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Chitin synthases containing myosin motor-like domain are required for cell wall integrity and virulence of vascular wilt pathogen *Verticillium dahliae*

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Abstract

Verticillium wilt (VW) of cotton poses a serious threat to the quality and yield of cotton. *Verticillium dahliae* is the primary causal agent of cotton VW. Moreover, *V. dahliae* can infect more than 200 species of dicotyledonous plants. The fungal cell wall plays a crucial role in its growth, development and pathogenicity. However, the mechanism of cell wall synthesis in *V. dahliae* and its role in pathogenesis remains unclear. In this study, we identified two chitin synthase (CHS) genes *VdChs5* and *VdChs7* containing myosin motor-like domain (MMD) and characterized their role in virulence of *V. dahliae*. The results showed that the functions of *VdChs5* and *VdChs7* were largely redundant, and target deletion of both *VdChs5* and *VdChs7* in *V. dahliae* did not affect vegetative growth, but reduced conidial production. *ΔVdChs5Chs7* deletion mutant failed to colonize and proliferate in cotton vascular tissue, and exhibited significantly reduced virulence on cotton, suggesting that VdChs5 and VdChs7 are necessary for pathogenesis. In addition, the thickness of the cell wall in *ΔVdChs5Chs7* showed significantly decreased, and *ΔVdChs5Chs7* mutant exhibited hypersensitivity to cell wall perturbing agents and reactive oxygen species (ROS), indicating that VdChs5 and VdChs7 play key roles in cell wall integrity. Further, host-induced gene silencing (HIGS) silenced transcripts of *VdChs5* and *VdChs7* in susceptible cotton (*Gossypium hirsutum* L. acc. TM-1) enhanced resistance to cotton VW. Taken together, our data demonstrated that VdChs5 and VdChs7 play pivotal roles in proliferation, cell wall integrity, and pathogenicity, and provided a novel strategy to improve *Verticillium* wilt resistance in cotton and other susceptible host plants.

Keywords *Gossypium hirsutum*, *Verticillium dahliae*, CHS, Cell wall integrity, Virulence, HIGS

Background

Verticillium wilt (VW) is a serious threat to the quality and yield of cotton. The main causal agent of *Verticillium* wilt is the soil-borne pathogenic fungus *Verticillium dahliae*, which affects more than 200 dicotyledonous plant species, including many economically important crops (Fradin and Thomma 2006). After stimulated by host root exudates, the microsclerotia (MS), melanized resting structure of *V. dahliae*, germinate and produce invading hyphae to penetrate the root epidermis and cortex, and then colonize in the xylem (Klosterman et al. 2009; Luo et al. 2014; Klimes et al. 2015; Tian and Kong

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2022). Microsclerotia can survive in soil for more than ten years (Klosterman et al. 2009), so the cotton VW is difficult to control in the field. In recent years, the pathogenic mechanism of *V. dahliae* has become a research hotspot in cotton disease resistance. Some critical effectors involved in the host immune response (such as VdRTX1, VdPevD1, VdIsc1), and the transcription factors regulated fungal morphology and development (such as VdSge1, VdMRTF1, VdFTF1) have been successively explored in *V. dahliae* (Santhanam and Thomma 2013; Liu et al. 2014; Zhang et al. 2018; Zhang et al. 2021; Lai et al. 2022; Yin et al. 2022). However, the mechanism of cell wall synthesis in *V. dahliae* and its role in pathogenesis remains unclear.

Fungal cell wall plays important roles in cell viability, development, and pathogenicity (Gow et al. 2017). Generally, the inner layer of the fungal cell wall is mainly composed of cross-linked chitin-glucan matrix, while the outer layer is mainly glycoproteins. The composition of fungal cell wall is highly regulated in response to environmental conditions and imposed stresses (Geoghegan et al. 2017; Gow et al. 2017; Latgé et al. 2017). Chitin, a β-(1,4)-linked polymer of N-acetylglucosamine (GlcNAc), is synthesized by a huge family of chitin synthase (CHS) enzymes (Roncero 2002). In fungi, CHSs were grouped into eight discernable classes, of which classes III, V, VI, and VII are specific for filamentous fungi. Most interestingly, class V and class VII CHS contain N-terminal myosin motor-like domain (MMD) fused to the C-terminal CHS domain. In *Aspergillus nidulans*, the interaction between the MMD and actin is essential for the proper localization and function of CsmA (Takeshita et al. 2005). Class V chitin synthase CsmA and class VII chitin synthase CsmB play compensatory roles and are essential for cell wall integrity and hyphal tip growth in *A. nidulans* (Takeshita et al. 2006). In the corn smut fungus *Ustilago maydis*, Mcs1, a CHS containing the domain of the myosin-17 motor, travel along MT and F-actin mediated by kinesin-1 and myosin-5 (Schuster et al. 2012). Class VII CHS lacks the MMD domain at the N terminus, and Class VII CHS requires the motor domain of class V CHS for polar localization in *U. maydis* (Schuster et al. 2016). Two MMD-containing class V and class VII chitin synthase genes affect fungal asexual growth, stress resistance, cell wall integrity, and virulence in *Neurospora crassa*, *Magnaporthe oryzae, Gibberella zeae*, *Metarhizium acridum*, and *Trichoderma atroviride* (Kim et al. 2009; Kong et al. 2012; Fajardo-Somera et al. 2015; Zhang et al. 2019; Kappel et al. 2020). Nonetheless, the role of two MMD-containing chitin synthase genes in development and pathogenicity of *V. dahliae* remains unclear.

In this study, we identified two MMD-containing chitin synthase genes in *V. dahliae* and found that they play important roles in cell wall integrity and the virulence of the cotton pathogen *V. dahliae*. Further, host-induced gene silencing (HIGS) of *VdChs5* and *VdChs7* in cotton showed significantly enhanced VW resistance. Therefore, this study provided a novel strategy to enhance *Verticillium* wilt resistance in cotton and other sensitive host plants.

Results

Identification of classes V and VII chitin synthase in *V. dahliae*

According to the known chitin synthase sequences in *A. nidulans*, *U. maydis, N. crassa,* and *M. oryzae*, we identified eight *CHS* genes in the *V. dahliae VdLs.17* genome, named as *VdChs1-8* (Additional file 1: Figure S1). All chitin synthases have multiple transmembrane (TM) domains in *V. dahliae*. VdChs5 (VDAG_00420) consists of 1869 amino acid residues, which contains an N-terminal MMD (approximately 739 amino acids) and a C-terminal Chitin_synth_2 domain (approximately 508 amino acids), as based on the results of an InterProScan analysis (https://www.ebi.ac.uk/interpro/) and pfam (https://pfam.xfam.org). VdChs7 (VDAG_00419) consists of 1793 amino acid residues, which contains an N-terminal MMD (approximately 575 amino acids) and a C-terminal Chitin_synth_2 domain (approximately 508 amino acids). The N-terminal MMD domain of VdChs5 is class XVII myosin motor domain, while the MMD domain of VdChs7 is myosin and kinesin motor domain. VdChs5 has several other domains such as P-loop, switch I region, and switch II region, while VdChs7 does not, which is similar to the situation in *A. nidulans, N. crassa*, and *M. oryzae*. VdChs5 displayed 37.95% overall amino acid identity to VdChs7. *VdChs5* and *VdChs7* are in adjacent genomic proximity, are positioned head-to-head, and most likely share a bidirectional promoter, as has been shown in other fungi (Kappel et al. 2020). The phylogenetic analysis illustrated that the class V and VII chitin synthases of *V. dahliae* are highly conserved with other filamentous fungi homologs (Fig. 1).

