Journal of Cotton Research

RESEARCH Open Access

Isolation and characterization of the *GbVIP1* gene and response to Verticillium wilt in cotton and tobacco

ZHANG Kai†, ZHAO Pei†, WANG Hongmei*, ZHAO Yunlei, CHEN Wei, GONG Haiyan, SANG Xiaohui and CUI Yanli

Abstract

Background: Verticillium wilt is a serious soil-borne vascular disease that causes major losses to upland cotton (*Gossypium hirutum* L.) worldwidely every year. The protein VIP1 (VirE2 interaction protein 1), a bZIP transcription factor, is involved in plant response to many stress conditions, especially pathogenic bacteria. However, its roles in cotton response to Verticillium wilt are poorly understood.

Results: The *GbVIP1* gene was cloned from resistant sea-island cotton (*G. barbadense*) cv. Hai 7124. Expression of *GbVIP1* was up-regulated by inoculation with *Verticillium dahliae* and exogenous treatment with ethylene. Results of virus-induced gene silencing suggested that silencing of *GbVIP1* weakened cotton resistance to Verticillium wilt. The heterologous expression of *GbVIP1* in tobacco showed enhanced resistance to Verticillium wilt. The *PR1, PR1-like* and *HSP70* genes were up-regulated in GbVIP1 transgenic tobacco after Verticillium wilt infection.

Conclusion: Our results suggested that *GbVIP1* increased plant resistance to Verticillium wilt through up-regulating expressions of *PR1, PR1-like,* and *HSP70*. These results provide new approaches to improving resistance to Verticillium wilt in upland cotton and also have great potential for disease-resistance breeding of cotton.

Keywords: Cotton, VIP1, Verticillium wilt

Background

Over 200 kinds of dicotyledonous plant species are susceptible to Verticillium wilt, a serious soil-borne vascular disease (Fradin and Thomma 2006). Among them, upland cotton (*Gossypium hirsutum* L) is the most economically important crop for natural textile fiber and oil in the world. Cotton infected with Verticillium wilt shows several symptoms including leaf wilting or defoliation and vascular discoloration or necrosis. Verticillium wilt causes enormous losses in cotton yield and fiber quality every year in many couZntries, especially China (Cai et al. 2009). There are no effective chemical and biological methods to control the contagion of Verticillium wilt because of its highly aggressive pathogenicity and many years of survival in soil as resting structures without a host (Gong et al. 2018). After expending great efforts on control of Verticillium wilt, it was suggested that cultivating resistant varieties was an economical and effective method of control. Unfortunately, upland cotton, the most widely cultivated cotton species, is usually susceptible to Verticillium wilt (Zhang et al. 2012b). The related sea-island cotton (*G. barbadense*) is often highly resistant or tolerant to Verticillium wilt (Zhou et al. 2014). Nevertheless, *G. barbadense* cannot be vigorously promoted for cultivation due to yield limitations. Due to difficulty in performing interspecific crosses of *G.hirsutum* and *G. barbadense* and the low efficiency of traditional breeding, development of Verticillium disease-resistant cultivar breeding has been slow (Zhang et al. 2014). Therefore, many researchers have focused on the key genes involved in the disease-resistance process and bred disease-resistant cotton cultivars via modern genetic engineering and molecular breeding methods. Genes associated with Verticillium wilt resistance were identified using map-based cloning, genome-wide association study and high-throughput sequencing, including NBS-LRR genes (e.g. *GbaNA1* and *GbRVd*) (Li et al. 2018; Yang et al. 2016), kinase and receptor-like protein genes (e.g.

* Correspondence: aywhm@163.com
†Kai ZHANG and Pei ZHAO contributed equally to this work.
Institute of Cotton Research of Chinese Academy of Agricultural Sciences / State Key Laboratory of Cotton Biology, Anyang 455000, Henan, China

© The Author(s). 2019 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

GhMKK2 and *GbRLK*) (Jun et al. 2015) and synthesis genes of antitoxin and antifungal protein (e.g. *GhPAO* and *GhAFP4*) (Mo et al. 2015; Wang et al. 2016). The resistance process to Verticillium wilt in cotton is very complex and a series of genes and proteins are regulated to from the defense response. As the cotton genome was sequenced, increasing numbers of genes involved in the process of resistance to Verticillium wilt were identified and cloned (Zhao et al. 2018). However, it is important to find the major resistance gene. Transcription factors associated with disease resistance, which regulate expression of resistance genes might play a key role in plant defense against pathogens.

The protein VIP1 (VirE2 interacting protein1), was first identified in *Arabidopsis* as a host protein that specifically interacted with the VirE2 protein of *Agrobacterium tumefaciens* (Tzfira et al. 2000). VIP1 is involved in the formation of T-DNA complexes and assists T-DNA in cytoplasm transporting, nuclear importing and nuclear locating (Liu et al. 2010). The VIP1 is a plant transcription factor belonging to subfamily I of the bZIP (basic-zipper protein) transcription factor family and contains a leucine zipper domain (Jakoby et al. 2002). A nuclear locating signal is located in the leucine zipper domain of VIP1 (van der Krol and Chua 1991). As a transcription factor, VIP1 regulates a series of gene expressions via combining the VIP1 response element region and response to biotic and abiotic stresses including pathogenic bacteria, drought, touch, low-sulfur and hyperosmotic stress (Lacroix and Citovsky 2013). Zaltsman et al. (2010) suggested that VIP1 was a defense-related transcription factor which up-regulated the expression of PR1 (pathogenesis-related protein 1). When the plant suffered from pathogenic bacteria, Ser^{79} of VIP1 was phosphorylated by mitogen-activated protein kinase in the cell cytoplasm. Phosphorylated VIP1 then translocated to the cell nucleus and activated PR1 transcription, which finished the plant disease-resistance response. Tsugama et al. (2014) found that AtVIP1 accumulated in the nucleus during hypo-osmotic stress and confirmed the VIP1-binding sequences (AGCTGT/G) by gel shift assays. The VIP1 regulated the expression pattern of hypo-osmolarity-responsive genes *CYP707A1* and *CYP707A3*, which are involved in the plant response to hypo-osmotic conditions. The VIP1 interacted with heterotrimeric G proteins β subunit (AGB1) during hypotonic conditions but not in mannitol-containing hypertonic conditions, implicating VIP1 and AGB1 in response of *Arabidopsis thaliana* to osmolarity and/or turgor pressure (Tsugama et al. 2013). Additionally, *AtVIP1* acted as a downstream gene of *AGB1* and *AtVIP1* overexpression in *Arabidopsis* increased its sensitivity to abscisic acid treatment and enhanced its drought tolerance (Xu et al. 2015). Researchers used a specific transgenic plant in which the genes up-regulated by *VIP1* were repressed, to examine the AtVIP1 physiological roles and suggested that AtVIP1 suppressed the root waving induced by touch stress and the phenomenon was influenced by ethylene and auxin (Tsugama et al. 2016a, b). Li et al. (2014) cloned the *Agvip1* gene from three celery cultivars and found that *Agvip1* was expressed differently in different organs and cultivars. Results suggested that *Agvip1* was up-regulated under cold, drought, hot, salt and metal ion stresses in different cultivars. Wu et al. (2010) isolated a low-sulfur-tolerant mutant with a recessive locus in *AtVIP1* which improved sulfur utilization efficiency and understanding of the underlying molecular mechanism of sulfur tolerance using *VIP1*.

It is clear that VIP1, as one kind of bZIP transcription factor, is involved in many responses to stress, especially of pathogenic bacteria. Thus, we planned to utilize *VIP1* to increase the resistance of upland cotton to Verticillium wilt. In this study, *GbVIP1* which encodes a bZIP transcription factor protein was cloned in *G. barbadense*. The structure, expression pattern and hereditary character of *GbVIP1* were investigated. The primary biological function of GhVIP1 was ascertained in cotton and tobacco using VIGS and transformation strategy. Our output will provide new approaches to improve resistance to Verticillium wilt in upland cotton and will also have much potential in disease-resistance breeding of cotton.

