cut into 2-mm pieces, homogenized, moved to PDA medium plates, and cultured in an incubator at 25 °C. Three days later, the phenotype was recorded by photographs taken using a camera (D7100, Nikon, Japan). The stems were dissected medially around the cotyledonary node and photographed under a stereoscopic microscope (MZFLIII, LEICA, Germany). DNA extracted from the above-mentioned stems was used as a template for the RT-qPCR-based detection of fungal biomass.

B. cinerea was cultured on plates with PDA at 25 °C for 4 days and then gathered using a hole puncher (5 mm). The punched discs were then inoculated into leaves of the GhPUB17 transgenic lines to assess their resistance. The pieces of PDA medium with *B. cinerea* spores were moved to the middle of fresh and healthy cotton leaves and cultured under moist and dark conditions at 25 °C for pathogen inoculation. Forty-eight hours later, the morbid phenotype was photographed, and the mean lesion area was calculated using Digimizer Image Analysis Software (Li et al. 2014a).

Agrobacterium tumefaciens-mediated transient protein expression in *Nicotiana benthamiana*

The constructed expression vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 through electroporation. The expression cultures and p19 (*A. tumefaciens* cells expressing the silencing suppressor) were cultured, and the cells were centrifuged at 1500×*g* for 10 min. The pellets were dissolved in resuspension buffer (10 mM MES, 10 mM $MgCl_2$ and 0.15 mM acetosyringone). Each expression culture was mixed with the same amount of p19, and the combined solutions were placed at room temperature (25 °C) for 2 h for the activation of infestation activity. Leaves from 4- to 6-week-old *Nicotiana benthamiana* plants were infiltrated with the agrobacterial mix using a needle-less syringe

and harvested 3 days post-inoculation from the infiltrated area for BiFC and SLC analyses (He et al. 2015; Liao et al. 2017). Six hours prior to imaging, the proteasome inhibitor MG132 (50 μM) (M7749, SIGMA, USA) was infiltrated into the leaves.

SLC assay

The infiltrated leaves were sprayed with 1 mM beetle luciferin (P1041, Promega, USA), and the resulting signal was captured using a Photek camera (Lumazone PyLoN 2048B, Roper, USA) for 15 min (Liao et al. 2017).

BiFC analysis

The fluorescence in the infiltrated leaves was imaged using a confocal laser-scanning microscope (FV1200, Olympus, Japan) (Liao et al. 2017). ICE1 is a promoter element involved in the cold induction of CBF genes in Arabidopsis (Lee et al. 2015; Zarka et al. 2003). No studies conducted to date have revealed that homologues of ICE1 are involved in the management of immunity in plant species. The homologue in cotton, GhICE1, was used as the negative control in this assay to elucidate the specific interactions between GhCyP3 and GhPUB17ARM and between GhCyP3 and GhPUB17.

Prokaryotic expression of GhPUB17 and GhCyP3 proteins

Full-length *GhPUB17* and *GhCyP3* genes were used for vector construction. HIS and GST tags were fused to the N terminus of GhCyP3, and the MBP tag was fused to the N terminus of GhPUB17. The vectors were transfected into *E. coli* BL21 for expression. HIS-GST was expressed and used as a control. The positive clones were detected and stored in a fridge at − 70 °C in the presence of 15% glycerinum. The expression of the proteins was induced in the presence of 3% IPTG (0.1 mol/L) at 18 °C for 6 h. The induced *E. coli* colonies were collected for detection of the fused proteins on an SDS-PAGE gel. The proteins were extracted using suitable kits (GST, Promega V8603; HIS, Promega V8500; MBP, Biolabs, #E8021V) and stored at − 70 °C or extracted when needed.

Identification of MBP-GhPUB17 fragments by mass-spectrometric assay

MBP-GhPUB17 protein extracted from the prokaryotic expression system was separated by SDS-PAGE and stained with Coomassie blue. The three different fragments in the gel were separately collected and subjected to a mass-spectrometric assay (Shanghai Applied Protein Technology Co., Ltd. China). The detected peptides matching each fragment are listed in Table S3.