The deletion of *VdChs5* and *VdChs7* genes affected aerial mycelial growth and conidial production

To investigate the roles of *VdChs5* and *VdChs7,* we generated single knockout mutants *ΔVdChs5* and *ΔVdChs7* for class V and VII chitin synthase of *V. dahliae* by homologous recombination (Wang et al. 2016). The deletion mutants were verified by PCR (Additional file 1: Figure S2) and RT-qPCR (Fig. 2b). The knockout mutants *ΔVdChs5* and *ΔVdChs7* had significantly less white aerial mycelium and less conidial production than the wild-type strain V592, but the growth rate and conidial germination rate in potato dextrose agar (PDA) medium were not

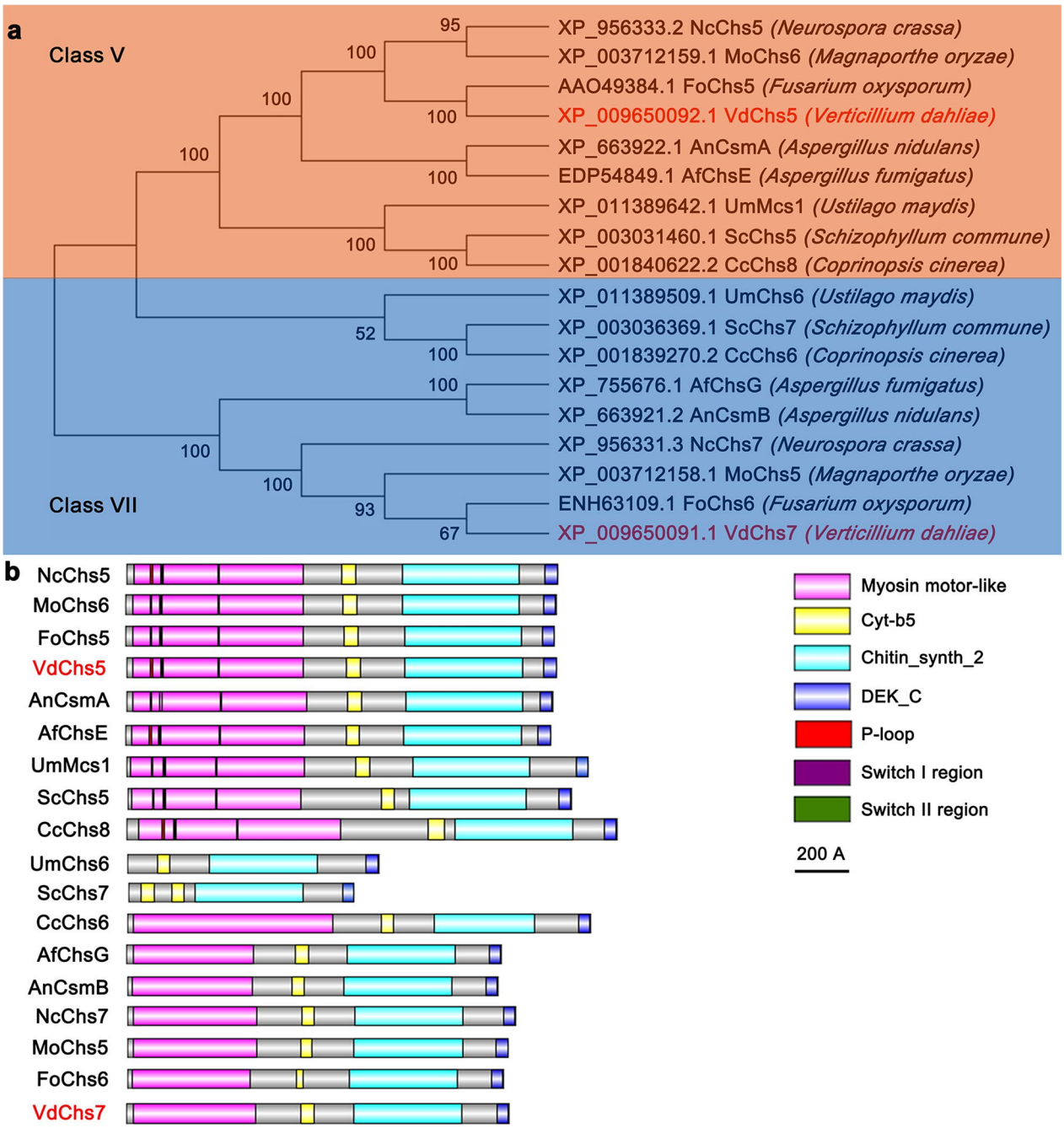

Fig. 1 Phylogenetic tree analysis and domain structures of class V and class VII chitin synthases in fungi. **a** The phylogenetic tree of class V and class VII CHSs in filamentous fungi is based on the full-length protein and was calculated in MEGA 7.0 software. Bootstrap values from 1000 replications are shown at the tree nodes. **b** The domain structure of class V and class VII CHSs. The boxes in different color show the myosin motor-like domains (MMD, PF00063), chitin_synth_2 domain (CS2, PF03142), cytochrome b5-like Heme/Steroid binding domain (b5, PF00173), and the DEK_C terminal domain (PF08766) in Pfam database

significantly different from those of the wild-type strain (Fig. 2a, c, d, e). To further determine whether the two genes have functional redundancy, we generated double knockout mutant *ΔVdChs5Chs7* in the same method. The double mutant *ΔVdChs5Chs7* showed significantly lower conidial production than *ΔVdChs5*, *ΔVdChs7,* and wild-type strain V592, but there are still no significant differences in growth rate and conidial germination rate