Results

GbVIP1 cloning and structure analysis

The AtVIP1 protein sequence (Q9MA75) was applied as a seed sequence to BLAST NCBI databases with the organism selected as cotton. A predicted transcription factor VIP1-like gene (XM016838286) was identified. According to sequence information, we designed primers to amplify the *VIP1* from *G. barbadense* cv. Hai 7124. A 1 014 bp fragment was obtained and sequence analysis indicated that *GbVIP1* encoded a polypeptide of 337 amino acid residues. The protein sequence of *GbVIP1* had 49% similarity with *AtVIP1*. Mapping the 1 014 bp cDNA sequence identified two copies of *GbVIP1* in the *G. barbadense* genome database. One was mapped to chromosome D02 and localized to the reference genome within positions 10 550 650–10 553 567. The other copy was mapped to chromosome A02 and localized within positions 10 056 074–10 062 958. The alignment results showed that *GbVIP1* contained four exons and three introns. To determine the sequence differences among cultivars resistant and susceptible to Verticillium wilt, *VIP1* was also amplified from *G. hirsutum* cv. TM-1 and *G. barbadense* cv. Pima. We found eight amino acid differences among VIP1 protein sequences from cv. TM-1 and Hai 7124 (Fig. 1a). The VIP1 protein sequences of other plant species were downloaded from NCBI to investigate the phylogenetic relationships and conservatism of the bZIP domain among different species. The phylogenetic tree showed *VIP1* from tetraploid and diploid cotton clustered into a subgroup,

and *VIP1* from *Glycine max* and *Citrus sinensis* had a close phylogenetic relationship with *VIP1* from cotton (Additional file 1). The conserved domain of VIP1 from different plant species was predicted by CD-Search software and the results suggested that all examples of the VIP1 protein had one bZIP domain (Fig. 1b). We extracted the protein sequences of the VIP1 conserved domain and analyzed the conservation of amino acids using WebLogo software. The bZIP domain from different plant species was had highly conserved and there was one leucine every six amino acids (Fig. 1c).

Expression patterns of *VIP1* gene

Quantitative real-time PCR (qRT-PCR) was used to determine the expression profile of *VIP1* in different tissues (roots, stems and leaves) of TM-1 and Hai 7124. Results indicated that *VIP1* was expressed in all of the tested tissues but was highly expressed in roots and little expressed in stems. Tissue specific expression results from the two cultivars were consistent (Fig. 2a). To investigate the expression pattern of *VIP1*induced by *Verticillium dahliae*, the relative expression level of *VIP1* was determined after inoculation with *V. dahliae*. Expression of *VIP1* was up-regulated at 6 h after inoculation. At 24 h after inoculation, *VIP1* expression began to return to its original level (Fig. 2b). Using qRT-PCR to evaluate the effect of plant hormone (salicylic acid (SA, Ethylene (ET) and Jasmonic acid (MeJA) treatments showed that *VIP1* expression was up-regulated under exogenous treatment of ET but the other hormones had no significant effect on expression (Fig. 2c).

Silencing of *GbVIP1* reduced Verticillium wilt resistance in cotton

A tobacco rattle virus (TRV)-based VIGS system was used to study the function of *GbVIP1* in cotton responses to Verticillium wilt. We designed the appropriate primer and amplified a *GbVIP1* fragment of about 300 bp. The selected fragment was integrated into pTRV2 vector to generate *VIP1*-knockdown cotton lines. After *Agrobacterium* infection, silencing efficiency was assessed using qRT-PCR. The relative expression level of VIP1 was reduced severely in *TRV: VIP1* compared with the control, and silencing efficiency exceeded 70% (Fig. 3a). When *TRV:PDS* seedlings showed an albino phenotype for new leaves, all the silenced cotton seedlings were inoculated with *V. dahliae* (Fig. 3b). About 15 days after inoculation, symptoms of Verticillium wilt were observed in *TRV:VIP1* seedlings, including yellow,

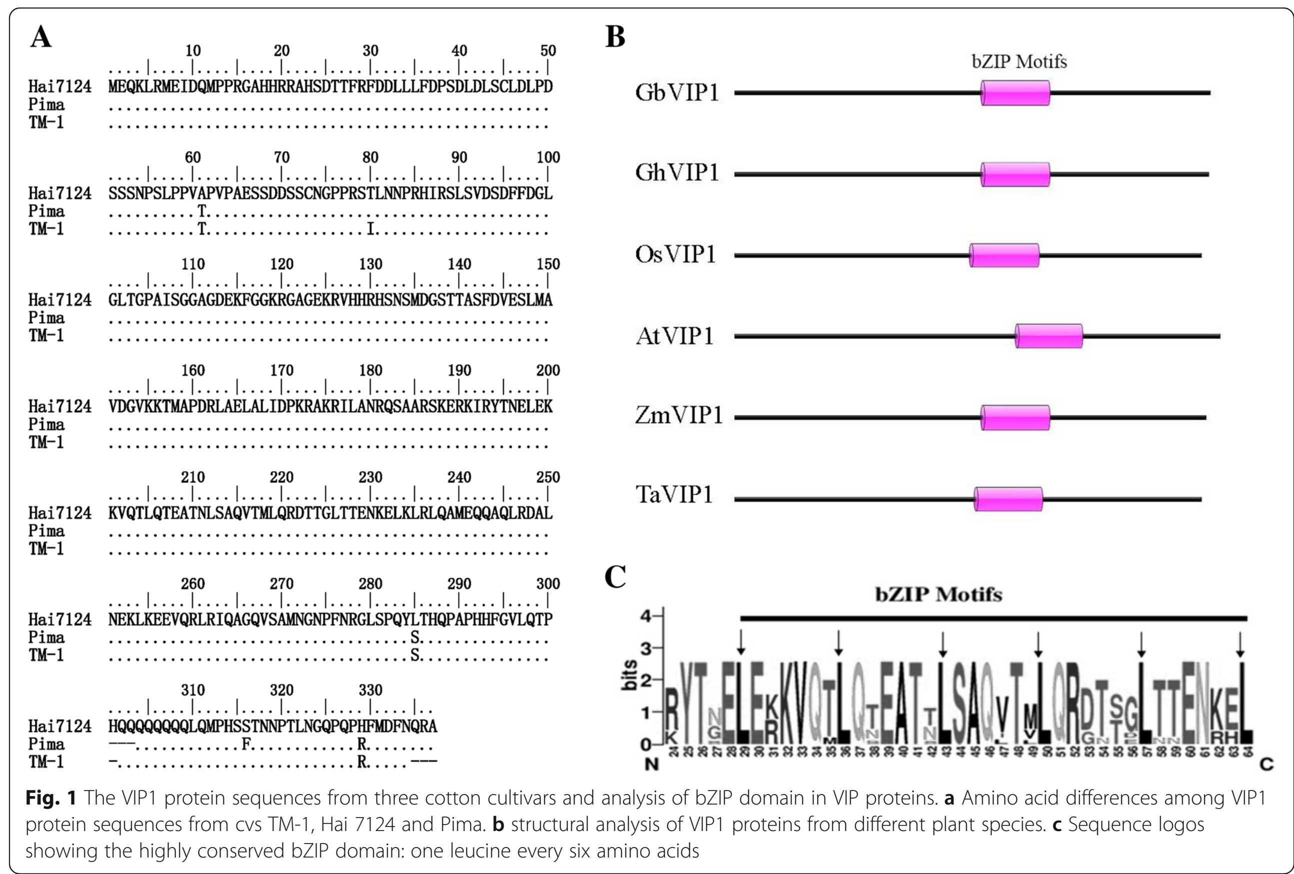

Fig. 1 The VIP1 protein sequences from three cotton cultivars and analysis of bZIP domain in VIP proteins. **a** Amino acid differences among VIP1 protein sequences from cvs TM-1, Hai 7124 and Pima. **b** structural analysis of VIP1 proteins from different plant species. **c** Sequence logos showing the highly conserved bZIP domain: one leucine every six amino acids