Y2H screen

The Y2H screen was performed using a cDNA library constructed with RNAs from YZ1 roots inoculated with *V. dahliae*. The ARM domain (assumed to be the substrate-interacting domain) of GhPUB17 was used as the bait to screen for potential target(s), as described in a previous study (Orosa et al. 2017). The recombinants were filtered on SD-4 medium (-His/-Leu/-Ser/-Ade/+x-α-gel), and the surviving colonies were used for information analysis. Full-length GhPUB17 was subsequently used to confirm the interaction with GhCyP3 as described above.

GST pulldown assay

GST-GhCyP3 and MBP-GhPUB17 proteins were used for the GST pulldown assays, and a GST-Tag was used as the negative control. Proteins stored at − 70 °C were mixed gently on ice, incubated at room temperature for 30 min and pulled down by GST-binding beads. The samples were separated by SDS-PAGE and subsequently detected by immunolabelling through western blotting with anti-GST and anti-MBP antibodies. The immunolabelling results were recorded on X-ray film.

E3 Ub ligase activity assay and analysis of the repression of GhPUB17 by GhCyP3

The ubiquitination assays were performed with an Auto-ubiquitinoylation Kit (Instruction Manual BML-UW0970, Enzo Life Sciences, USA), following the protocols supplied by the manufacturer. Anti-Ubiquitin antibody was used in the following immunoblotting steps (He et al. 2015).

GST-GhCyP3 protein was used as the only variable factor to confirm the suppression of MBP-GhPUB17 by GST-GhCyP3. A GST-GhCyP3 gradient was set up as follows. Pre-purified proteins maintained at − 70 °C in a freezer were thawed on ice. GST-GhCyP3 protein was diluted with 50 ml of column buffer (CB; 1 M Tris–HCl (pH 7.4), 1 ml; NaCl, 0.585 g; and 0.5 M EDTA, 100 μl) into six tubes containing 0.05, 0.5, 5, 50, 100, or 200 μg of GST-GhCyP3 (final volume, 20 μl) and gently mixed with 5 μg of MBP-GhPUB17. The mixtures were incubated in a water bath at 37 °C for 30 min and then used as components for the ubiquitination assays. The protocols used for these assays are similar to those of the E3 Ub ligase activity assay. Anti-ubiquitin and anti-GST antibodies were used in the subsequent immunoblotting steps.

The recombinant proteins MBP-GhPUB17 and GST-GhCyP3 were co-incubation in E3 ligase reaction buffer

for up to 3 and 6 h at 25 °C, and their stability was then assessed by immunoblotting. Five micrograms of GST-GhCyP3 and 5 µg of MBP-GhPUB17 were combined and maintained in E3 ligase reaction buffer (total volume, 50 µl). Each group was prepared in triplicate: one was used as the control at 0 h, and the other two were maintained in a water bath at 25 °C for 3 and 6 h. The three samples (20 µl) were loaded onto SDS-PAGE gels and then detected by immunoblotting using anti-GST and anti-MBP antibodies (Ma et al. 2017).

Antifungal activity of HIS-GhCyP3

Previously described methods (Lee et al. 2007) and agar dilution method (Fernandes et al. 2007) were modified for this assay. *V. dahliae* strain V991 was cultured in Czapek liquid medium at 25 °C for 3 days, and fresh spores were collected and diluted in Czapek medium to a proper concentration (1×10^3/ml) for incubation with the recombinant proteins HIS-GhCyP3 and HIS-GST. Fresh HIS-GhCyP3 and HIS-GST proteins were immediately extracted from IPTG-induced *E. coli* BL21 using HIS extraction kits (Promega V8500) to maintain their activity. The concentration of the extracted solutions was measured using the Bradford method and adjusted by lyophilization to obtain a high concentration of 300 µg/ml. A concentration gradient starting from the high concentration of 300 µg/ml was then obtained by obtaining 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 dilutions with the solution buffer supplied with the kit. One hundred microliters of the prepared protein sample was mixed with 100 µl of V991 spore solution, and the mixtures were incubated in a water bath at 25 °C for 1 h. The incubated samples were separately inoculated on plates with PDA medium and cultivated at 25 °C. Three days later, photographs of the PDA plates were obtained to record the phenotype. Another group of protein-spore mixtures (prepared using the same previously described method) was mixed with 300 µl of Czapek solution to a final volume of 500 µl, and the mixture was then transferred to 2-ml sterilized PE tubes and cultured at 25 °C with shaking at 200 rpm/min. Three days later, the number of spores was calculated under a microscope using a blood counting chamber.

Acknowledgements This work was financially supported by the Nature Science Foundation of Hubei Province (2016CFA054) and the International Science and Technology Cooperation Program of China (Grant No. 2015DFA30860).

Author Contributions Professor LZ and Professor XZ designed the main thoughts of this study, TQ proceeded this study and finished the manuscript, SL and ZZ acted as the assistants in materials preparing and study proceeding, LS and XH provided significant suggestions in experiment designment, KL commented the research and revised the manuscript.

References

Andreas B, Gottfried W, Heinrich A, Antal R, Peter P (1997) Presence of cyclophilin A in synovial fluids of patients with rheumatoid arthritis. J Exp Med 185:975–980

Boller T, He SY (2009) Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science 324:742–744

Cao S, Yan L (2013) Construction of a high-quality yeast two-hybrid (Y2H) library and its application in identification of interacting proteins with key vernalization regulator TaVRN-A1 in wheat. BMC Res Notes 6:81

Dalio RJD, Magalhaes DM, Rodrigues CM, Arena GD, Oliveira TS, Souza-Neto RR, Picchi SC, Martins PMM, Santos PJC, Maximo HJ, Pacheco IS, De Souza AA, Machado MA (2017) PAMPs, PRRs, effectors and R-genes associated with citrus-pathogen interactions. Ann Bot 119:749–774

Dodds PN, Rathjen JP (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. Nat Rev Genet 11:539–548

Du Z, Zhou X, Li L, Su Z (2009) plantsUPS: a database of plants' Ubiquitin Proteasome System. BMC Genom 10:227

Fernandes CJ, O'Sullivan MV, Cai Y, Kong F, Zeng X, Gilbert GL, Kotsiou G (2007) Agar dilution method for detection of inducible clindamycin resistance in Staphylococcus spp. J Clin Microbiol 45:4018–4020

Gao W, Long L, Zhu LF, Xu L, Gao WH, Sun LQ, Liu LL, Zhang XL (2013) Proteomic and virus-induced gene silencing (VIGS) analyses reveal that gossypol, brassinosteroids, and jasmonic acid contribute to the resistance of cotton to *Verticillium dahliae*. Mol Cell Proteomics 12:3690–3703

Godoy AV, Lazzaro AS, Casalongué CA, Segundo BS (2000) Expression of a Solanum tuberosum cyclophilin gene is regulated by fungal infection and abiotic stress conditions. Plant Sci 152:123–134

Gonzalez-Lamothe R, Tsitsigiannis DI, Ludwig AA, Panicot M, Shirasu K, Jones JD (2006) The U-box protein CMPG1 is required for efficient activation of defense mechanisms triggered by multiple resistance genes in tobacco and tomato. Plant cell 18:1067–1083

Göthel SF, Marahiel MA (1999) Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. Cell Mol Life Sci 55:423

He Q, McLellan H, Boevink PC, Sadanandom A, Xie C, Birch PR, Tian Z (2015) U-box E3 ubiquitin ligase PUB17 acts in the nucleus to promote specific immune pathways triggered by *Phytophthora infestans*. J Exp Bot 66:3189–3199

Horowitz DS, Lee EJ, Mabon SA, Misteli T (2002) A cyclophilin functions in pre-mRNA splicing. EMBO J 21:470–480

Jones JD, Dangl JL (2006) The plant immune system. Nature 444:323–329

Kan YC, Liu SW, Guo ZJ, Li DB (2002) Characterization of a cyclophilin cDNA from soybean cells. Acta Bot Sin 44:173–176

Kong HY, Lee SC, Hwang BK (2001) Expression of pepper cyclophilin gene is differentially regulated during the pathogen infection and abiotic stress conditions. Physiol Mol Plant Pathol 59:189–199

Kullertz G, Liebau A, Rucknagel P, Schierhorn A, Diettrich B, Fischer G, Luckner M (1999) Stress-induced expression of cyclophilins in proembryonic masses of Digitalis lanata does not protect against freezing/thawing stress. Planta 208:599–605

Kwon SJ, Choi EY, Choi YJ, Ahn JH, Park OK (2006) Proteomics studies of post-translational modifications in plants. J Exp Bot 57:1547–1551

Lee JR, Park SC, Kim JY, Lee SS, Park Y, Cheong GW, Hahm KS, Lee SY (2007) Molecular and functional characterization of a cyclophilin with antifungal activity from Chinese cabbage. Biochem Biophys Res Commun 353:672–678

Lee JH, Jung JH, Park CM (2015) INDUCER OF CBF EXPRESSION 1 integrates cold signals into FLOWERING LOCUS C-mediated flowering pathways in Arabidopsis. Plant J 84:29–40

Li C, He X, Luo X, Xu L, Liu L, Min L, Jin L, Zhu L, Zhang X (2014a) Cotton WRKY1 mediates the plant defense-to-development transition during infection of cotton by *Verticillium dahliae* by activating JASMONATE ZIM-DOMAIN1 expression. Plant Physiol 166:2179–2194

Li M, Ma X, Chiang YH, Yadeta KA, Ding P, Dong L, Zhao Y, Li X, Yu Y, Zhang L, Shen QH, Xia B, Coaker G, Liu D, Zhou JM (2014b) Proline isomerization of the immune receptor-interacting protein RIN4 by a cyclophilin inhibits effector-triggered immunity in Arabidopsis. Cell Host Microbe 16:473–483

Liao D, Cao Y, Sun X, Espinoza C, Nguyen CT, Liang Y, Stacey G (2017) Arabidopsis E3 ubiquitin ligase PLANT U-BOX13 (PUB13) regulates chitin receptor LYSIN MOTIF RECEPTOR KINASE5 (LYK5) protein abundance. New Phytol 214:1646–1656

Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP (2002) Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J 30:415–429

Ma Z, Zhu L, Song T, Wang Y, Zhang Q, Xia Y, Qiu M, Lin Y, Li H, Kong L, Fang Y, Ye W, Wang Y, Dong S, Zheng X, Tyler BM, Wang Y (2017) A paralogous decoy protects *Phytophthora sojae* apoplastic effector PsXEG1 from a host inhibitor. Science 355:710–714

Mainali HR, Chapman P, Dhaubhadel S (2014) Genome-wide analysis of cyclophilin gene family in soybean (Glycine max). BMC Plant Biol 14:282

Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JD (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. Plant Physiol 135:1113–1128

Oh K, Ivanchenko MG, White TJ, Lomax TL (2006) The diageotropica gene of tomato encodes a cyclophilin: a novel player in auxin signaling. Planta 224:133–144

Orosa B, He Q, Mesmar J, Gilroy EM, McLellan H, Yang C, Craig A, Bailey M, Zhang C, Moore JD, Boevink PC, Tian Z, Birch PR, Sadanandom A (2017) BTB-BACK domain protein POB1 suppresses immune cell death by targeting ubiquitin E3 ligase PUB17 for degradation. PLoS Genet 13:e1006540

Piechota-Polanczyk A, Demyanets S, Nykonenko O, Huk I, Mittlboeck M, Domenig CM, Neumayer C, Wojta J, Nanobachvili J, Klinger M (2013) Decreased tissue levels of cyclophilin A, a cyclosporine a target and phospho-ERK1/2 in simvastatin patients with abdominal aortic aneurysm. Eur J Vasc Endovasc Surg 45:682–688

Sang Y, Wang Y, Ni H, Cazale AC, She YM, Peeters N, Macho AP (2018) The Ralstonia solanacearum type III effector RipAY targets plant redox regulators to suppress immune responses. Mol Plant Pathol 19:129–142

Shi H, Liu Z, Zhu L, Zhang C, Chen Y, Zhou Y, Li F, Li X (2012) Overexpression of cotton (*Gossypium hirsutum*) dirigent1 gene enhances lignification that blocks the spread of *Verticillium dahliae*. Acta Biochim Biophys Sin 44:555–564

Stone SL, Callis J (2007) Ubiquitin ligases mediate growth and development by promoting protein death. Curr Opin Plant Biol 10:624–632

Sun L, Zhu L, Xu L, Yuan D, Min L, Zhang X (2014) Cotton cytochrome P450 CYP82D regulates systemic cell death by modulating the octadecanoid pathway. Nat Commun 5:5372

Thomma BP, Nurnberger T, Joosten MH (2011) Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant cell 23:4–15

Trujillo M (2018) News from the PUB: plant U-box type E3 ubiquitin ligases. J Exp Bot 69:371–384

Trujillo M, Shirasu K (2010) Ubiquitination in plant immunity. Curr Opin Plant Biol 13:402–408

Vierstra RD (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. Nat Rev Mol Cell Biol 10:385–397

Xu L, Zhang W, He X, Liu M, Zhang K, Shaban M, Sun L, Zhu J, Luo Y, Yuan D, Zhang X, Zhu L (2014) Functional characterization of cotton genes responsive to *Verticillium dahliae* through bioinformatics and reverse genetics strategies. J Exp Bot 65:6679–6692

Yang CW, Gonzalez-Lamothe R, Ewan RA, Rowland O, Yoshioka H, Shenton M, Ye H, O'Donnell E, Jones JD, Sadanandom A (2006) The E3 ubiquitin ligase activity of arabidopsis PLANT U-BOX17 and its functional tobacco homolog ACRE276 are required for cell death and defense. Plant cell 18:1084–1098

Yee D, Goring DR (2009) The diversity of plant U-box E3 ubiquitin ligases: from upstream activators to downstream target substrates. J Exp Bot 60:1109–1121

You Q, Zhai K, Yang D, Yang W, Wu J, Liu J, Pan W, Wang J, Zhu X, Jian Y, Liu J, Zhang Y, Deng Y, Li Q, Lou Y, Xie Q, He Z (2016) An E3 ubiquitin ligase-BAG protein module controls plant innate immunity and broad-spectrum disease resistance. Cell Host Microbe 20:758–769

Zarka DG, Vogel JT, Cook D, Thomashow MF (2003) Cold induction of Arabidopsis CBF genes involves multiple ICE (inducer of CBF expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. Plant Physiol 133:910–918

Zhou B, Zeng L (2016) Conventional and unconventional ubiquitination in plant immunity. Mol Plant Pathol 18:1313–1330

Zhou B, Mural RV, Chen X. Oates ME, Connor RA, Martin GB, Gough J, Zeng L (2017) A subset of ubiquitin-conjugating enzymes is essential for plant immunity. Plant Physiol 173:1371–1390

Zhu C, Wang Y, Li Y, Bhatti KH, Tian Y, Wu J (2011) Overexpression of a cotton cyclophilin gene (GhCyp1) in transgenic tobacco plants confers dual tolerance to salt stress and *Pseudomonas syringae* pv. tabaci infection. Plant Physiol Biochem 49:1264–1271

Zipfel C, Oldroyd GE (2017) Plant signalling in symbiosis and immunity. Nature 543:328–336