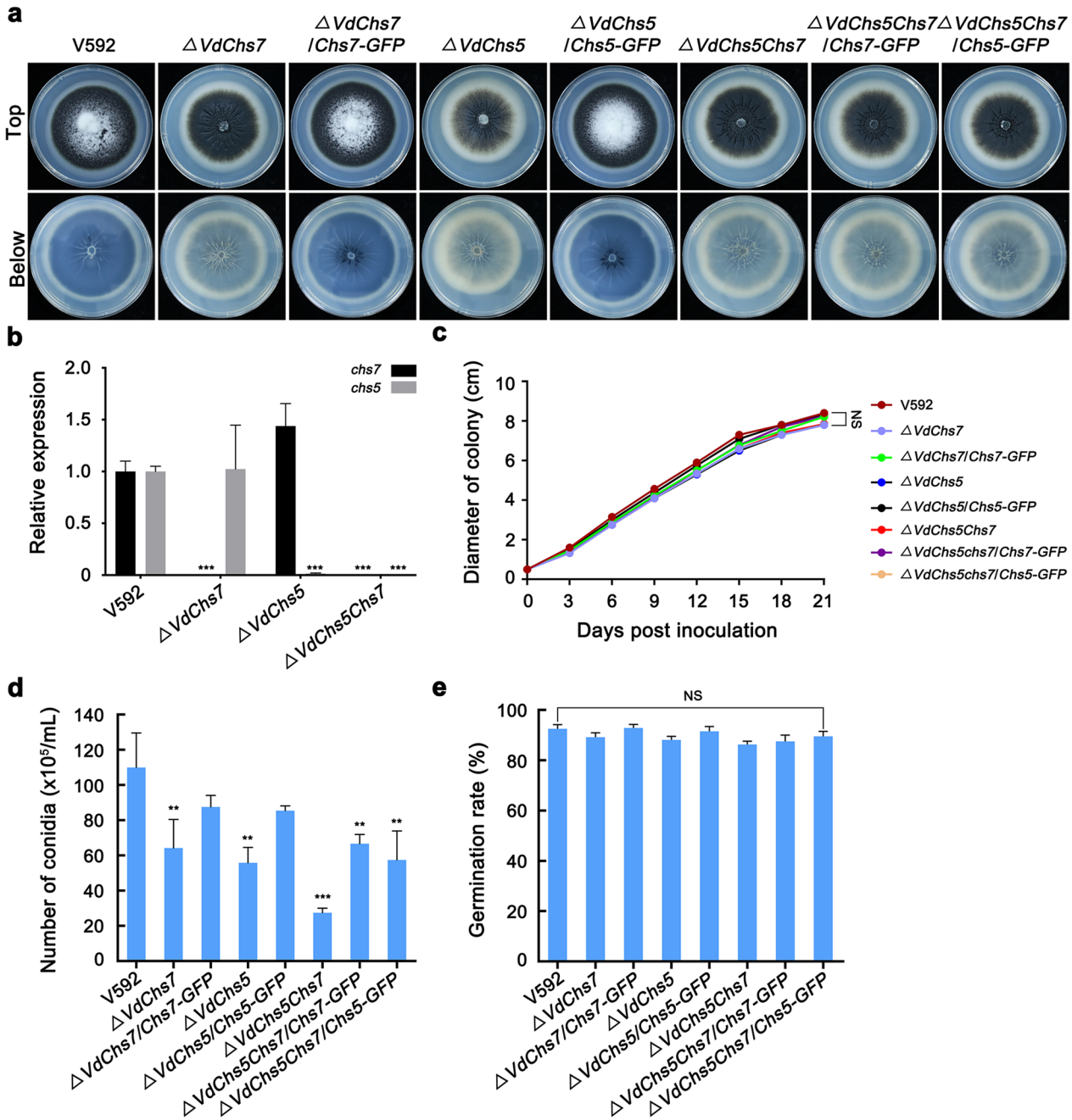

Fig. 2 Morphological and physiological characterization of *ΔVdChs7*, *ΔVdChs5*, and *ΔVdChs5Chs7*. **a** Colony morphology of *ΔVdChs7*, *ΔVdChs5*, *ΔVdChs5Chs7* and complemented strains *ΔVdChs7/VdChs7-GFP*, *ΔVdChs5/VdChs5-GFP*, *ΔVdChs5Chs7/VdChs7-GFP*, and *ΔVdChs5Chs7/VdChs5-GFP* at 15 days post inoculation (dpi) on PDA plates. **b** The relative expression of *VdChs7* and *VdChs5* in V592, *ΔVdChs7*, *ΔVdChs5*, and *ΔVdChs5Chs7* by RT-qPCR. **c** Radial growth of tested strains on PDA medium. **d** Conidia production of individual strains on PDA at 7 dpi. **e** Germination rate of each strain on PDA. Data represent means and standard deviation from three independent biological replicates. ** and *** indicate significant differences at $P < 0.01$ and $P < 0.001$, respectively, according to Student's *t*-test

(Fig. 2c, d, e). *ΔVdChs7*, *ΔVdChs5*, and *ΔVdChs5Chs7* mutants colonies exhibit reduced melanin production than wild-type strain V592 (Fig. 2a). It indicated that the roles of the *VdChs5* and *VdChs7* genes may be partially redundant, and VdChs5 and VdChs7 are required for conidial production.

VdChs5 and *VdChs7* played vital roles in virulence on cotton

To address the functions of the *VdChs5* and *VdChs7* in pathogenicity, two-week-old susceptible cotton (*Gossypium hirsutum* L. acc. TM-1) seedlings were inoculated with conidial suspensions of wild-type strain V592, *ΔVdChs7*, *ΔVdChs5*, *ΔVdChs5Chs7*, and the complemented strains using the unimpaired root dip-inoculation method (Feng et al. 2018). The cotton plants infected with the wild-type strain V592 showed typical *Verticillium* wilt symptoms, such as tissue chlorosis, wilting and vascular discoloration at 30 days post inoculation (dpi) (Fig. 3a). By contrast, cotton plants infected with the *ΔVdChs5Chs7* mutant kept healthy and did not show wilting and defoliation symptoms at 30 dpi, like uninfected (mock) control plants (Fig. 3a). The double mutant *ΔVdChs5Chs7* was significantly less pathogenic than the wild-type strain (Fig. 3a). Stem segments harvested from cotton plants inoculated with the *ΔVdChs5Chs7* strain

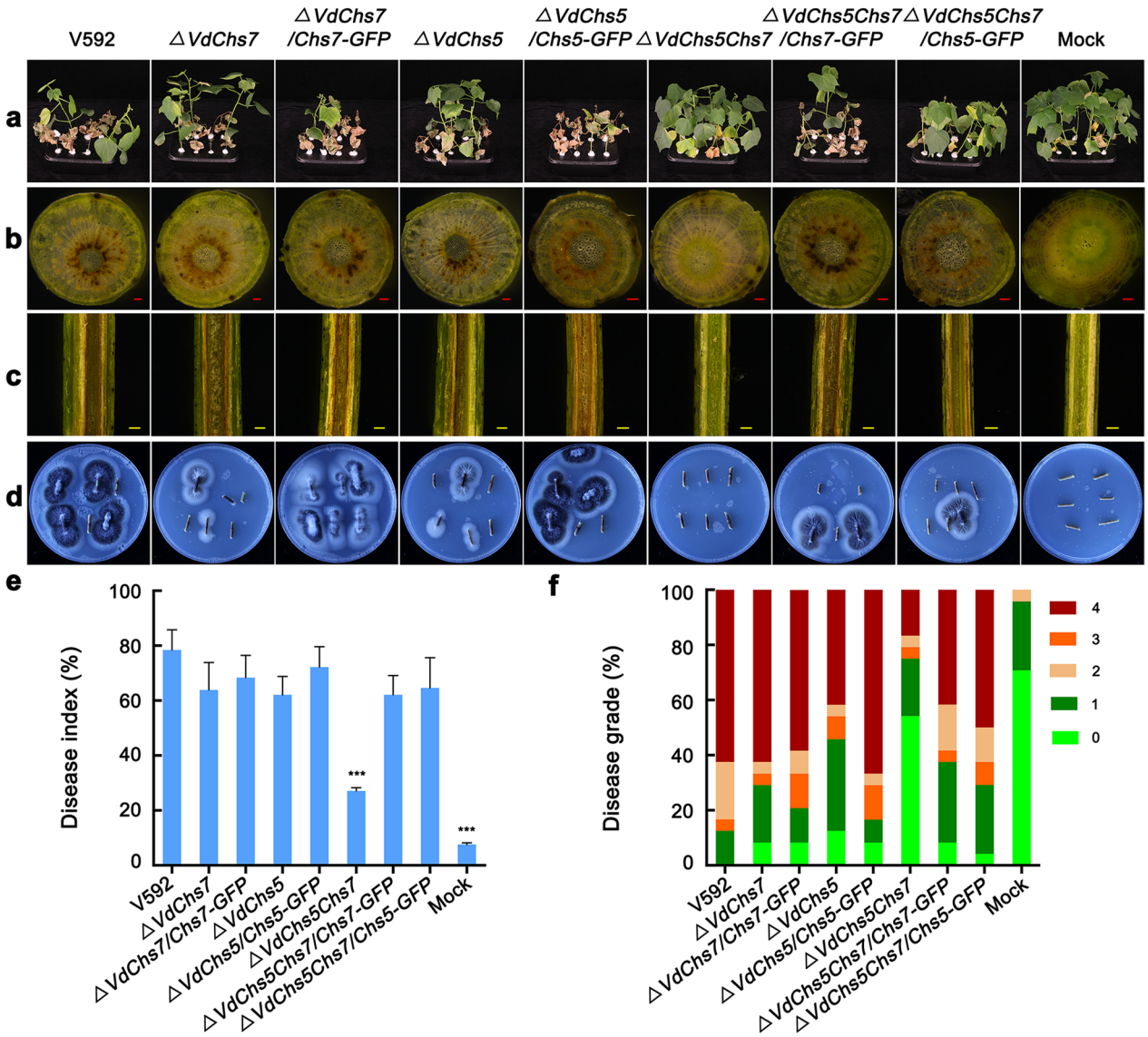

Fig. 3 Pathogenicity assays of *ΔVdChs7*, *ΔVdChs5*, and *ΔVdChs5Chs7* on cotton. **a** Disease symptoms of infected cotton plants at 30 days post inoculation (dpi). **b** Stem transverse sections of infected cotton plants at 30 dpi. Scale bars, 0.1 mm. **c** Stem longitudinal section of infected cotton plants at 30 dpi. Scale bars, 0.3 mm. **d** Fungal recovery from infected cotton stems at 30 dpi. **e** The disease index of infected cotton plants at 30 dpi. **f** The disease grade of infected cotton plants at 30 dpi. The disease index and disease grades were calculated with three replicates of 24 cotton plants. Data represent means and standard deviation from three independent biological replicates. *** indicates significant differences at $P < 0.001$ as calculated with Student's *t*-test

showed no vascular discoloration (Fig. 3b, c). Fungal recovery assays revealed that fungal mycelium could be recovered from cotton plants infected by wild-type strain and fully complementary strain, but not from *ΔVdChs5Chs7*-infected plants (Fig. 3d). In addition, the disease index was significantly lower in cotton plants inoculated with the *ΔVdChs5Chs7* strain compared to plants inoculated with the wild-type strain (Fig. 3e, f). We also observed that the expression of *VdChs5* and *VdChs7* was substantially up-regulated during cotton infection by *V. dahliae* (Additional file 1: Figure S3). Therefore, we concluded that the chitin synthase genes *VdChs5* and *VdChs7* act together to affect the pathogenicity of *V. dahliae* on cotton.