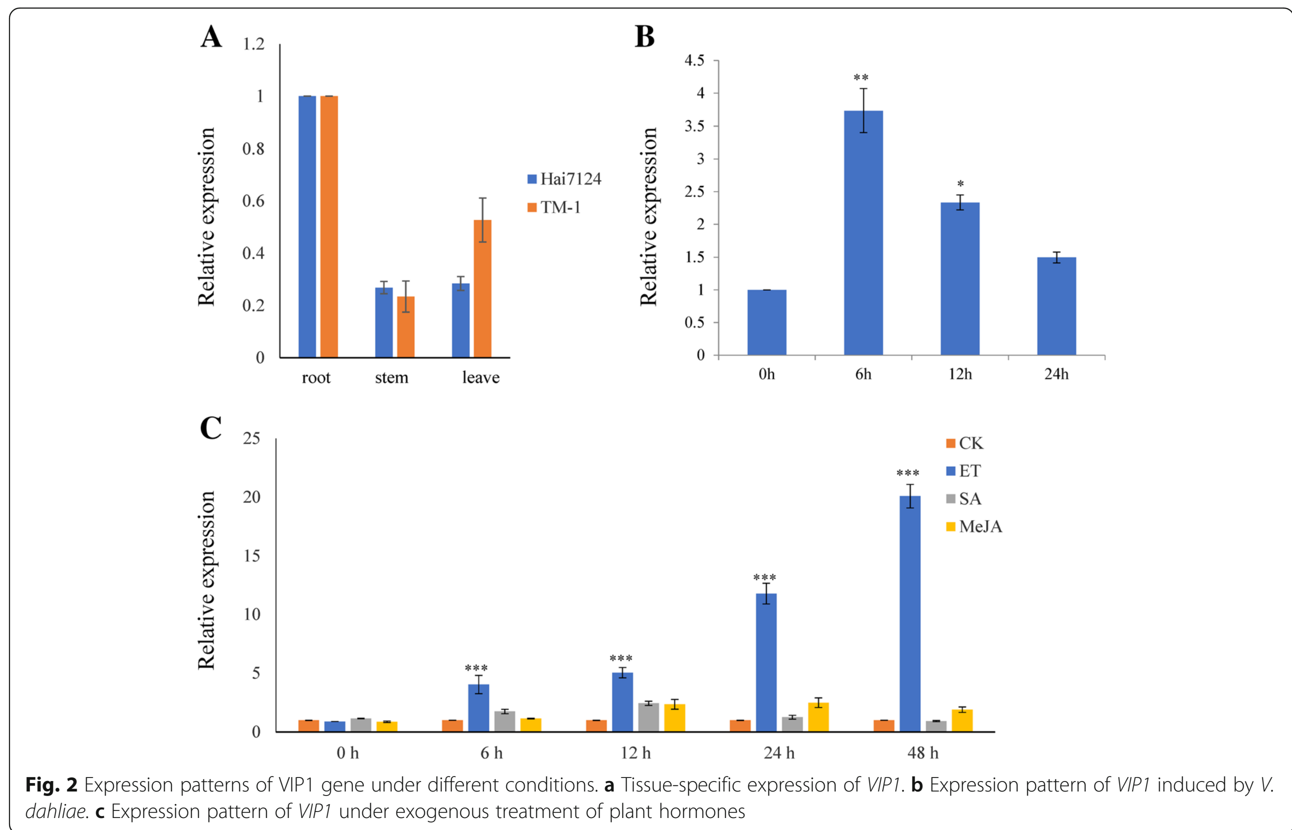

Fig. 2 Expression patterns of VIP1 gene under different conditions. **a** Tissue-specific expression of *VIP1*. **b** Expression pattern of *VIP1* induced by *V. dahliae*. **c** Expression pattern of *VIP1* under exogenous treatment of plant hormones

wilting and falling leaves, whereas the control showed no symptoms (Fig. 3b). We investigated the disease index (DI) values of *TRV:VIP1* and control seedlings at 25 and 35 days after inoculation. The DI values of TRV:*GbVIP1* and TRV:00 did not significantly differ at 25 days after inoculation. However, the DI value of *TRV:VIP1* seedlings was about 30 at 35 days after inoculation, which was remarkably higher than 23.45 for control (Fig. 3c). Leaves of silenced and control cotton seedlings were picked off and showed more disease speckles from silenced seedlings than control (Fig. 4a). We extracted the stems of silenced and control seedlings and cut through the middle of the stem vascular bundle. The xylem of *TRV:VIP1* seedlings showed more brown and necrotic areas than control because *V. dahliae* infected the plants through vascular bundles (Fig. 4b). These stems were also used in recovery experiments to analyze the level of *V. dahliae* colonization. Results indicated that more fungal growth around stems from *TRV:VIP1* than control seedlings and suggested that *TRV:VIP1* seedlings suffered more severe disease (Fig. 4c). Leaves of silenced cotton seedlings were dipped in trypan blue dye to assay the cell state of plants after *V. dahliae* inoculation. The leaves of *TRV:VIP1* seedlings had larger and darker blue areas, gathered around veins than controls (Fig. 4d), indicating that *V. dahliae* inoculation caused more dead cells in leaves of *TRV:VIP1* seedlings. The above results suggest that *VIP1* silencing weakened cotton resistance to Verticillium wilt.

Overexpression of *GbVIP1* increased resistance to Verticillium wilt in tobacco

To further study the function of VIP1, tobacco cv. NC89 was used to overexpress cotton *VIP1*. The *VIP1* was inserted into the pBI121 vector driven by 35S promoter and transformed into tobacco by *Agrobacterium*-mediated transformation. Then PCR and qRT-PCR were used to verify transgenic plants and the results showed that *GbVIP1* was significantly up-regulated in transgenic tobacco plants (Additional file 2). Two approaches were used to determine the disease-resistance capacity of transgenic tobacco. Detached leaves from transgenic and control tobacco plants were inoculated with *V. dahliae* and, 16 days after inoculation, leaves from control plants showed obvious disease symptoms compared with transgenic plants (Fig. 5a). The transgenic tobacco plants had a lower disease grade of detached leaves than control (Fig. 5b). Additionally, following treatment with *V. dahliae*, transgenic tobacco showed more resistance to Verticillium wilt than control seedlings (Fig. 5c and d). Hence, *GbVIP1* from cotton conferred resistance to Verticillium wilt in tobacco plants. To find the possible mechanism underlying *VIP1* increasing the disease resistance in cotton, qRT-PCR was used to determine expression levels of several possible

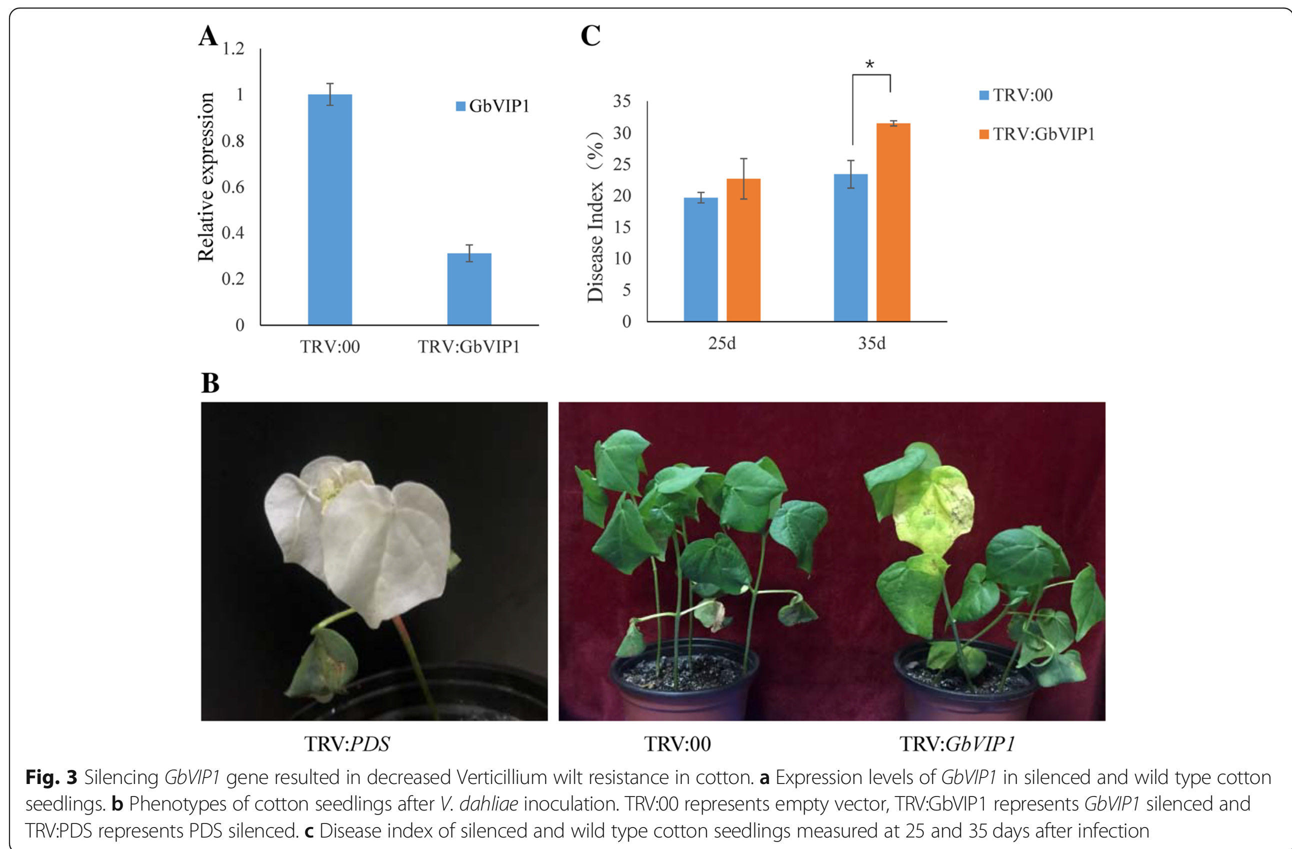

Fig. 3 Silencing *GbVIP1* gene resulted in decreased Verticillium wilt resistance in cotton. **a** Expression levels of *GbVIP1* in silenced and wild type cotton seedlings. **b** Phenotypes of cotton seedlings after *V. dahliae* inoculation. TRV:00 represents empty vector, TRV:GbVIP1 represents *GbVIP1* silenced and TRV:PDS represents PDS silenced. **c** Disease index of silenced and wild type cotton seedlings measured at 25 and 35 days after infection

resistance-related genes in transgenic and control tobacco: *PR1*, *PR1-like*, *RAR1*, *HSP70* and *RPP13*. All selected resistance-related genes showed increased expression at 6 h after inoculation and *PR1* and *PR1-like* were significantly up-regulated in *GbVIP1*-overexpressing tobacco seedlings (Fig. 6). The transcript abundances of *PR1* and *PR1-like* increased over 10-fold in transgenic tobacco after inoculation with *V. dahliae* compared with control, suggesting that *GbVIP1* overexpression activated expression of defense genes.

Discussion

Verticillium wilt has become the most serious and devastating disease of cotton in China and causes heavy losses of cotton production. Many years of practicing disease prevention has shown that breeding and application of disease-resistant cotton cultivars are the most effective and economical methods to reduce damage from Verticillium wilt to cotton. However, upland cotton cultivars with high resistance to Verticillium wilt are rare. The common cultivated cotton varieties are allotetraploid and the cotton genome is huge, both of which limit development of *Verticillium* disease-resistant cultivars. Screening of resistant genes and their utilization using modern molecular biology has accelerated the breeding of disease-resistant cultivars. The function and underlying mechanisms of VIP1 protein in disease and stimuli responses have been researched in model plants such as *Arabidopsis thaliana*, but the function of VIP1 in responding to Verticillium wilt is little understood. In this study, we found new roles of *GbVIP1* in defense responses to Verticillium wilt and studied the mechanisms underlying the functions of *GbVIP1* in cotton.

The *GbVIP1* gene was cloned from a resistant *G. barbadense* variety using the homologous cloning method. The *GhVIP1* was also cloned from susceptible *G.hirsutum* and base differences were found among *VIP1* nucleotide sequences from resistant and susceptible varieties, which we speculated resulting in the different disease resistance of varieties. In our research, *GbVIP1* was obviously up-regulated by Verticillium wilt inoculation and exogenous treatment of ET in cotton. The process of plant response to pathogenic bacteria is regulated by multiple signals, in which plant hormones play a key role (Katagiri and Tsuda 2010). Previous studies suggested that ET had a multiple effect on the interaction between plants and Verticillium wilt, with several genes activated by ET in response to Verticillium wilt, including *ethylene responsive factor* (*ERF6*), *ERF1* and *GbERF1-like* (Robison et al. 2001; Yang et al. 2015; Guo et al. 2016). These transcription factors regulated the expression of downstream resistance proteins and

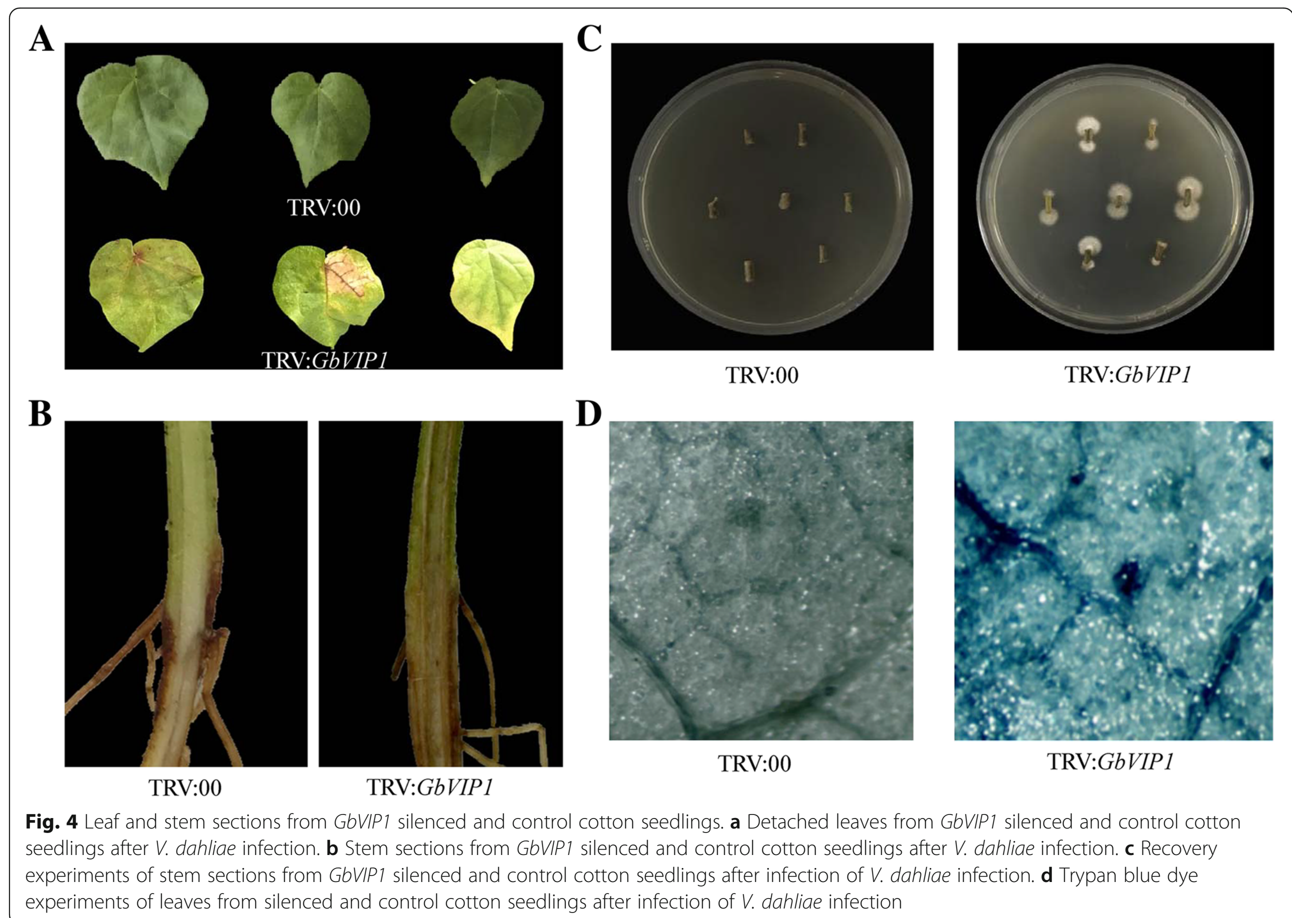

Fig. 4 Leaf and stem sections from *GbVIP1* silenced and control cotton seedlings. **a** Detached leaves from *GbVIP1* silenced and control cotton seedlings after *V. dahliae* infection. **b** Stem sections from *GbVIP1* silenced and control cotton seedlings after *V. dahliae* infection. **c** Recovery experiments of stem sections from *GbVIP1* silenced and control cotton seedlings after infection of *V. dahliae* infection. **d** Trypan blue dye experiments of leaves from silenced and control cotton seedlings after infection of *V. dahliae* infection