The deletion of *VdChs5* and *VdChs7* genes affected the proliferation/colonization of *V. dahliae* in the vascular tissues of cotton

To explore the specific reasons that account for the significant reduction in virulence of *ΔVdChs5Chs7*, penetration abilities of *ΔVdChs5Chs7* were firstly evaluated by inoculating the wild-type strain and the mutant strains on cellophane membrane laid on PDA medium. The result showed that all mutants were able to penetrate through the cellophane membrane, indicating deletion of *VdChs5* and *VdChs7* genes in *V. dahliae* did not affect penetration into the host (Fig. 4a). After 18 days inoculation with V592-GFP and *ΔVdChs5Chs7-GFP*, the longitudinal section of cotton stem was observed by confocal microscopy. A few mycelia were found in the vascular

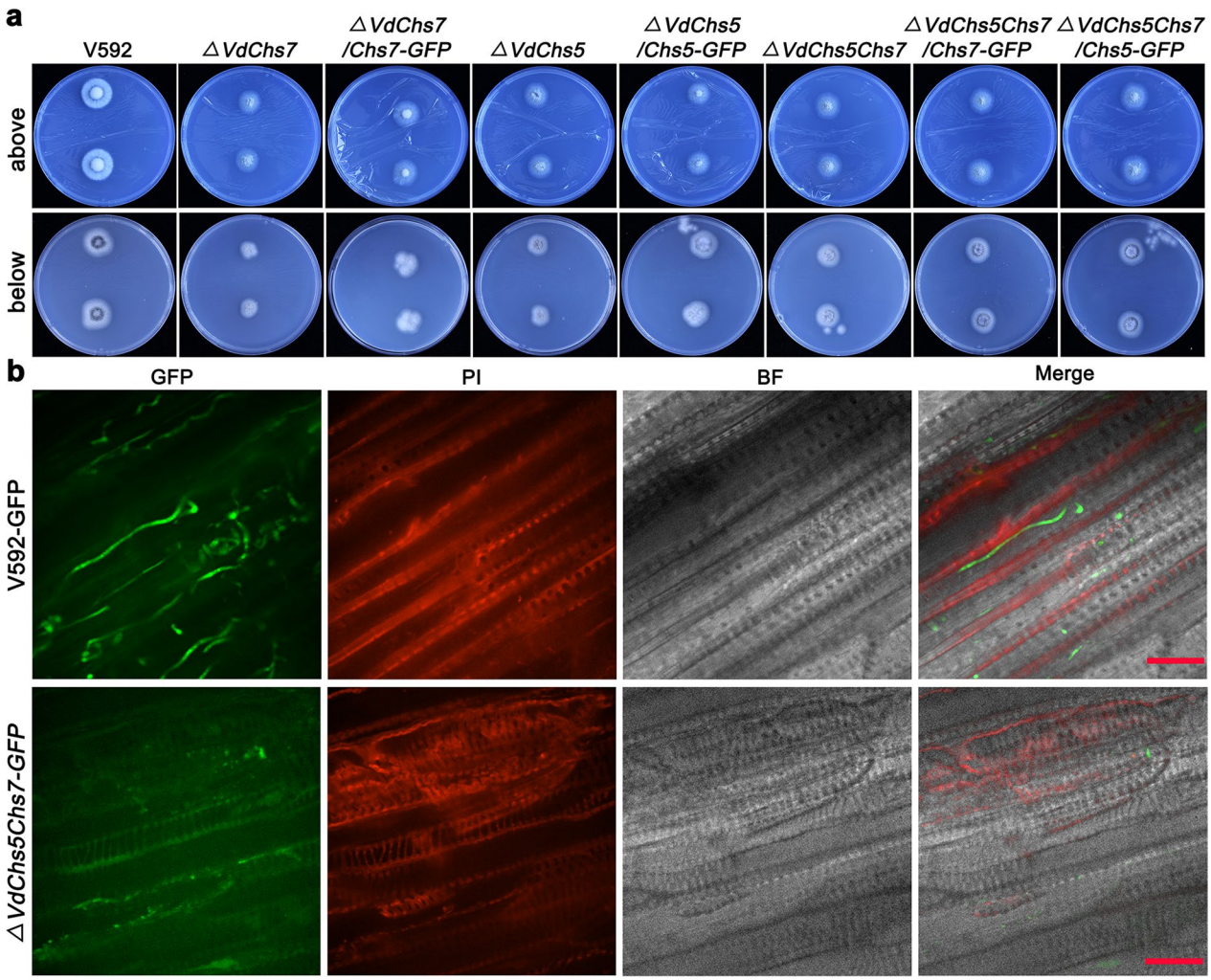

Fig. 4 Deletion of *VdChs5* and *VdChs7* affected the propagation of *Verticillium dahliae* in cotton. **a** Penetration assays of *ΔVdChs7*, *ΔVdChs5*, and *ΔVdChs5Chs7*. All strains were inoculated in PDA plates for 5 days, the cellophane membranes were removed, and the culture was continued for 3 days. An aliquot of 10 µL conidial suspension (1×10^6 conidia/mL) was placed on a cellophane membrane in the center of the plate. **b** The longitudinal sections of the stem vascular tissue in infected cotton inoculated by V592-GFP and *ΔVdChs5Chs7-GFP*. Scale bars, 24 µm

tissue of infected cotton inoculated with *ΔVdChs5Chs7-GFP* inoculation, while abundant mycelia were detected with the V592-GFP (Fig. 4b). Hence, we suggest that the *ΔVdChs5Chs7* mutant affects the colonization and propagation of *V. dahliae* in cotton. Combined with the evidence that deletion of *VdChs5* and *VdChs7* genes leads to impaired conidial production, we hypothesize that knockout of the *VdChs5Chs7* gene may not affect the invasion process, but specifically impairs the colonization and proliferation in host vascular tissues.

ΔVdChs5Chs7 mutant increased sensitivity to environmental stresses

Chitin is an important component of the fungal cell wall. To investigate whether deletion of *VdChs5* and *VdChs7* affect cell wall integrity in *V. dahliae*, we compared the growth inhibition rates of *ΔVdChs5*, *ΔVdChs7*, and *ΔVdChs5Chs7* mutants and wild-type strain V592 on PDA containing the cell wall perturbing agents and H_2O_2. As shown in Fig. 6, the growth inhibition rates of *ΔVdChs5*, *ΔVdChs7*, and *ΔVdChs5Chs7* mutants were significantly higher in calcofluor white (CFW), Congo red (CR), and H_2O_2, compared with the wild-type strain V592. Therefore, we suggest that these chitin synthase mutants are more sensitive to cell wall stress and ROS pressure.

In addition, we observed the transverse sections and longitudinal sections of hyphal cell wall in wild-type strain V592 and double mutant *ΔVdChs5Chs7* by transmission electron microscopy (TEM). The cell wall thickness of double mutant *ΔVdChs5Chs7* hyphae was significantly lower than that of the wild-type strain V592 (Fig. 5). The chitin contents of *ΔVdChs5*, *ΔVdChs7*, and *ΔVdChs5Chs7* mutants were significantly lower than wild-type strain (Additional file 1: Figure S4). Therefore, we verified that the knockdown of two genes, *VdChs5* and *VdChs7*, results in *V. dahliae* being more sensitive to external stresses, possibly by impairing the fungal cell wall integrity.

HIGS of *VdChs5Chs7* in cotton enhanced resistance to *Verticillium* wilt

To examine whether silencing of *V. dahliae* chitin synthase genes in cotton could improve resistance to VW, we constructed TRV silencing vectors against *VdChs5* and *VdChs7*. We then injected TRV:*VdChs5*, TRV:*VdChs7*, respectively, and injected TRV:*VdChs5* and TRV:*VdChs7* together into susceptible cotton (*Gossypium hirsutum* L. acc. TM-1) before V592 inoculation. The injections of the silencing vectors have no effect on the growth of cotton (Additional file 1: Figure S5). Then all HIGS treated cotton plants were inoculated with wild-type strain V592. The results showed that the TRV:*VdChs5* and TRV:*VdChs7* co-silenced plants showed fewer *Verticillium* wilt phenotypes than the TRV:*VdChs5*, TRV:*VdChs7* and TRV:00 seedlings (Fig. 7). The disease index and fungal biomass also indicated that simultaneous silencing of *VdChs5* and *VdChs7* in cotton enhanced resistance to VW (Fig. 7e–g). We also tested the effect of target gene silencing (Fig. 7h, i) to assure that silencing of both *VdChs5* and *VdChs7* in cotton significantly improved resistance to *V. dahliae*.