increased the plant disease resistance. We speculated that *GbVIP1* was regulated by ET, which played a key role in plant tolerance to Verticillium wilt.

We studied the resistance function of VIP1 through positive and negative sides: the positive was *GbVIP1* overexpression in tobacco using *Agrobacterium* mediated transformation and the negative was silencing of *GbVIP1*in cotton using VIGS. Results consistently indicated that *GbVIP1* overexpression in tobacco increased resistance to Verticillium wilt, but silencing *GbVIP1* in cotton decreased the resistance. Thus, *GbVIP1* had a positive role in Verticillium wilt resistance.

As described previously, VIP protein is a bZIP transcription factor and regulates expression of a series of stress-related genes. Notably, VIP1 up-regulated *PR1* expression in plants infected by pathogenic bacteria. We consistently found that *PR1* was obviously up-regulated in transgenic GhVIP1 tobacco at 6 h after incubation of Verticillium wilt. The PR protein family was ubiquitous in plant and play roles in multiple growth and development processes (Kaur et al. 2017). Among these, *PR1* is a marker gene of disease-resistance response, and *PR1* was also up-regulated by some disease-resistance genes during incubation of Verticillium wilt in cotton (Lu et al. 2011). For instance, *Arabidopsis* plants overexpressing *GhSNAP33* and *Gbvdr6* showed resistance to *V. dahliae* with elevated expression of PR1, and endogenous cAMP induced rapid increases of PR1 transcription in plant defense responses against pathogen Verticillium (Yang et al. 2017; Jiang et al. 2005; Wang et al. 2018). It is possible that GbVIP1 might increase resistance to Verticillium wilt in transgenic tobacco by up-regulating expression of *PR1*. In addition to *PR1*, *RAR1*, *HSP70* and *RPP13* were also up-regulated during incubation of Verticillium wilt at different levels and different time points. The expression pattern of *HSP70* was similar to that of *PR1* was significantly up-regulated in GbVIP1 transgenic tobacco at 6 h after infection and expression level was reduced after 12 h. The HSP70 proteins are evolutionarily conserved molecular chaperones and play a key role in correct protein folding, plant growth and development process and biotic and abiotic stress responses in plants (Lin et al. 2001). HSP70 is rapidly up-regulated and accumulates in plants experiencing stress and HSP70 can reduce the damage to plant cells, which improves plant stress tolerance (Sung 2001). In our studies, the *GbVIP1* transgenic tobacco may have increased the resistance to Verticillium wilt through up-regulating HSP70. The expression levels of *RAR1-like* and

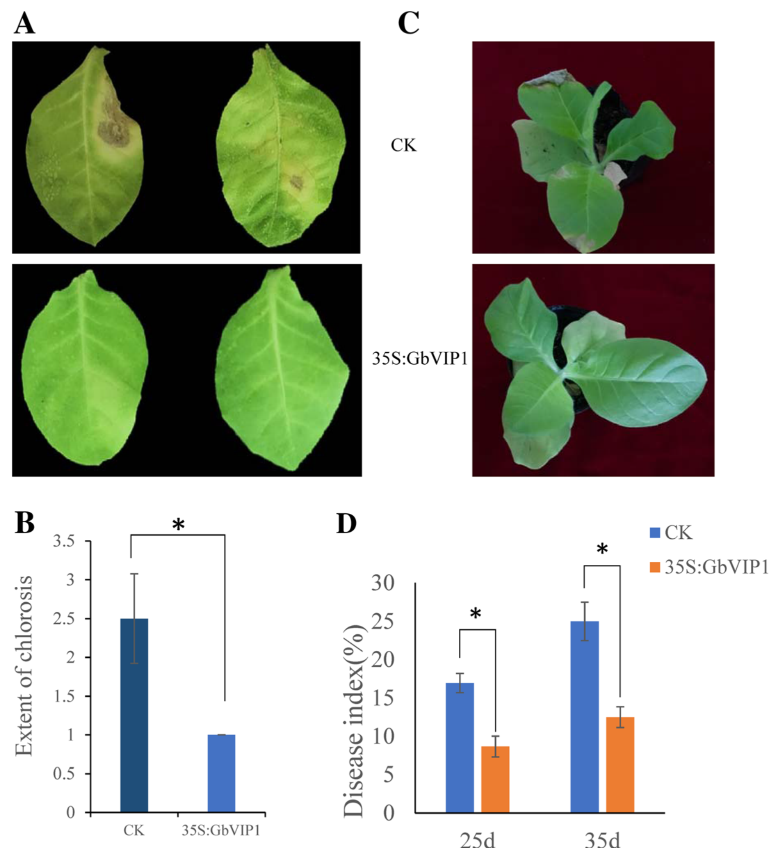

Fig. 5 GbVIP1 gene from cotton conferred resistance to Verticillium wilt in transgenic tobacco plants. **a** Disease phenotype of leaves from *GbVIP1* transgenic tobacco and control at 16 days after inoculation with *V. dahliae.* 35S:GbVIP1 represents transgenic tobacco lines and CK represents the control tobacco lines. **b** Extent of chlorosis of leaves. **c** Whole plant disease phenotype of *GbVIP1* transgenic tobacco and control at 25 days after infection of *V. dahliae.* **d** Disease index of *GbVIP1* transgenic tobacco and control seedlings measured at 25 and 35 days after infection

RPP13 were slightly up-regulated after 6 h infection and reached maxima after 12 h in transgenic tobacco. The *RAR1* protein is a eukaryotic zinc-binding protein and RPP13 contains CC, NB-ARC and LRR domains, which play a crucial part in resistance to various plant diseases(Cheng et al. 2018; Wang et al. 2017). In our results, *RAR1-like* and *RPP13* were not significantly up-regulated by GbVIP1 and we deduced that RAR1-like and RPP13 proteins might have little relationship with GbVIP1 and Verticillium wilt.

Conclusions

We cloned *GbVIP1* from a resistant *G. barbadense* variety and verified the resistance function of GbVIP1 in plant defense against Verticillium wilt. Our results suggested that *GbVIP1* increased plant resistance to Verticillium wilt through up-regulating the expression levels of *PR1*, *PR1-like* and *HSP70*. Our output will provide new approaches to improve disease resistance to Verticillium wilt in *G. hirsutum* and also have much potential for disease-resistance breeding of cotton.

Methods

Plant materials and growth conditions

The cotton cv. Hai 7124 (resistant), Pima (resistant) and TM-1(susceptible) were obtained from the Institute of Cotton Research of Chinese Academy of Agricultural Sciences. The tobacco line NC89 was kindly granted by Dr. Pei Xinwu from the Biotechnology Research Institute of Chinese Academy of Agricultural Sciences. Cotton seedlings were grown in incubators at 25 °C during the day and 20 °C at night, 60% relative humidity, under a 16/8 h light/dark photoperiod. The tobacco aseptic seedlings were grown in another incubator at 25 °C and 60% relative humidity conditions under a 16/8 h light/dark photoperiod in culture bottles. Transgenic tobacco seedlings were grown in a greenhouse under the same conditions as cotton seedlings.