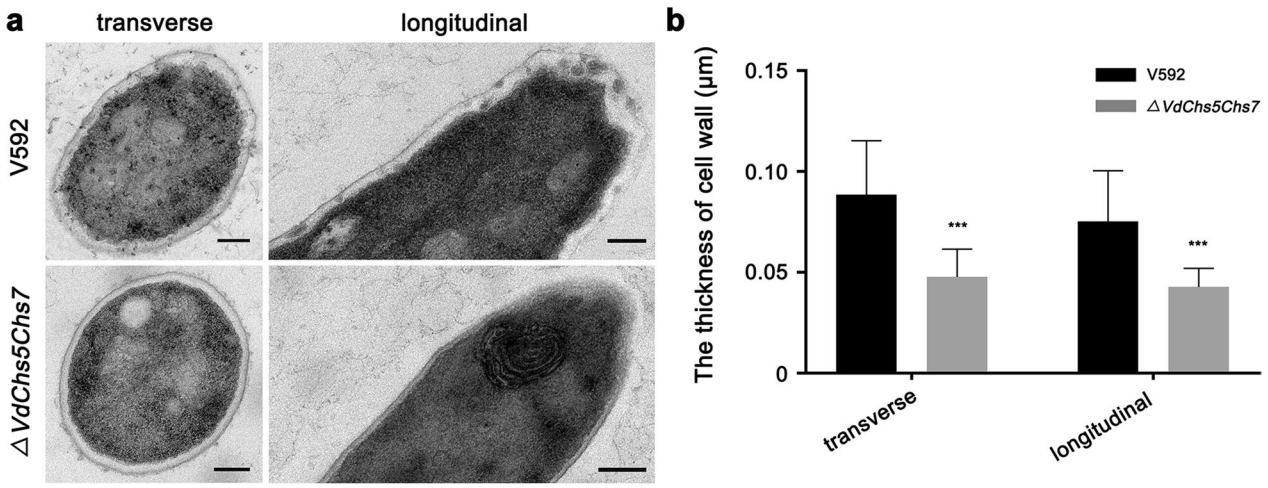

Fig. 5 Deletion of *VdChs5* and *VdChs7* affected the cell wall thickness of hyphae in *Verticillium dahliae*. **a** Transmission electron microscope images of the transverse section and longitudinal section in wild-type strain V592 and *ΔVdChs5Chs7* mutants. The *ΔVdChs5Chs7* mutant cell wall is thinner than wild-type strain V592. Scale bars, 0.2 μm. **b** Statistical analysis on cell wall thickness of hyphae transverse section and longitudinal section in wild-type strain V592 and *ΔVdChs5Chs7* mutants. Data represent means and standard deviation from three independent biological replicates. *** indicates significant differences at $P < 0.001$ as calculated with Student's *t*-test

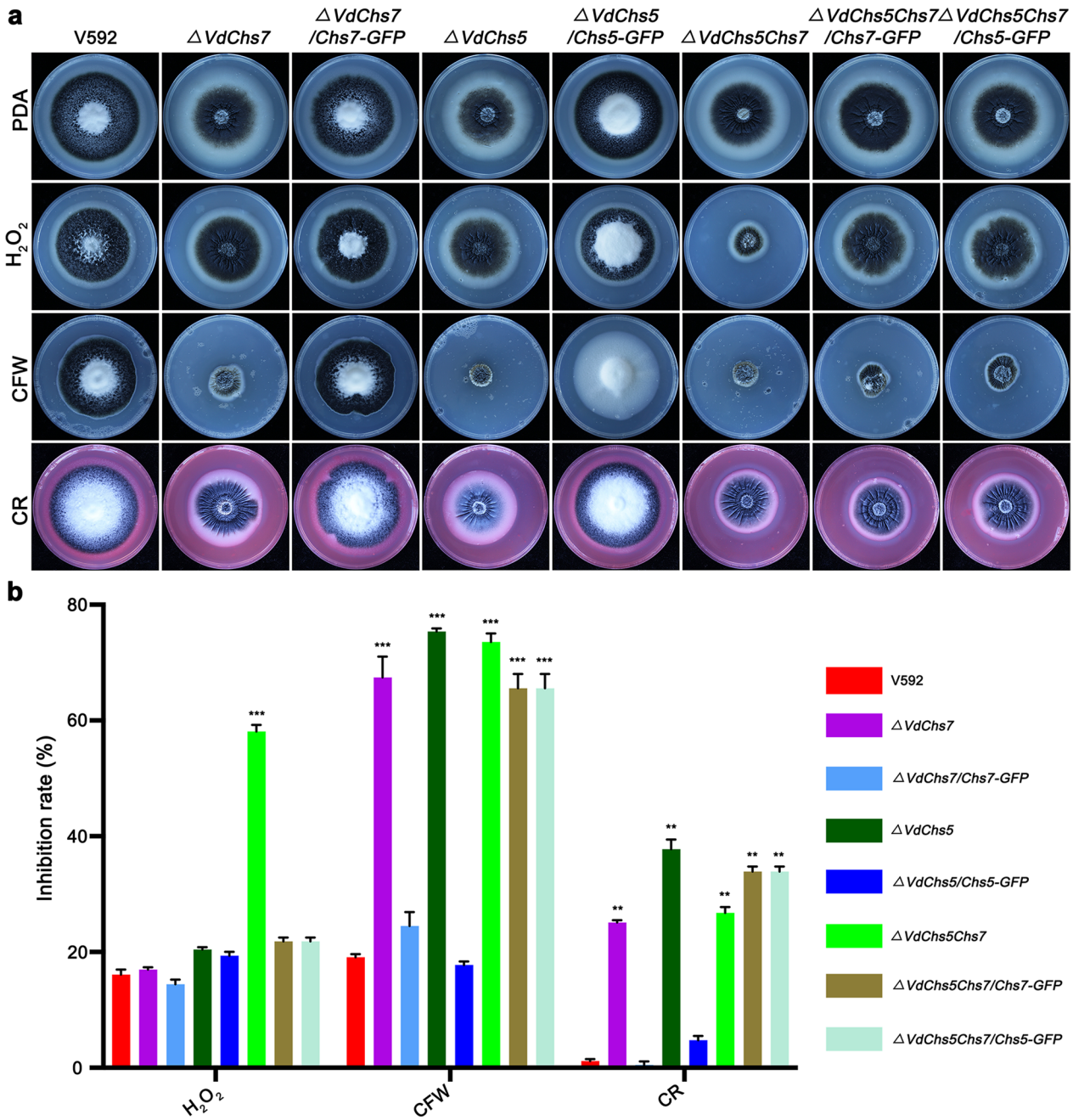

Fig. 6 Sensitivity of *ΔVdChs7*, *ΔVdChs5*, and *ΔVdChs5Chs7* to cell wall perturbing agents and reactive oxygen species. **a** Colony morphology of the indicated strains grown on PDA containing H_2O_2 (2.5 μM), Calcofluor white (150 μg/mL), or Congo red (100 μg/mL). Photographs were taken at 15 days post inoculation (dpi). An aliquot of 10 μL conidial suspension (1×10^6 conidia/mL) was spotted on medium. **b** Growth inhibition rates of the indicated strains on different growth medium at 15 dpi. Data represent the mean ± standard deviation from three independent biological replicates. ** and *** indicate significant differences at $P < 0.01$ and $P < 0.001$, respectively, as calculated with Student's *t*-test

Discussion

In this study, we identified two important genes, *VdChs5* and *VdChs7*, encoding chitin synthases containing myosin motor-like domain. Targeted deletion both of *VdChs5* and *VdChs7* in *V. dahliae* reduced conidial production and impaired pathogenicity. In addition, the double knockout mutant *ΔVdChs5Chs7* had thinner cell walls and increased sensitivity to stress. More interestingly, upland cotton silenced with TRV:*VdChs5* and TRV:*VdChs7* simultaneously by HIGS showed

considerably increased resistance to *Verticillium* wilt. The results demonstrate that *VdChs5* and *VdChs7* play key roles in pathogenicity, conidiation and cell wall integrity.

Fungi CHS could be usually divided into eight classes based on phylogenetic analysis (I to VIII). These eight CHS classes are divided into three divisions, each having its structural domain. Division 1 contains classes I, II, and III, which contain CS1N domain (PF08407) and CS1 domain (PF01644). Classes IV, V, VII, and VIII in Division 2 include MMD domain (PF00063), b5 domain (PF00173), CS2 domain (PF03142), and DEK_C domain (PF08766). Division 3 only contains class VI, which consists of CS2 domain (PF03142) (Rogg et al. 2012; Zhang et al. 2016b; Liu et al. 2017b). In this study, we classified the chitin synthases (CHS) of *V. dahliae* into eight classes, class I-VIII, by conserved protein domains and motifs. Among them, VdCHS5 is a class V chitin synthases, while VdCHS7 belongs to class VII, and both of which contain N-terminal MMD domains and C-terminal chitin synthesis domains (Fig. 1 and Additional file 1: Figure S1). The N-terminal myosin motor-like domain (MMD) of Class V CHSs is conserved in fungi and three types of ATP-binding motifs exist in the MMD, including a P-loop motif, two switch I region motifs, and a switch II region motif. In contrast, the MMD in class VII CHSs is absent and has lost ATP-binding motifs.

Previous studies on *A. nidulans*, *M. oryzae*, *G. zeae*, *U. maydis*, and *N. crassa* have shown that class V and VII chitin synthases play key functions in mycelial growth and host–pathogen interactions (Takeshita et al. 2006; Kim et al. 2009; Treitschke et al. 2010; Kong et al. 2012; Fajardo-Somera et al. 2015). In *M. oryzae*, CHS5 and CHS6, homologous to VdCHS7 and VdCHS5, respectively, have an N-terminal myosin motor-like domain and are closely linked in the genome. The *chs5* mutant exhibited no detectable phenotype, and the *chs6* mutant was defective in differentiation and growth of invasive hyphae. However, the *chs5chs6* double mutant had more severe defects than the *chs6* mutant, indicating that CHS5 and CHS6 may have overlapping functions (Kong et al. 2012). In *V. dahliae*, we found that the virulence of *ΔVdChs5* and *ΔVdChs7* mutant was slightly weaker than that of wild-type strain V592, but double *ΔVdChs5Chs7* mutants showed significantly reduced virulence in cotton (Fig. 3). Therefore, we speculate that the functional redundancy existing between class V and class VII chitin synthases may be widespread in fungi.

Fungal cell wall synthesis plays an important role in both polar growth and conidia production (Gow et al. 2017; Steinberg et al. 2017). Genes encoding chitin synthase influence not just conidia morphology but also conidial production. *A. nidulans* class III chitin synthase ChsB is required for conidial development and localizes at polarized cell wall synthesis sites (Fukuda et al. 2009). In *M. oryzae*, *CHS1* deletion resulted in severe morphological abnormalities in more than 90% of conidia. The spore production of the *chs5chs6* double mutant was significantly lower than that of the wild-type strain and the class V chitin synthase *chs6* mutant in *M. oryzae* (Kong et al. 2012). Con7p, a transcription factor, is involved in conidial morphology in *M. oryzae*, and chitin content is decreased in *Con7* deletion mutant conidia (Odenbach et al. 2007). Our results found that conidial production of the double mutant *ΔVdChs5Chs7* decreased to 25% of the wild-type strain V592 in *V. dahliae* (Fig. 2d). These findings suggest that chitin synthase is involved in asexual reproduction of filamentous fungi.

The cell wall of fungi plays a key function in the interaction between pathogens and hosts and chitin is an important component of the cell wall (Lenardon et al. 2010). The chitin antagonists calcofluor white and 1,3-β-glucan-binding stain Congo red cause cell wall stress and activate CWI signaling (Levin 2005). Plants recognize pathogens and produce ROS to defend against pathogen invasion (Daub et al. 2013). The *ΔVdChs5*, *ΔVdChs7*, and *ΔVdChs5Chs7* mutants were more sensitive to cell wall stress agents and ROS stress compared with wild-type strain V592 (Fig. 6), suggesting that chitin synthase mutants are more sensitive to external environmental stresses. We presumed that *ΔVdChs5Chs7* mutants have difficulty adapting to complicated environmental conditions during invading hosts, leading to unable to develop and reproduce.

HIGS has been extensively employed to improve disease resistance in plants, such as the cotton, tomato and *Arabidopsis* (Liu et al. 2002; Zhang et al. 2016a; Song and Thomma 2018; Xu et al. 2018). The 35S-VdH1i-3,

(See figure on next page.)

Fig. 7 Effects of *VdChs5* and *VdChs7* HIGS on cotton resistance to *Verticillium dahliae* V592 infection. **a** Disease symptoms of cotton seedlings (TM-1) infiltrated with different HIGS constructs. The seedlings were photographed at 30 days post inoculation (dpi) with V592. **b** The transverse section of stems of different HIGS-treated cotton plants at 30 dpi. Scale bars, 0.5 mm. **c** The longitudinal section of stems of different HIGS-treated cotton plants at 30 dpi. Scale bars, 0.5 mm. **d** Fungal recovery from infected stems of different HIGS-treated cotton plants at 30 dpi. **e** The disease grade of different HIGS-treated cotton plants at 30 dpi. **f** The disease index of different HIGS-treated cotton plants at 30 dpi. **g** The relative fungal biomass of different HIGS-treated cotton plants at 30 dpi. **h** The relative expression levels of *VdChs7* in V592-infected cotton plants injected with diverse TRV constructs. **i** The relative expression levels of *VdChs5* in V592-infected cotton plants inoculated with distinct TRV constructs. For **e**–**i**, data represent means and standard deviations from three independent biological replicates. *** indicates significant differences at $P < 0.001$ as calculated with Student's *t*-test

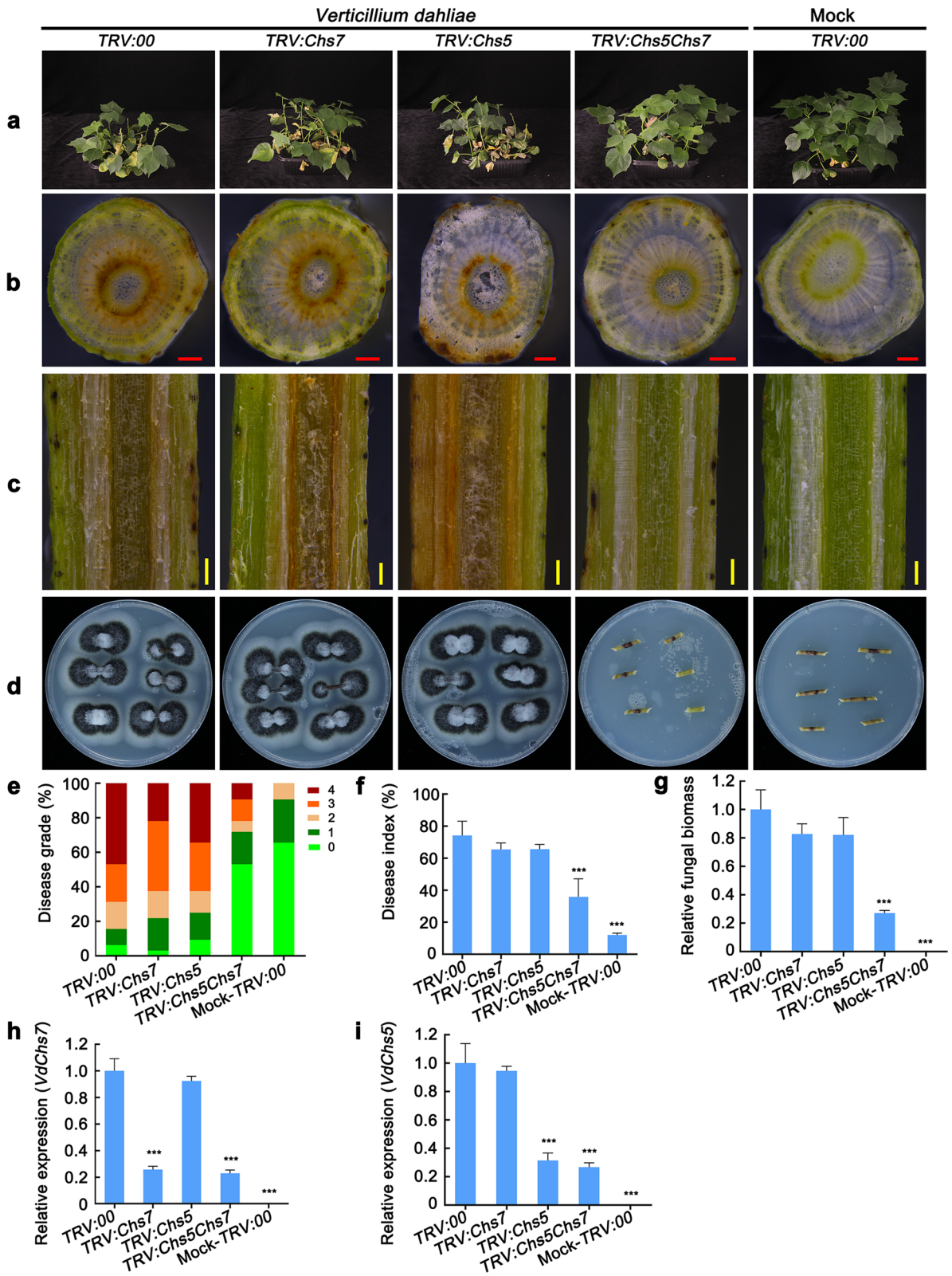

Fig. 7 (See legend on previous page.)

6, 14, and 16 lines of transgenic cotton demonstrated differing degrees of resistance to V592 infection, with considerably lower disease grade in inoculated seedlings (Zhang et al. 2016a). Tobacco rattle virus-mediated HIGS in cotton plants repressed *VdRGS1* (regulator of G protein signaling) transcripts in invading *V. dahliae* strains and improved broad-spectrum resistance to cotton *Verticillium* wilt (Xu et al. 2018). In this study, cotton silenced with both TRV:*VdChs5* and TRV:*VdChs7* using the HIGS approach enhanced resistance to *V. dahliae* (Fig. 7). Chitin is a structural component of fungal cell walls but is not present in plants, vertebrates, mammals, and humans. Therefore, chitin synthase is an attractive molecular target for the development of effective agents for disease control (Maertens and Boogaerts 2000). In the future, we can design and synthesize chitin synthase inhibitors as potent fungicides.

Conclusions

In conclusion, our study revealed that two important pathogenic genes, chitin synthase *VdChs5* and *VdChs7*, play key roles in the fungal conidiation, virulence, and tolerance to environmental stresses. The resistance of upland cotton to *Verticillium* wilt was significantly improved by silencing *VdChs5* and *VdChs7* based on HIGS technology. These two genes may be used as important targets to develop biological or chemical agents for the green control of cotton *Verticillium* wilt.

Methods

Fungal strains and culture conditions

The virulent defoliating *V. dahliae* strain V592 was used as the wild-type strain in this study. The mycelium stored in 30% glycerol at −80°C was recovered on potato dextrose agar (PDA) plates (200 g potato, 20 g glucose, 15 g agar) at 26 °C in an incubator for 7 days. The Czapek-Dox liquid culture medium was used as extraction of conidiophores ($NaNO_3$ at 2 g/L, $K_2HPO_4{\cdot}3H_2O$ at 1.32 g/L, $MgSO_4{\cdot}7H_2O$ at 1 g/L, KCl at 1 g/L, $FeSO_4{\cdot}7H_2O$ at 0.01 g/L, sucrose at 30 g/L) with shaking at 150 rpm for 7 days, 26°C. These conidial suspensions were used for penetration assays.

Bioinformatic analysis

The gene of *VdChs5* (VDAG_00420) and *VdChs7* (VDAG_00419) was identified by BLASTP program with AnCsmA (XP_663922.1) and AnCsmB (XP_663921.2) of *A. nidulans* in homology search of the database of NCBI. Multiple full-sequence alignments were performed with ClustalX 2.0, and the phylogenetic tree was generated with MEGA 7.0 using the neighbor-joining method and bootstrap test was replicated 1000 times (Kumar et al. 2016). Domain prediction was performed using IBS 1.0.3 software (Liu et al. 2015) based on Pfam (Mistry et al. 2021) and InterProScan.

Vector construction and fungal transformation

To generate the plasmids knockout *VdChs5*, *VdChs7*, and *VdChs5Chs7*, about 1 kb upstream and downstream sequences flanking the coding regions were amplified from V592 genomic DNA with the following primer pairs VdChs5-5'-F/R, VdChs5-3'-F/R, VdChs5-Hyg-F/R, VdChs7-5'-F/R, VdChs7-3'-F/R, VdChs7-Hyg-F/R, VdChs5Chs7-5'-F/R, VdChs5Chs7-3'-F/R, and VdChs5Chs7-Hyg-F/R (Additional file 2: Table S1). The hygromycin resistance fragment was obtained from the pUC-Hyg plasmid. These fragments and linearized pGKO2 plasmid were cloned together using recombinase (ClonExpress MultiS One Step Cloning Kit, Vazyme, Nanjing, China) to generate knockout plasmids pGKO-VdChs5, pGKO-VdChs7, and pGKO-VdChs5Chs7. To generate the vectors for complementation assays, the coding sequences for *VdChs5* and *VdChs7* and their native promoters were amplified using primer pairs promoter-VdChs5-F/R, VdChs5-F/R, promoter-VdChs7-F/R, and VdChs7-F/R (Additional file 2: Table S1). The resulting fragments and linearized pSul-VisG plasmid were cloned together in the same way to obtain the pSul-p*VdChs5*::*VdChs5*-GFP and pSul-p*VdChs7*::*VdChs7*-GFP vectors. The *Agrobacterium tumefaciens*-mediated transformation method was used to generate the knockout mutant as previously described (Wang et al. 2016). Transformants were verified by serial subculture to PDA plates supplemented with 30 μg/mL of hygromycin and PCR using the primer set Test-Hyg-F/R. The pSul-p*VdChs5*::*VdChs5*-GFP and pSul-p*VdChs7*::*VdChs7*-GFP vectors were transformed into the mutant strains *ΔVdChs5*, *ΔVdChs7*, and *ΔVdChs5Chs7* and the transformants were selected on PDA plates containing chlorimuron-ethyl (100 μg/mL).

Plant infection assays

Gossypium hirsutum cultivar TM-1 was used to perform pathogenicity assays as host. Two-week-old cotton seedlings were inoculated with conidial suspension (1×10^7 CFU/mL) by the unimpaired root-dip inoculation method (Feng et al. 2018). The disease index was calculated as previously described (Liu et al. 2014). The disease grade was classified as follows: 0 (no symptoms), 1 (0–25% wilted leaves), 2 (25–50% wilted leaves), 3 (50–75% wilted leaves), and 4 (75–100% wilted leaves). The disease index was calculated for each treatment according to the following formula: $DI = [(\sum \text{disease grades} \times \text{number of infected plants})/(\text{total checked plants} \times 4)] \times 100$. The fungus was recovered from infected cotton by surface sterilizing stem sections of infected cotton plants in

70% ethanol followed by 10% H_2O_2 for 60 min. The samples were rinsed three times with sterile water, placed on PDA medium and cultured at 26°C (Zhao et al. 2016).

Confocal microscopy

The image of cotton infected by fungi was taken under a spinning disk confocal microscope (UltraView Vox, PerkinElmer, UK). All the images were processed and analyzed using Volocity (PerkinElmer) and image J.

RT-qPCR and Quantitative Real-Time PCR

For RT-qPCR analysis of gene expression, RNA was extracted using an EASY spin Plus kit (Aidlab, Beijing, China). Then, isolated total RNA was reverse-transcribed with HiScript II Q RT SuperMix (Vazyme, Nanjing, China). The RT-qPCR was performed in a Bio-Rad CFX96 Real-Time system using SYBR Premix ExTaq II (TOYOBO). Cycling conditions were 1 min 30 s at 95°C, followed by 45 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The β-tubulin gene (VDAG_10074) was used as an internal reference for all RT-qPCR analyses. Relative expression levels were calculated with $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Primers are listed in Additional file 2: Table S1. To determine fungal biomass, DNA was extracted from the ground powder using Plant Rapid Genomic DNA kit (Biomed, Beijing, China), about 100 ng total DNA was subjected to qPCR (quantitative real-time PCR) analysis with plant-specific (*18S* gene) and fungal-specific (*VdEF-1α*) primer pairs. Ratios of fungal DNA to plant ($2^{-\Delta Ct}$) were calculated for each sample (Schmittgen and Livak 2008; Wang et al. 2021).

TEM analysis

For transmission electron microscope (TEM; JEOL, JEM-1400, Tokyo, Japan) observation, the hyphae *V. dahliae* were fixed immediately in 2.5% glutaraldehyde, buffered with PBS (pH 7.0) at 4˚C overnight, washed with the same buffer four times and post-fixed with 1% osmium tetroxide for 1 h. Dehydration was then performed in an acetone series (50, 75, 85, 95, 100%), and the slices were embedded in Spurr's resin mixture (Zhou et al. 2017). Ultrathin serial sections (70 nm thickness) were cut from resin blocks using a microtome (Leica, EM-UC7, Wetzlar, Germany), followed by uranyl acetate staining, and observed with a TEM.

TRV treatment

The pTRV1 and pTRV vectors were used to construct TRV:*VdChs5* and TRV:*VdChs7* for HIGS analysis. These vectors were transformed into *Agrobacterium tumefaciens* strain GV3101. Subsequently, all the TRV vectors were agroinfiltrated as previously described (Gao et al. 2013; Xu et al. 2018). To test HIGS efficiency, *GhCHLI*, encoding magnesium chelatase subunit I, was used as a positive control (Xiong et al. 2020). The cotyledons of 10-day-old TM-1 cotton seedlings were infiltrated with 1:1 mixtures of pTRV1 and pTRV constructs. Two weeks after TRV:*GhCHLI* inoculation, the plants showed highly uniform bleaching in newly emerged leaves. Next, control and HIGS treated plants were inoculated with V592 conidia suspension (1×10^7 conidia/mL). About 4 weeks after inoculation, control plants displayed obvious leaf-yellowing symptoms. Next, we randomly and repeatedly cut the stems and incubated the sliced stems on PDA for 6 days for RNA extraction.

Different environmental stresses

Fungal stress assays were performed as follows: conidia from 7 day-old cultures on PDA plates were harvested and suspended in doubled-distilled H_2O. The final concentration of conidial suspension was adjusted to 1×10^6 conidia/mL. The conidial suspensions (10 μL) were spotted onto the center of various plates including PDA amended with Congo red (CR; 100 μg/mL), calcofluor white (CFW; 150 μg/mL), H_2O_2 (2.5 μmol/L). Plates were incubated at 26°C for 15 days and colony diameters were quantified. The growth inhibition rate (GI) was calculated as follows: GI = (control colony diameter—treatment colony diameter)/control colony diameter × 100% (Liu et al. 2017a). Six replicate plates were used for each condition/experiment and the entire experiment were repeated with 3 independent batches of conidia.

Cell wall chitin analysis

Conidia were inoculated into 100 mL liquid complete medium (CM) at a concentration of 1×10^6 conidia/mL and incubated at 26°C with shaking (150 rpm) for 3 days. The mycelia were harvested, washed with deionized water, and frozen with liquid nitrogen. The cell wall chitin was separated and assayed as previously described (Lee et al. 2005). Three aliquots of 10 mg lyophilized mycelia were used as independent samples for cell wall analysis and the experiment was repeated three times from different biological samples.

Abbreviations

CFW	Calcofluor white
CHS	Chitin synthase
CM	Complete medium
CR	Congo red
HIGS	Host-induced gene silencing
MMD	Myosin motor-like domain
MS	Microsclerotia
PDA	Potato dextrose agar
ROS	Reactive oxygen species
TEM	Transmission electron microscopy
VW	*Verticillium* Wilt

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00175-z.

Additional file 1: Figure S1. Domain structures of class I-VIII chitin synthases in Verticillium dahliae. Class V and class VII CHSs have an N-terminal myosin motor-like domainand a C-terminal Chitin_synth_2 domain. Meanwhile, other CHSs contain at least a Chitin_synth_1, Chitin_synth_2, or Chitin_synth_1N domain. All chitin synthases have multiple transmembranedomains in V. dahliae. The boxes in different colors show the myosin motor-like domains, chitin_synth_1 domain, chitin_synth_1N domain, chitin_synth_2 domain, cytochrome b5-like Heme/Steroid binding domain, and the DEK_C terminal domainin Pfam database. The transmembrane helicesdomain was indicated by black box. **Figure S2**. Identification of ΔVdChs7, ΔVdChs5, and ΔVdChs5Chs7 knockout mutants. a Strategy for gene knockout and primers used for testing mutants. HPH represents hygromycin phosphotransferase encoding gene. b PCR confirmation of the knockout mutants and the complemented strains with the primers Test-Hyg-F/R. Lanes 1, 2, 3, 4, 5, 6, 7, and 8 indicate V592, ΔVdChs7, ΔVdChs7/VdChs7-GFP, ΔVdChs5, ΔVdChs5/VdChs5-GFP, ΔVdChs5Chs7, ΔVdChs5Chs7/VdChs7-GFP, and ΔVdChs5Chs7/VdChs5-GFP strains, respectively. c PCR confirmation of the knockout mutants and the complemented strains with the primers Test-VdChs7-F/R. d PCR confirmation of the knockout mutants and the complemented strains with the primers Test-VdChs5-F/R. e PCR confirmation of the ΔVdChs5Chs7 knockout mutant and the wild-type strain V592 with the primers Test-1F/R. f PCR confirmation of the ΔVdChs5Chs7 knockout mutant and the wild-type strain V592 with the primers Test-2F/R. Lanes 1 and 2 indicate wild-type strain V592 and ΔVdChs5Chs7, respectively. **Figure S3**. RT-qPCR analyses of the VdChs5and VdChs7expression during Verticillium dahliae infection into cotton. The samples were collected from taproot of the infected cotton at 12 h, 24 h, 48 h, 3 d, 5 d, and 8 d post inoculation with conidial suspension of wild-type strain V592. Conidial suspension for infectionand the mycelia 12 h, 24 h, and 48 h post inoculation with conidia on Czapek-Dox mediumwere set as control. The V. dahliae β-tubulinwas used as an endogenous control for gene expression analysis. The error bars represent the standard deviation. ** indicates the statistical significancecompared with 0 h by Student's t-test. **Figure S4**. The chitin content of the V592, ΔVdChs7, ΔVdChs5, and ΔVdChs5Chs7 knockout mutants and the complemented strains. Conidia were inoculated into 100 mL liquid CM at a concentration of 106 conidia/mL and incubated at 26 °C with shakingfor 3 days. Three aliquots of 10 mg lyophilized mycelia were used as independent samples for cell wall analysis and the experiment was repeated three times from different biological samples. The valuesshown are micrograms of cell wall component per 10 mg dry mycelia. ** and *** indicate significant differences at $P < 0.01$ and $P < 0.001$, respectively, according to Student's t-test. **Figure S5**. HIGS silencing of VdChs5Chs7 had no impact on the cotton growth. a Growth phenotype of different HIGS-treated cotton plants without inoculating V592. The seedlings were photographed at 30 days post inoculation. b The transverse section of stems of different HIGS-treated cotton plants without inoculating V592 at 30 dpi. Scale bars, 0.5 mm. c The longitudinal section of stems of different HIGS-treated cotton plants without inoculating V592 at 30 dpi. Scale bars, 0.5 mm.

Additional file 2: Table S1. Primers used in this study.

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Authors' contributions
BC performed most of the experiments, analysed the data, and wrote the manuscript. JT, ZF, HW, and JS provided some essential data, interpreted the data, and refined the ideas. ZK conceived the project, interpreted the data, and revised the article. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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