V. dahliae materials and inoculation methods

The *V. dahliae* (Vd853) was kindly provided by Professor Zhu Heqin of the Institute of Cotton Research of Chinese Academy of Agricultural Sciences. The Vd853 was cultured on potato dextrose broth at 25 °C for 6 days

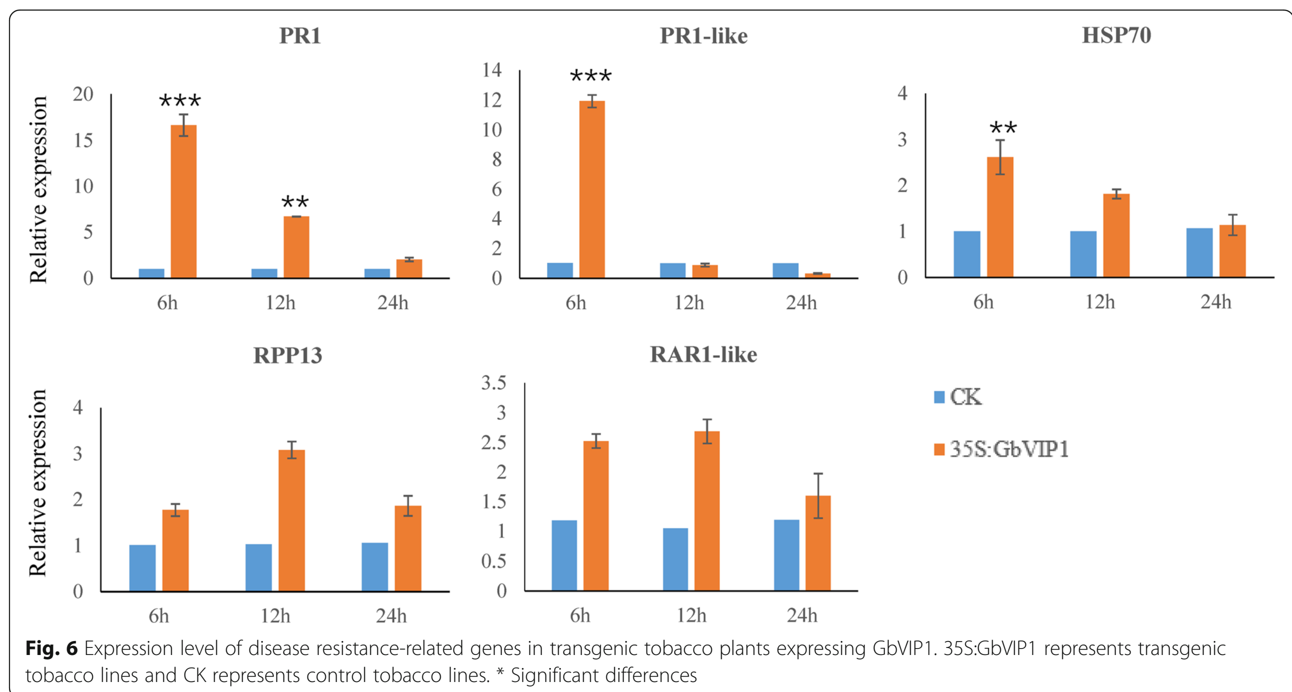

Fig. 6 Expression level of disease resistance-related genes in transgenic tobacco plants expressing GbVIP1. 35S:GbVIP1 represents transgenic tobacco lines and CK represents control tobacco lines. * Significant differences

with shaking. Then, conidia were harvested and grown in liquid Czapek's medium at 25 °C for 7 days with shaking. Czapek's medium comprised 3% sucrose, 0.2% $NaNO_3$, 0.131% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.002% $FeSO_4 \cdot 7H_2O$ (all *w*/*v*). The conidia concentration was verified by counting conidia using a hemocytometer under a microscope. For *V. dahliae* infection, the conidia working concentration was 1×10^{10} conidia·L^{-1}. Roots of the cotton or tobacco seedlings were uprooted gently, dipped in 10 mL of conidial suspensions for 5 min and replanted in pots. The *V. dahliae* infection of tobacco leaves was performed as described previously (Munis et al. 2010). Leaves from transgenic and wild type tobacco plants were harvested at the same position. The detached leaves were inoculated with conidial suspensions (1×10^{10} conidia·L^{-1}) for 5 s and then put in sterile Petri dishes with moistened sterile filter paper at 25 °C for 48 h. After incubation, infected leaves were washed three times with sterile demineralized water and then placed in new sterile Petri dishes with moistened sterile filter paper at 25 °C.

Extraction of RNA and gene cloning

Plant total RNA was extracted using EASYspin Plus Plant RNA kit (Aidlab, Beijing, China) according to the manufacturer's instructions. The cDNA was synthesized using a PrimeScript™ II 1st strand cDNA Synthesis Kit (TakaRa, Dalian, China). For cloning *GbVIP1*, a 20 μL reaction system was used, containing 10 × PCR buffer for KOD-Plus-Neo, 0.2 mmol·L^{-1} dNTPs, 1.5 mmol·L^{-1} $MgSO_4$, 0.3 μmol·L^{-1} forward primer and 0.3 μmol·L^{-1} reverse primer, 0.4 U of KOD-Plus-Neo (TOYOBO, Osaka, Japan) and 200 mmol·L^{-1} cDNA. The PCR amplification was performed on a Bio-Rad PCR thermal cycler (C1000) and the procedure consisted of 94 °C for 4 min, 34 cycles of 98 °C for 10 s, 60 °C for 1 min, 68 °C for 30 s/kb, and 68 °C for 5 min. The PCR product was segregated by agarose gel electrophoresis and purified by TIANgel Maxi Purification Kit (TIANGEN, Beijing, China). Purified product was cloned into the pEASY-Blunt Zero Cloning vector (TransGen, Beijing, China) and sequenced by GENEWIZ (Suzhou, China). The *GhVIP1* from upland cotton was cloned and sequenced using the same method. All primers used in this paper are listed in Additional file 3 and were synthesized by GENEWIZ.

Vector construction for virus-induced gene silencing (VIGS) in cotton and VIGS experiments

The *GbVIP1* fragment (272 bp) was amplified by VIP1-V-F/ VIP1-V-R primers as described in previous steps. The *GbVIP1* fragment was inserted into pYL156 vector, a TRV based vector used for VIGS through ClonExpress™ II One Step Cloning Kit (Vazyme, Nanjing, China). The pYL156–*GhPDS* used as a positive control vector was constructed using the same method. The plasmids containing pYL156–*GbVIP1*, pYL156–*GhPDS*, pYL156 and pYL192 were transformed into *Agrobacterium tumefaciens* strain GV3101 respectively using the freeze–thaw method (Dupadahalli 2007). For VIGS, *Agrobacterium* was harvested and injected into two fully expanded cotyledons of cotton seedlings as previously described (Gao and Shan 2013). The VIGS experiments

were performed with at least three biological repeats and for each repeat there were more than ten plants per constructed vector.

Vector construction for overexpression in tobacco and *Agrobacterium*-mediated transformation

The full-length *GbVIP1* coding sequence was inserted into pBI121, a plant overexpression vector through homologous recombination. The *GbVIP1* was expressed by 35S promoter and selected by *NPTII*. The conducted pBI121–GbVIP1 vector was transformed into *Agrobacterium* strain GV3101. For *Agrobacterium*-mediated transformation, sterile leaves derived from tissue-cultured tobacco plants were cut into 1 cm^2 squares. The leaf explants were pre-cultured on MS solid medium for 3 days in darkness. *Agrobacterium tumefaciens* harboring the pBI121–GbVIP1 vector was cultured at 28 °C overnight and when the OD_{600} value reached 0.6, *Agrobacterium* solution was harvested using a centrifuge (3 500 r·min^{-1}, 10 min) and re-suspended in MS liquid medium. The pre-cultured tobacco leaves were placed in *Agrobacterium* suspensions for 20 min, dried in sterile filter paper, and then co-cultured on solid MS medium at 25 °C in darkness for 2 days. The infected leaf disks were transferred to the selection medium (MS solid medium containing 300 mg·L^{-1} carbenicillin, 100 mg·L^{-1} cefotaxime, 100 mg·L^{-1} kanamycin, 1.0 mg·L^{-1} 6-BA and 0.1 mg·L^{-1} IAA) at 25 °C in light conditions for several days until putative transgenic shoots were regenerated. Selection medium was changed every 2 weeks. The regenerated shoots were transferred into rooting medium (MS solid medium containing 300 mg·L^{-1} carbenicillin, 100 mg·L^{-1} cefotaxime and 100 mg·L^{-1} kanamycin) for taking roots. Transgenic detection was performed using PCR and real-time PCR.

Morbidity situation analysis

The DI was used to measure the morbidity situation of cotton seedlings after *V. dahliae* infection. A higher DI value indicates less, disease resistance. According to leaf chlorosis symptoms, cotton seedlings were classified into five grades: 0 (healthy plants), 1 (25% of leaves showing infection symptoms), 2 (25%~50% of leaves showing symptoms), 3 (50%~75% of leaves showing symptoms) and 4 (more than 75% of leaves showing symptoms) (Zhang et al. 2012a). The DI was calculated using the following formula:

$$DI = \frac{\sum number\ of\ infected\ plant \times Disease\ grade}{total\ number\ of\ infected\ plant \times 4} \times 100\%$$

The incidence of detached tobacco leaves was investigated at 16 days after inoculation with *V. dahliae*. Disease grade was according to 0–3 scale: 0 (healthy leaves), 1 (=10%~20% leaf area showing infection symptoms), 2 (=20%~50% leaf area showing symptoms), and 3 (= > 50% leaf area showing symptoms).

Trypan blue test

Trypan is a kind of cell dye that stains dead cells blue. Leaves from infected cotton seedlings were dipped in trypan blue dye solution containing 15 mg of trypan blue, 10 mL of 85% lactic acid, 10 mL of glycerol, 10 mL of phenol, 10 mL of sterile water for 15 min in vacuum conditions. Then the leaves were placed in boiling water for 10 min in order to fix the dye. Finally, 2.5 g·mL^{-1} chloral hydrate solution was used to decolorize (Choi and Hwang 2011).

qRT-PCR

Tissue-specific expression of *VIP1* and its differential expression patterns in different conditions were investigated using qRT-PCR. SYBR Primix Ex Taq™ II (Tli RNaseH Plus), Bulk (TaKaRa) were used for qRT-PCR and a 20 μL reaction volume including 10 μL 2× SYBR Premix Ex Taq II, 2 μL of cDNA template, 0.8 μL of PCR forward primer (10 μmol·L^{-1}), 0.8 μL of PCR reverse primer (10 μmol·L^{-1}), 0.4 μL of ROX and 6 μL of sterile water was used. The qRT-PCR was performed on an ABI 7500 qRT-PCR System (Applied Biosystems). The qRT-PCR procedure consisted of 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The dissociation curves of each reaction were checked and all reactions were performed with three biological replicates. Cotton and tobacco *actin* genes were used as an internal control for normalization of expression values. Results of qRT-PCR were calculated by $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) and statistical analysis of qRT-PCR results was conducted using DPS software (IBM, USA). All primers used in qRT-PCR were synthesized by GENEWIZ and are listed in Additional file 3.

Bioinformatics analysis

The BLAST tool in the National Center for Biotechnology Information website was used to find *VIP1* genes from different plants. BioEdit software was applied for alignment of nucleotide and protein sequences. Conserved domains were analyzed using WebLogo software and CD-Search software. Mega 6.0 software was used to construct the phylogenetic tree.

Additional files

Additional file 1: Figure S1. Phylogenetic tree of VIP1 gene from different species. (DOCX 170 kb)

Additional file 2: Figure S2. Transgenic detection using PCR and qRT-PCR methods. A: Transgenic detection using PCR methods. Lane 1: Marker; Lane 2: H_2O; Lane 3: Positive control; Lane 4: Negative control; Lane5-11: transgenic tobacco plants. B: Transgenic detection using qRT-PCR methods. (DOCX 318 kb)

Additional file 3: Table S1. The primer sequences used in our study. (DOCX 16 kb)

Abbreviations

DI: Disease index; ET: Ethylene; HSP: Heat shock protein; MeJA: Jasmonic acid; PR: Pathogenesis related; SA: Salicylic acid; TRV: Tobacco rattle virus; VIGS: Virus induced gene silence; VIP1: VirE2 interaction protein 1

Acknowledgements

Not applicable.

Funding

This work was supported by the National Key R&D Program of China (2018YFD0100300) and State Key Laboratory of Crop Biology Open Fund (2018KF09).

Availability of data and materials

Not applicable.

Authors' contributions

Wang HM conceived and designed the experiments, Zhang K and Zhao P performed the experiments and analyzed the data, Zhao P wrote the paper, Zhao YL revised the manuscript, Chen W, Gong HY, Sang XH and Cui YL prepared the materials, All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 21 December 2018 Accepted: 20 February 2019
Published online: 08 March 2019

References

Cai YF, He XH, Mo JC, et al. Molecular research and genetic engineering of resistance to Verticillium wilt in cotton: a review. Afr J Biotechnol. 2009;8(25):7363–72.

Cheng J, Fan H, Li L, et al. Genome-wide identification and expression analyses of RPP13-like genes in barley. BioChip J. 2018;12(2):102–13. https://doi.org/10.1007/s13206-017-2203-y

Choi DS, Hwang BK. Proteomics and functional analyses of pepper abscisic acid-responsive 1 (ABR1), which is involved in cell death and defense signaling. Plant Cell. 2011;23(2):823–42. https://doi.org/10.1105/tpc.110.082081

Dupadahalli K. A modified freeze-thaw method for the efficient transformation of *Agrobacterium tumefaciens*. Curr Sci. 2007;93(6):770.

Fradin EF, Thomma BP. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Mol Plant Pathol. 2006;7(2):71–86. https://doi.org/10.1111/j.1364-3703.2006.00323.x

Gao X, Shan L. Functional genomic analysis of cotton genes with *Agrobacterium*-mediated virus-induced gene silencing. Methods Mol Biol. 2013;975:157–65. https://doi.org/10.1007/978-1-62703-278-0_12

Gong Q, Yang Z, Chen E, et al. A phi-class glutathione S-transferase gene for Verticillium wilt resistance in *Gossypium arboreum* identified in a genome-wide association study. Plant Cell Physiol. 2018;59(2):275–89. https://doi.org/10.1093/pcp/pcx180

Guo W, Jin L, Miao Y, et al. An ethylene response-related factor, GbERF1-like, from *Gossypium barbadense* improves resistance to *Verticillium dahliae* via activating lignin synthesis. Plant Mol Biol. 2016;91(3):305–18. https://doi.org/10.1007/s11103-016-0467-6

Jakoby M, Weisshaar B, Droge-Laser W, et al. bZIP transcription factors in *Arabidopsis*. Trends Plant Sci. 2002;7(3):106–11. https://doi.org/10.1016/S1360-1385(01)02223-3

Jiang J, Fan LW, Wu WH. Evidences for involvement of endogenous cAMP in *Arabidopsis* defense responses to Verticillium toxins. Cell Res. 2005;15(8):585–92. https://doi.org/10.1038/sj.cr.7290328

Jun Z, Zhang Z, Gao Y, et al. Overexpression of GbRLK, a putative receptor-like kinase gene, improved cotton tolerance to Verticillium wilt. Sci Rep. 2015;5:15048. https://doi.org/10.1038/srep15048

Katagiri F, Tsuda K. Understanding the plant immune system. Mol Plant-Microbe Interact. 2010;23(12):1531–6. https://doi.org/10.1094/mpmi-04-10-0099

Kaur A, Pati PK, Pati AM, et al. *In-silico* analysis of *cis*-acting regulatory elements of pathogenesis-related proteins of *Arabidopsis thaliana* and *Oryza sativa*. PLoS One. 2017;12(9):e0184523. https://doi.org/10.1371/journal.pone.0184523

Lacroix B, Citovsky V. Characterization of VIP1 activity as a transcriptional regulator *in vitro* and *in planta*. Sci Rep. 2013;3:2440. https://doi.org/10.1038/srep02440

Li NY, Zhou L, Zhang DD, et al. Heterologous expression of the cotton NBS-LRR gene GbaNA1 enhances Verticillium wilt resistance in *Arabidopsis*. Front Plant Sci. 2018;9:119. https://doi.org/10.3389/fpls.2018.00119

Li Y, Chen YY, Wang F, et al. Isolation and characterization of the Agvip1 gene and response to abiotic and metal ions stresses in three celery cultivars. Mol Biol Rep. 2014;41(9):6003–11. https://doi.org/10.1007/s11033-014-3478-x

Lin BL, Wang JS, Liu HC, et al. Genomic analysis of the Hsp70 superfamily in *Arabidopsis thaliana*. Cell Stress Chaperones. 2001;6(3):201–8. https://doi.org/10.1379/1466-1268(2001)00660;0201:gaoths62;2.0.co;2

Liu Y, Kong X, Pan J, et al. VIP1: linking *Agrobacterium*-mediated transformation to plant immunity? Plant Cell Rep. 2010;29(8):805–12. https://doi.org/10.1007/s00299-010-0870-4

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods. 2001;25(4):402–8. https://doi.org/10.1006/meth.2001.1262

Lu S, Friesen TL, Faris JD. Molecular characterization and genomic mapping of the pathogenesis-related protein 1 (PR-1) gene family in hexaploid wheat (*Triticum aestivum* L.). Mol Gen Genomics. 2011;285(6):485–503. https://doi.org/10.1007/s00438-011-0618-z

Mo HJ, Wang XF, Zhang Y, et al. Cotton polyamine oxidase is required for spermine and camalexin signalling in the defence response to *Verticillium dahliae*. Plant J. 2015;83(6):962–75. https://doi.org/10.1111/tpj.12941

Munis MF, Tu L, Deng F, et al. A thaumatin-like protein gene involved in cotton fiber secondary cell wall development enhances resistance against *Verticillium dahliae* and other stresses in transgenic tobacco. Biochem Biophys Res Commun. 2010;393(1):38–44. https://doi.org/10.1016/j.bbrc.2010.01.069

Robison MM, Griffith M, Pauls KP, et al. Dual role for ethylene in susceptibility of tomato to Verticillium wilt. J Phytopathol. 2001;149(7–8):385–8. https://doi.org/10.1111/j.1439-0434.2001.tb03867.x

Sung DY. Comprehensive expression profile analysis of the *Arabidopsis* Hsp70 gene family. Plant Physiol. 2001;126(2):789–800. https://doi.org/10.1104/pp.126.2.789

Tsugama D, Liu S, Takano T. A bZIP protein, VIP1, interacts with *Arabidopsis* heterotrimeric G protein beta subunit, AGB1. Plant Physiol Biochem. 2013;71:240–6. https://doi.org/10.1016/j.plaphy.2013.07.024

Tsugama D, Liu S, Takano T. Analysis of functions of VIP1 and its close homologs in osmosensory responses of *Arabidopsis thaliana*. PLoS One. 2014;9(8):e103930. https://doi.org/10.1371/journal.pone.0103930

Tsugama D, Liu S, Takano T. The bZIP protein VIP1 is involved in touch responses in *Arabidopsis* roots. Plant Physiol. 2016a;171(2):1355–65. https://doi.org/10.1104/pp.16.00256

Tsugama D, Liu S, Takano T. VIP1 is very important/interesting protein 1 regulating touch responses of *Arabidopsis*. Plant Signal Behav. 2016b;11(6):e1187358. https://doi.org/10.1080/15592324.2016.1187358

Tzfira T, Rhee Y, Chen MH, et al. Nucleic acid transport in plant-microbe interactions: the molecules that walk through the walls. Annu Rev Microbiol. 2000;54:187–219. https://doi.org/10.1146/annurev.micro.54.1.187

Van der Krol AR, Chua NH. The basic domain of plant B-ZIP proteins facilitates import of a reporter protein into plant nuclei. Plant Cell. 1991;3(7):667–75. https://doi.org/10.1105/tpc.3.7.667

Wang P, Sun Y, Pei Y, et al. GhSNAP33, a t-SNARE protein from *Gossypium hirsutum*, mediates resistance to *Verticillium dahliae* infection and tolerance to drought stress. Front Plant Sci. 2018;9:896. https://doi.org/10.3389/fpls.2018.00896

Wang X, Wang Y, Liu P, et al. TaRar1 is involved in wheat defense against stripe rust pathogen mediated by YrSu. Front Plant Sci. 2017;8:156. https://doi.org/10.3389/fpls.2017.00156

Wang Y, Liang C, Wu S, et al. Significant improvement of cotton Verticillium wilt resistance by manipulating the expression of Gastrodia antifungal proteins. Mol Plant. 2016;9(10):1436–9. https://doi.org/10.1016/j.molp.2016.06.013

Wu Y, Zhao Q, Gao L, et al. Isolation and characterization of low-Sulphur-tolerant mutants of *Arabidopsis*. J Exp Bot. 2010;61(12):3407–22. https://doi.org/10.1093/jxb/erq161

Xu DB, Chen M, Ma YN, et al. A G-protein beta subunit, AGB1, negatively regulates the ABA response and drought tolerance by down-regulating AtMPK6-related pathway in *Arabidopsis*. PLoS One. 2015;10(1):e0116385. https://doi.org/10.1371/journal.pone.0116385

Yang CL, Liang S, Wang HY, et al. Cotton major latex protein 28 functions as a positive regulator of the ethylene responsive factor 6 in defense against *Verticillium dahliae*. Mol Plant. 2015;8(3):399–411. https://doi.org/10.1016/j.molp.2014.11.023

Yang J, Ma Q, Zhang Y, et al. Molecular cloning and functional analysis of GbRVd, a gene in *Gossypium barbadense* that plays an important role in conferring resistance to Verticillium wilt. Gene. 2016;575(2):687–94. https://doi.org/10.1016/j.gene.2015.09.046

Yang Y, Chen T, Ling X, et al. Gbvdr6, a gene encoding a receptor-like protein of cotton (*Gossypium barbadense*), confers resistance to Verticillium wilt in *Arabidopsis* and upland cotton. Front Plant Sci. 2017;8:2272. https://doi.org/10.3389/fpls.2017.02272

Zaltsman A, Krichevsky A, Kozlovsky SV, et al. Plant defense pathways subverted by agrobacterium for genetic transformation. Plant Signal Behav. 2010;5(10):1245–8. https://doi.org/10.4161/psb.5.10.12947

Zhang B, Yang Y, Chen T, et al. Island cotton Gbve1 gene encoding a receptor-like protein confers resistance to both defoliating and non-defoliating isolates of *Verticillium dahliae*. PLoS One. 2012b;7(12):e51091. https://doi.org/10.1371/journal.pone.0051091

Zhang J, Flynn R, Baral JB, et al. Germplasm evaluation and transfer of Verticillium wilt resistance from Pima (*Gossypium barbadense*) to upland cotton (*G. hirsutum*). Euphytica. 2012a;187(2):147–60. https://doi.org/10.1007/s10681-011-0549-0

Zhang JF, Fang H, Zhou HP, et al. Genetics, breeding, and marker-assisted selection for Verticillium wilt resistance in cotton. Crop Sci. 2014;54(4):1289–303. https://doi.org/10.2135/cropsci2013.08.0550

Zhao LJ, Yao JB, Chen W, et al. A genome-wide analysis of *SWEET* gene family in cotton and their expressions under different stresses. J Cotton Res. 2018;1:7. https://doi.org/10.1186/s42397-018-0007-9

Zhou HP, Fang H, Sanogo S, et al. Evaluation of Verticillium wilt resistance in commercial cultivars and advanced breeding lines of cotton. Euphytica. 2014;196(3):437–48. https://doi.org/10.1007/s10681-013-1045-5

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions