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Authors

Zhang, Wen-Qi
Gui, Yue-Jing
Short, Dylan PG
et al.

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Verticillium dahliae transcription factor VdFTF1 regulates the expression of multiple secreted virulence factors and is required for full virulence in cotton

WEN-QI ZHANG[1,†], YUE-JING GUI[1,†], DYLAN P. G. SHORT[2], TING-GANG LI[1], DAN-DAN ZHANG[1], LEI ZHOU[1], CHUN LIU[3], YU-MING BAO[1], KRISHNA V. SUBBARAO[2,*], JIE-YIN CHEN[1,*] AND XIAO-FENG DAI[1,*]

[1]*Laboratory of Cotton Disease, Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, China*
[2]*Department of Plant Pathology, University of California, Davis, CA 95616, USA*
[3]*BGI-Shenzhen, Shenzhen, Guangdong 518083, China*

SUMMARY

Fungal transcription factors (TFs) implicated in the regulation of virulence gene expression have been identified in a number of plant pathogens. In *Verticillium dahliae*, despite its agricultural importance, few regulators of transcription have been characterized. In this study, a T-DNA insertion mutant with significantly reduced virulence towards cotton was identified. The T-DNA was traced to *VdFTF1*, a gene encoding a TF containing a Fungal_trans domain. Transient expression in onion epidermal cells indicated that VdFTF1 is localized to the nucleus. The *VdFTF1*-deletion strains displayed normal vegetative growth, mycelial pigmentation and conidial morphology, but exhibited significantly reduced virulence on cotton, suggesting that VdFTF1 is required exclusively for pathogenesis. Comparisons of global transcription patterns of wild-type and *VdFTF1*-deletion strains indicated that VdFTF1 affected the expression of 802 genes, 233 of which were associated with catalytic processes. These genes encoded 69 potentially secreted proteins, 43 of which contained a carbohydrate enzyme domain known to participate in pathogenesis during infection of cotton. Targeted gene deletion of one VdFTF1-regulated gene resulted in significantly impaired vascular colonization, as measured by quantitative polymerase chain reaction, as well as aggressiveness and symptom severity in cotton. In conclusion, *VdFTF1*, which encodes a TF containing a Fungal_trans domain, regulates the gene expression of plant cell wall degradation enzymes in *V. dahliae*, which are required for full virulence on cotton.

Keywords: Fungal_trans domain containing protein, CAZymes, Fungal pathogenesis, RNA-seq, Secreted proteins, Transcriptomics, Verticillium wilt.

*Correspondence: Email: kvsubbarao@ucdavis.edu; chenjieyin@caas.cn; daixiaofeng_caas@126.com
†These authors contributed equally to this work.

INTRODUCTION

Verticillium dahliae is a soil-borne phytopathogenic fungus that causes a destructive vascular wilt in more than 200 dicotyledonous plant species, including a wide range of economically important crops (Inderbitzin and Subbarao, 2014; Pegg and Brady, 2002). The pathogen infects plants through the root tip or through sites on lateral roots. Subsequently, it reaches vascular tissues and proliferates, resulting in characteristic wilt symptoms, including wilting of leaves, stunting, necrosis, vein clearing and vascular discoloration (Fradin and Thomma, 2006). In agricultural fields, *V. dahliae* produces large amounts of microsclerotia in infected plant tissues, which are released into the soil with the progressive decomposition of crop residue and can survive in the soil environment for at least 15 years (Fradin and Thomma, 2006; Inderbitzin and Subbarao, 2014; Wilhelm, 1955). Few effective strategies are available for the consistent management of Verticillium wilt because little information exists on the crucial components of *V. dahliae* pathogenicity and virulence. Thus, the characterization of such mechanisms may ultimately inform disease management strategies.

Initial studies that attempted to elucidate the mechanisms of pathogenesis and virulence in *V. dahliae* were hindered by a lack of genomic information. As a result, only a few pathogenicity-related genes have been identified (Klimes and Dobinson, 2006; Rauyaree *et al.*, 2005; Tzima *et al.*, 2010; Wang *et al.*, 2004). Nevertheless, these studies documented that secreted proteins play an important role in *V. dahliae* pathogenicity (Fradin and Thomma, 2006). The publicly available *Verticillium* genomes have significantly increased the understanding of the molecular mechanisms underlying the pathogenicity and virulence of *V. dahliae* (de Jonge *et al.*, 2013; Klosterman *et al.*, 2011), and have provided evidence in favour of the previous hypotheses that a diverse arsenal of secreted proteins, including effectors, pectin and cellulase-degrading enzymes, are important for the infection process (Buchner *et al.*, 1982, 1989; Davis *et al.*, 1998; Mansoori *et al.*, 1995; Meyer *et al.*, 1994; Nachmias *et al.*, 1985). Several *V. dahliae* pathogenicity-related genes have been identified (Chen *et al.*,

2016; de Jonge *et al.*, 2012, 2013; Liu *et al.*, 2013, 2014; Maruthachalam *et al.*, 2011; Santhanam *et al.*, 2013; Zhou *et al.*, 2012). In addition, several pathogenicity-related genes have been identified using T-DNA insertion mutagenesis (Gao *et al.*, 2010; Li *et al.*, 2015; Luo *et al.*, 2016; Maruthachalam *et al.*, 2011; Santhanam *et al.*, 2017; Zhang DD *et al.*, 2016; Zhang YL *et al.*, 2015, 2016). Collectively, these studies indicate that the processes associated with pathogenicity are complex, and that a variety of *V. dahliae* gene products are required for host infection.

Transcription factors (TFs) regulate the rate of expression of genes required for disparate biological processes under different conditions and play important roles in fungal pathogenesis (Chen *et al.*, 2015; Cho *et al.*, 2012; Okmen *et al.*, 2014; Ramos *et al.*, 2007; de Vega-Bartol *et al.*, 2011; Wang *et al.*, 2011; Zhao *et al.*, 2011). The reference genome of *V. dahliae* strain VdLs.17 is predicted to encode 530 TFs classified into 42 TF families (Klosterman *et al.*, 2011; Park *et al.*, 2008). Like the TFs in other phytopathogens, *V. dahliae* TFs, including VdSge1 (Santhanam and Thomma, 2013), Vta2 (Tran *et al.*, 2014), VdCrz1 (Xiong *et al.*, 2015), Vdpf (Luo *et al.*, 2016) and Vst1 (Sarmiento-Villamil *et al.*, 2016), also play important roles in processes involved in pathogenicity, microsclerotium development and morphological development. In addition, gene activity during *V. dahliae* pathogenesis is regulated by several important signalling factors (Rauyaree *et al.*, 2005; Tian *et al.*, 2014; Tzima *et al.*, 2010, 2011, 2012) that positively regulate virulence factors.

The arsenal of key pathogenicity molecules possessed by *V. dahliae* includes potentially secreted proteins, such as cell wall-degrading enzymes and effectors (Bidochka *et al.*, 1999; Cooper and Wood, 1973; Dobinson *et al.*, 1997, 2004; Klosterman *et al.*, 2011). Further, there is evidence that TFs affect *V. dahliae* pathogenicity and virulence by the regulation of secreted enzymes involved in plant cell wall degradation. For example, *VdSge1* is required for pathogenicity by regulating the *in planta* expression of various effector genes (Santhanam and Thomma, 2013). In addition, Vta2 regulates the expression of 125 secreted proteins, including putative adhesins, effector molecules and a secreted catalase-peroxidase (Tran *et al.*, 2014). Similar results have occurred in other fungal pathogens, in which pathogenicity is significantly affected when the function of TFs is altered (Cho *et al.*, 2012; van der Does *et al.*, 2016; Okmen *et al.*, 2014; Tollot *et al.*, 2016). In the rice blast fungus *Magnaporthe oryzae*, systematic analysis of 104 Zn_2Cys_6 TFs revealed that at least seven were required for pathogenicity (Lu *et al.*, 2014). Together, these results strongly suggest that key TFs play important roles in fungal pathogenicity and virulence.

Although a few *V. dahliae* genes have been shown previously to be associated with pathogenicity (Luo *et al.*, 2016; Santhanam *et al.*, 2013; Tran *et al.*, 2014), which TFs participate in pathogenesis is largely unknown. In a previous study, we constructed an *Agrobacterium tumefaciens*-mediated transformation (ATMT) insertion mutant library of the highly virulent *V. dahliae* strain Vd991 and obtained 16 mutants with significantly reduced virulence on a susceptible cotton cultivar (Zhang DD *et al.*, 2016). One mutant, M06D06, was significantly less virulent towards cotton but exhibited normal radial growth under standard conditions on potato dextrose agar (PDA). Subsequently, we identified a T-DNA-tagged gene in M06D06, *VdFTF1*, which encodes a protein containing a fungal-specific TF domain (Fungal_trans). The Fungal_trans domain has also been found in several fungal TFs that regulate a variety of cellular and metabolic processes. The research reported here: (1) identified the putative TF gene *VdFTF1* as the gene affected by a T-DNA insertion in the mutant M06D06; (2) confirmed that VdFTF1 plays an important role in virulence, but not in fungal development; (3) elucidated the cellular processes potentially regulated by VdFTF1 by performing RNA-sequencing (RNA-seq) analyses; (4) added further evidence that the role of VdFTF1 in virulence is probably a result of its regulation of these processes by performing quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with selected genes under infection conditions; and (5) demonstrated that deletion of a gene putatively regulated by VdFTF1 resulted in a significant reduction in virulence in cotton.

RESULTS

Identification of a *V. dahliae* random T-DNA insertion mutant exhibiting reduced virulence towards cotton

In a previous study, we reported the generation of *V. dahliae* T-DNA insertion mutant M06D06 (Zhang DD *et al.*, 2016). To assess the virulence of M06D06, a root-dip inoculation assay was performed on susceptible cotton (*Gossypium hirsutum* L. 'Junmian No. 1'). Three weeks after inoculation with the wild-type strain Vd991, the cotton plants presented severe Verticillium wilt symptoms, including necrosis and wilting, as expected (Fig. 1A). In contrast, less severe chlorosis, necrosis and wilting were observed in cotton after inoculation with M06D06, relative to the vascular discoloration induced by the wild-type strain (Fig. 1A). Similarly, vascular discoloration was seldom observed in plants inoculated with the mutant M06D06 relative to the extensive vascular discoloration of plants inoculated with the wild-type strain (Fig. 1B). Quantification of fungal biomass in cotton using real-time quantitative polymerase chain reaction (qPCR) demonstrated that inoculation with mutant M06D06 resulted in significantly less *in planta* fungal biomass ($33\% \pm 2\%$) compared with inoculation with the wild-type strain (Fig. 1C). These results suggest that the ability of mutant M06D06 to colonize cotton is significantly reduced.

The M06D06 T-DNA-tagged gene encodes a Fungal_trans domain-containing protein

Flanking sequences of the exogenously integrated T-DNA were obtained using the thermal asymmetric interlaced-PCR (TAIL-PCR)

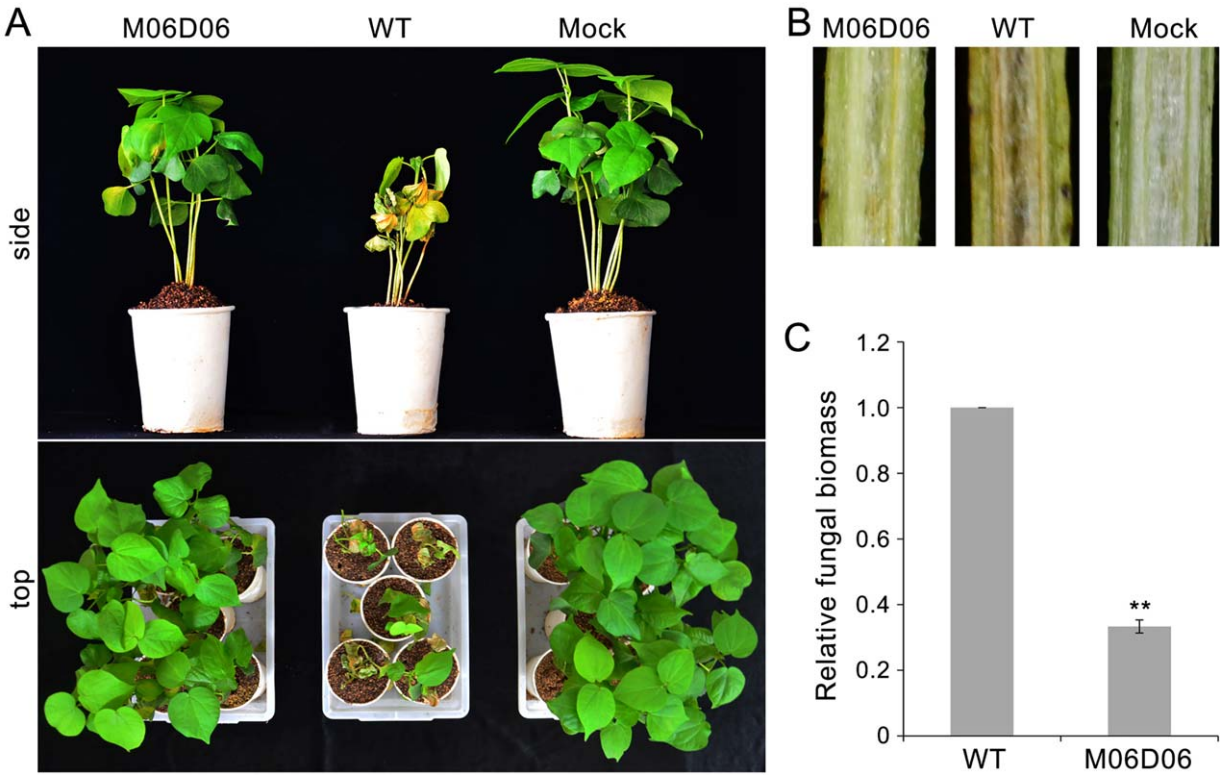

Fig. 1 Virulence of the *Verticillium dahliae* T-DNA mutant M06D06. (A) Virulence was assessed using the root-dipping method with a conidial suspension (2×10^9 conidia/mL) and 2-week-old seedlings of susceptible cotton (*Gossypium hirsutum* L., 'Junmian No. 1') at 21 days post-inoculation. M06D06 is a T-DNA random insertion mutant, WT refers to the wild-type strain Vd991 and Mock indicates the control that was treated with sterile water. The side and top images of Verticillium wilt symptoms were taken 3 weeks after inoculation. (B) Vascular discoloration of cotton plants inoculated with the T-DNA random insertion mutant (M06D06) and WT. Vertical sections of cotton hypocotyl were photographed at 21 days post-inoculation. (C) Fungal biomass of the T-DNA random insertion mutant M06D06 on cotton was determined by quantitative polymerase chain reaction (qPCR) from 100 mg of ground total plant tissue. Error bars represent the standard deviation, and a double asterisk indicates the statistical significance ($P \leq 0.01$) for the mutant compared with WT using an unpaired Student's *t*-test.

technique. After three continuous rounds, degenerate primers and nested primers amplified regions from the right border of the T-DNA (Fig. S1A, see Supporting Information), resulting in the recovery of three amplicons (LAD2/3/5) of 511, 258 and 524 bp flanking the T-DNA insertion site (Fig. S1B). BLAST analyses indicated that all three T-DNA flanking sequences matched a position located at bp 2 440 974 of Scaffold1 in the genome sequence assembly of Vd991 (data not shown). This position was located within a predicted gene (VEDA_01836) harbouring an open reading frame (ORF) of 1581 bp, according to the genome annotation (Figs 2A and S1C). The full-length cDNA of VEDA_01836 was obtained by RT-PCR using RNA isolated from infective conidia. The full-length gene was also amplified by PCR from genomic DNA purified from vegetative hyphae. Comparison of the full-length gene amplified from genomic DNA and the cDNA revealed that VEDA_01836 was predicted to contain one intron (84 bp) and encoded a 526-amino-acid protein; the T-DNA was integrated in the second exon of VEDA_01836 (Fig. 2A). Exploration of the protein domain architecture revealed that the protein encoded by VEDA_01836 contained a fungal-specific TF (Fungal_trans) domain (SMART Accession Number: SM00906; position at amino acids 174–253; *E*-value: 0.0000011) (Fig. 2B). Alignment of the homologous proteins indicated that the product of VEDA_01836 shared conserved domains with Fungal_trans domains from other fungi (Fig. 2C), suggesting that VEDA_01836 (named *VdFTF1* in this study) encodes a fungal-specific TF domain-containing protein. Taken together, these results strongly suggest that the virulence of the M06D06 mutant is significantly reduced on cotton because of the disrupted function of *VdFTF1*.

VdFTF1 is localized to the nucleus

VdFTF1 contains a Fungal_trans domain that is found in a number of fungal TFs that are likely to be localized to cell nuclei and act to regulate gene expression associated with a variety of cellular and metabolic processes. To investigate the subcellular location of the VdFTF1 protein, a VdTFT1-GFP fusion protein was generated (GFP, green fluorescent protein) and its localization was assessed

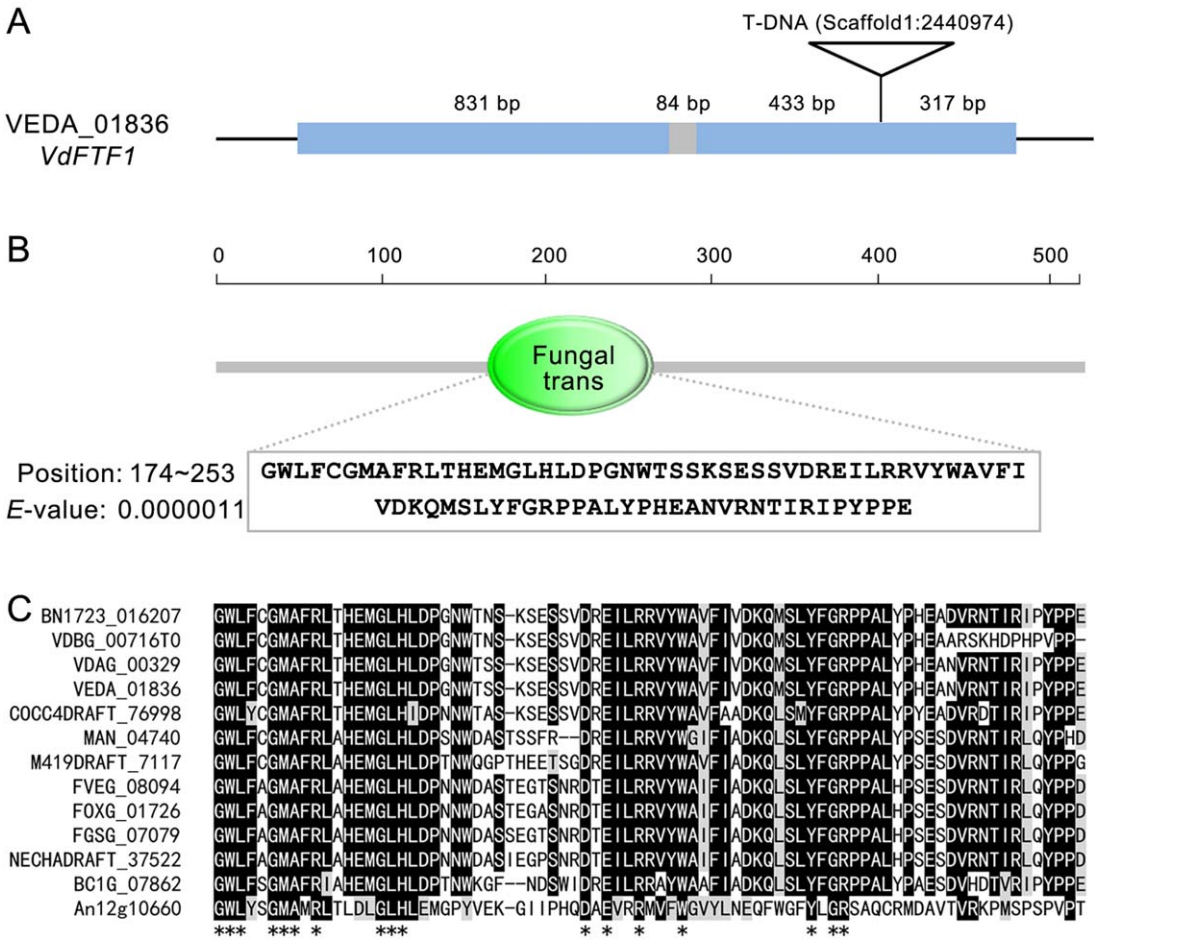

Fig. 2 Analysis of the T-DNA-tagged gene in the M06D06 mutant. (A) The physical location of the T-DNA insertion in the mutant genome. Scaffold 1 is the assembled sequence of the wild-type strain (Vd991) genome sequence (unpublished) and the T-DNA region integrated into the 2 440 974-bp position; the T-DNA-tagged gene is the predicted VEDA_01836 gene containing a fungal-specific transcription factor (TF) (*VdFTF1*). (B) Bioinformatics analysis of VdFTF1. The positions of putative fungal-specific TFs with conserved domains were determined using the SMART program (http://smart.embl-heidelberg.de/). (C) Alignment of the fungal-specific TF domain in fungi. Alignment of the encoded proteins, including BN1723_016207 (*Verticillium longisporum*), VDAG_00329 (*Verticillium dahliae* VdLs.17), NECHADRAFT_37522 (*Nectria haematococca* mpVI 77–13-4), FVEG_08094 (*Fusarium verticillioides* 7600), FOXG_01726 (*Fusarium oxysporum* f. sp. *lycopersici* 4287), FGSG_07079 (*Fusarium graminearum* PH-1), BC1G_07862 (*Botrytis cinerea* B05.10), VDBG_00716 (*Verticillium alfalfae* VaMs.102), MAN_04740 (*Metarhizium anisopliae* ARSEF 549), An12g10660 (*Aspergillus niger* strain CBS 513.88), M419DRAFT_7117 (*Hypocrea jecorina* strain ATCC 56765), COCC4DRAFT_76998 (*Cochliobolus heterostrophus* strain ATCC 48331) and VEDA_01836 (*Verticillium dahliae* Vd991). The asterisks are conserved amino acid residues.

by transient expression in onion epidermal cells. As expected, the fusion protein was clearly localized to cell nuclei (Fig. 3A). In contrast, the 35::GFP protein control encoded by the empty vector was prevalent throughout the onion epidermal cells (Fig. 3B). The nuclear localization of the fusion protein provided additional evidence that VdFTF1 encodes a TF with a Fungal_trans domain.

VdFTF1 is not required for radial growth and production of conidia, but plays an important role in virulence on cotton

To further study the function of *VdFTF1*, targeted replacement of *VdFTF1* was performed in the wild-type strain Vd991 via a hygromycin resistance (hygR) cassette introduced by homologous recombination (Fig. S2A–C, see Supporting Information). In three independent transformants (Δ*FTF1–1*/2/3), replacement of the VdFTF1 coding region with the hygR cassette was confirmed by PCR (Fig. S2D–F). It has been shown previously that Fungal_trans domain-containing proteins are associated with vegetative growth *in vitro* in other pathogens (Jonkers *et al.*, 2014; Lu *et al.*, 2014; Luo *et al.*, 2016). Unexpectedly, the colony morphology of the *VdFTF1*-deletion mutants on PDA did not differ from the morphology of the wild-type strain or the T-DNA insertion mutant M06D06 on PDA. All retained white mycelial development without the generation of additional pigmentation (Fig. 4A). The growth rates of the wild-type and mutants were comparable (Fig.

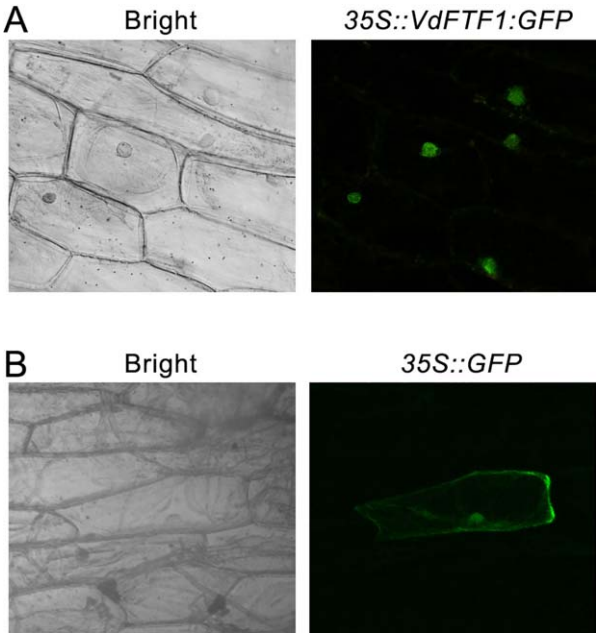

Fig. 3 Subcellular localization of the VdFTF1 protein. The *35S::VdFTF1:GFP* gene and *35S::GFP* control gene were introduced into onion epidermal cells via microprojectile bombardment technology and viewed with a laser scanning confocal microscope at 200× magnification. (A) Fluorescence images of the *35S::VdFTF1:GFP* fusion protein in onion epidermal cells. (B) Fluorescence images of the 35S::GFP control construct in onion epidermal cells. GFP, green fluorescent protein.

4B). In addition, the conidiation rates of the three *VdFTF1*-deletion strains and the T-DNA insertion mutant M06D06D were similar to the conidiation rate of the wild-type strain (Fig. 4C). Conidial morphology, assessed as the lengths of the major and minor axes, was similar for all strains (Fig. 4D,E). These results suggest that *VdFTF1* disruption does not alter vegetative growth, mycelial pigmentation or conidial morphology.

To assess the role of *VdFTF1* in Verticillium wilt, susceptible cotton plants were inoculated with one of the three independent mutant strains or the wild-type strain Vd991 using a root-dip method. As expected, the *VdFTF1*-deletion strains were significantly less virulent towards cotton. Typical Verticillium wilt symptoms, such as necrosis, wilting and vascular discoloration, were observed in plants inoculated with the wild-type strain Vd991 (Fig. 5A). Quantification of fungal biomass in cotton by qPCR revealed that *VdFTF1*-deletion mutant treatment resulted in approximately 40% less biomass *in planta* relative to the wild-type treatment (Fig. 5B). These results suggest that *VdFTF1* is required for full virulence of *V. dahliae* on cotton. The role of *VdFTF1* as an important virulence factor was supported by the restoration of virulence in deletion strains complemented with *VdFTF1*. Inoculation of cotton with two independent complementation transformants (EC^{Ko}-1 and EC^{Ko}-2) resulted in restored disease symptoms. The fungal biomass *in planta* resulting from inoculation with these two mutants was comparable with the fungal biomass resulting from inoculation with the wild-type (Fig. 5C,D). After the re-introduction of *VdFTF1* into M06D06 mutants, two ectopic transformants were obtained (EC^{Mu}-1 and EC^{Mu}-2). These two mutants caused severe symptoms after inoculation on cotton and the fungal biomass in cotton was similar to the fungal biomass of the wild-type treatment (Fig. 5E,F).

VdFTF1 modulates the expression of potential pathogenesis-related genes

The roles of VdFTF1 in the regulation of gene expression were investigated by RNA-seq analysis. A total of 802 genes putatively regulated by VdFTF1 were identified in comparisons between: (1) infective conidia of the wild-type strain and the wild-type strain grown on PDA; and (2) infective conidia of the wild-type strain and infective conidia of the Δ*FTF1* mutants (Fig. 6A). In total, 494 unique genes were identified that were up-regulated in infective conidia of the wild-type strain relative to growth on PDA, and which were also down-regulated in infective conidia of the Δ*FTF1* mutants compared with infective conidia of the wild-type. An additional 308 genes were identified that were down-regulated in the wild-type during infection (relative to growth on PDA), but up-regulated in the Δ*FTF1* mutants during cotton infection [false discovery rate (FDR) < 0.001, $P < 0.001$ and $|\log_2 \text{Ratio}| \geq 1.0$; Fig. 6A; Table S1, see Supporting Information].

Gene ontology (GO) annotation predicted that 233 of the 802 regulated genes belonged to the catalytic class (GO:0003824), and included 90 gene products with predicted hydrolase activity (GO:0016787) and 66 gene products with predicted oxidoreductase activity (GO:0016491) (Fig. 6B). Of the genes regulated by VdFTF1, annotation identified several that were potentially involved in processes related to pathogenesis, including secreted proteins, carbohydrate-active enzymes (CAZymes), pathogen–host interaction (PHI) homologues and TFs. Specifically, this subset of the genes regulated by VdFTF1 was predicted to encode 69 proteins with secretion characteristics, including 24 small cysteine-rich proteins (SCRPs, <400 amino acids, ≥4 cysteine residues), 43 proteins with CAZyme modules, including 19 with secretion characteristics, 90 homologues to PHI proteins, including 65 experimentally verified to be associated with pathogenicity (loss of pathogenicity or reduced virulence), and 15 TFs (Fig. 6C; Table S2, see Supporting Information). In addition, the expression levels of these putative pathogenicity and virulence-related genes mediated by VdFTF1 exhibited higher up-regulation during cotton infection than the total gene average during growth on PDA: the median values of expression changes for secreted proteins, SCRPs and secreted CAZymes were 5.28, 5.30 and 5.00, respectively, compared with 4.20 for the set of total genes during growth on PDA (Fig. 6D). Together, these results suggest that VdFTF1

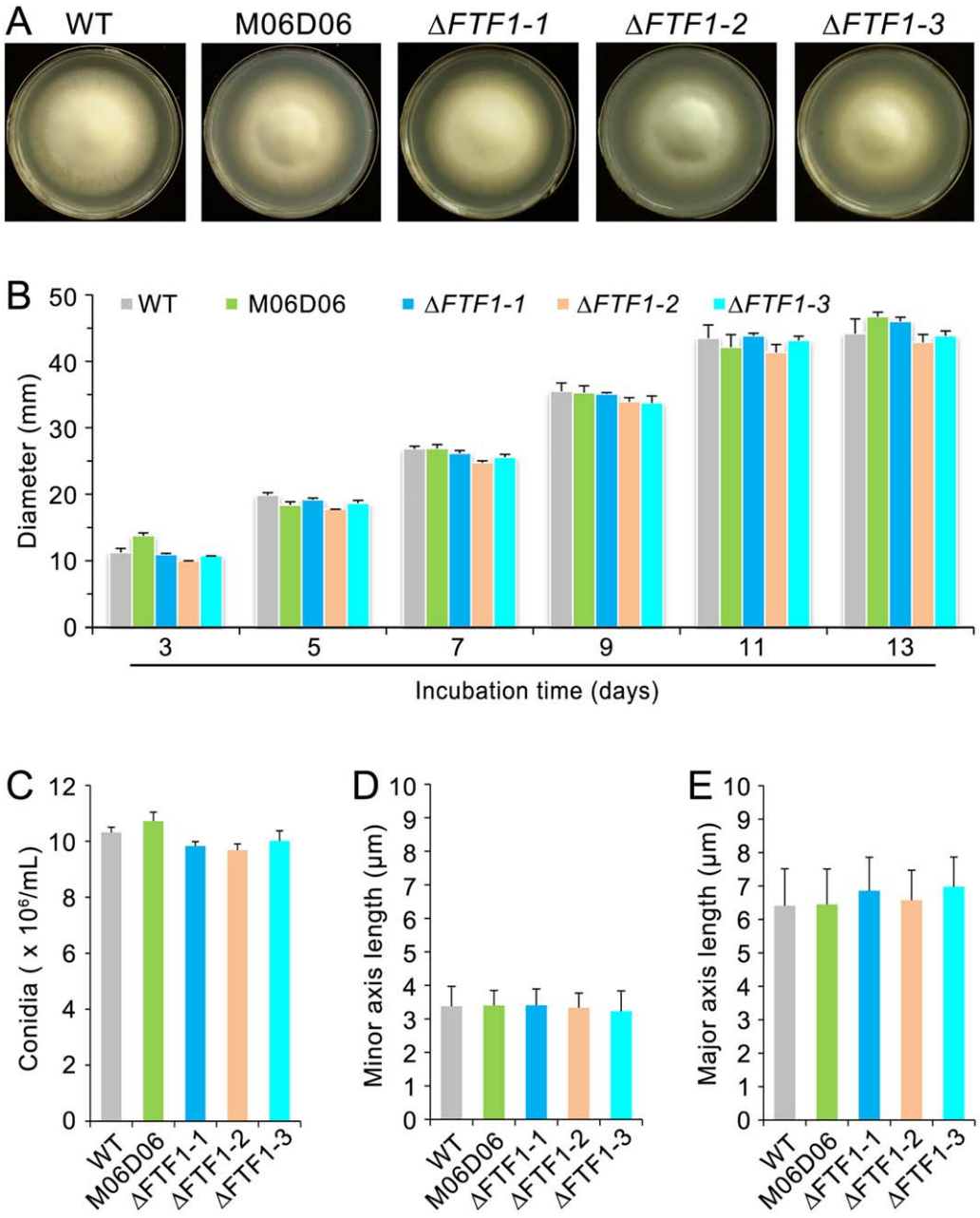

Fig. 4 Colony morphology and conidiospore production of strains carrying the *VdFTF1*-targeted deletion. All strains were incubated on potato dextrose agar (PDA) medium at 25 °C. (A) Colony morphology of the wild-type (WT), the T-DNA insertion mutant M06D06 and three independent *VdFTF1*-deletion stains (Δ*FTF1–1*, Δ*FTF1–2* and Δ*FTF1*-3) after 13 days. (B) Colony diameter (mm). Values shown are the average of three colony diameters, and the data were analysed using Student's *t*-test. Error bars represent standard deviations. (C) Quantification of conidiospore production ability based on a 5-mm-diameter agar plug of fungal colony mixed in 1 mL of water. Values represent the average number of conidia produced after 7 days of growth on PDA medium based on three independent experiments, and the data were analysed by Student's *t*-test ($P < 0.01$). Error bars indicate the standard deviation. (D) Major axis length of the conidiospore in the indicated strain. The conidiospores were collected after 7 days of incubation on PDA medium, and the conidiospore major axis lengths were measured via microscopy at ×100 magnification. Values are the average major axis length of 16 conidiospores with three replicates. The data were analysed using Student's *t*-test, and the error bars represent standard deviations. (E) Minor axis length of conidiospores in the indicated strain.

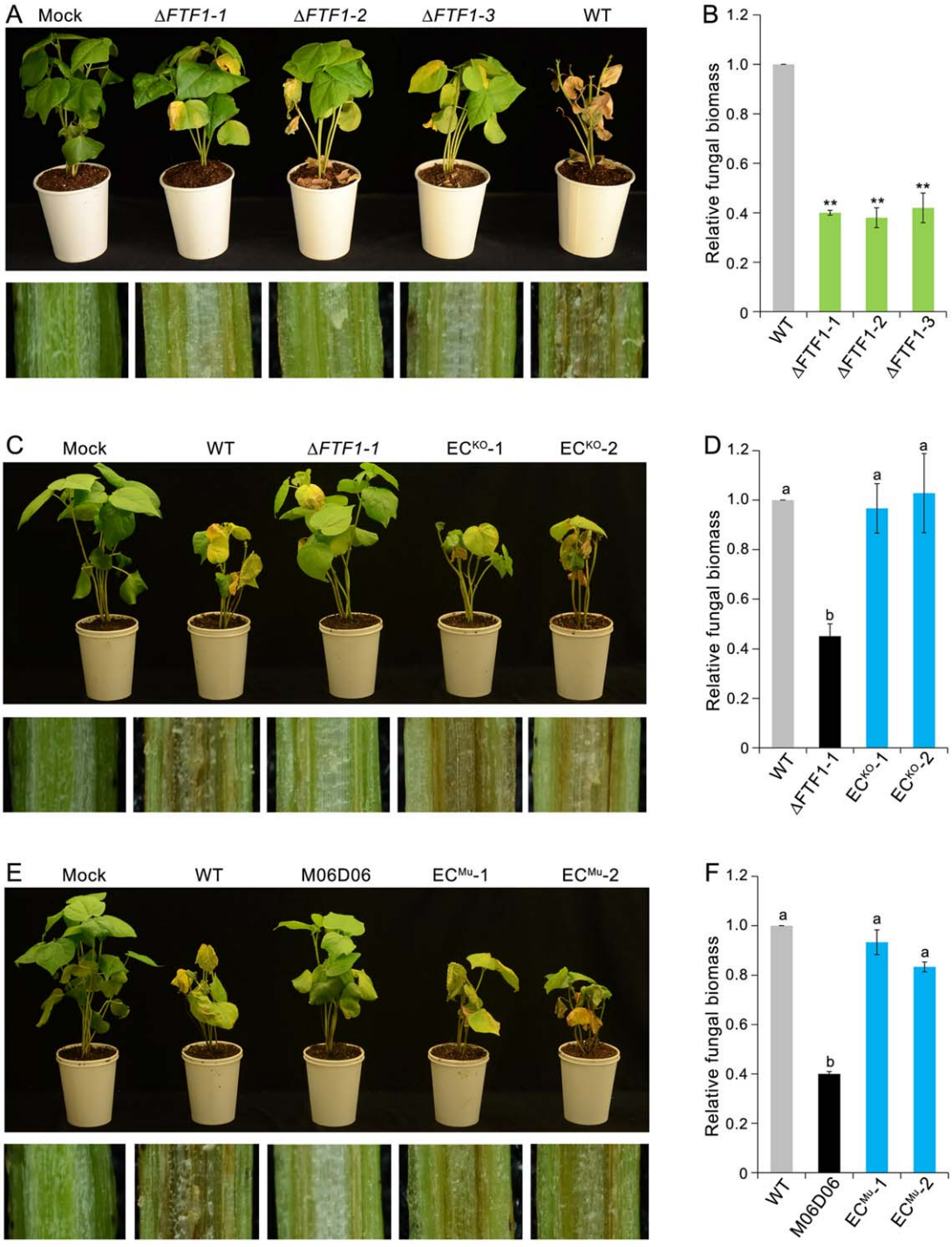

Fig. 5 *VdFTF1* is required for full virulence of *Verticillium dahliae* on cotton. (A) The disease symptom phenotypes of cotton seedlings inoculated with the *VdFTF1* gene-deletion strains at 21 days post-inoculation. Two-week-old seedlings of susceptible cotton (*Gossypium hirsutum* L. 'Junmian No. 1') were inoculated with sterile water (Mock), wild-type *V. dahliae* (WT) and the three independent *VdFTF1* gene-deletion strains (Δ*FTF1–1*, Δ*FTF1–2* and Δ*FTF1*-3). (B) The fungal biomass of the *VdFTF1* gene-deletion strain in cotton plants was determined via quantitative polymerase chain reaction (qPCR). Error bars represent the standard deviation, and a double asterisk indicates the statistical significance ($P \leq 0.01$) in the gene-deletion strains compared with WT using an unpaired Student's *t*-test. (C) The disease symptom phenotypes of cotton seedlings inoculated with the complemented strains with re-introduced *VdFTF1* observed at 21 days post-inoculation. EC^{Ko}-1 and EC^{Ko}-2 are two independent ectopic transformants of Δ*FTF1–1* strains. (D) The fungal biomass of the two independent complemented strains determined using qPCR. Error bars represent the standard deviations and different letters indicate significant differences at $P < 0.01$ as determined using an unpaired Student's *t*-test. (E) The disease symptom phenotypes of cotton seedlings inoculated with ectopic transformants with re-introduction of *VdFTF1* into the T-DNA insertion mutant (M06D06) at 21 days post-inoculation. EC^{Mu}-1 and EC^{Mu}-2 are two independent ectopic transformants of the M06D06 mutant. (F) qPCR quantification of fungal biomass from 100 mg of ground total plant tissue in two independent ectopic transformants (EC^{Mu}-1 and EC^{Mu}-2) on cotton. Error bars represent the standard deviation and different letters indicate significant differences at $P < 0.01$ as calculated using an unpaired Student's *t*-test.

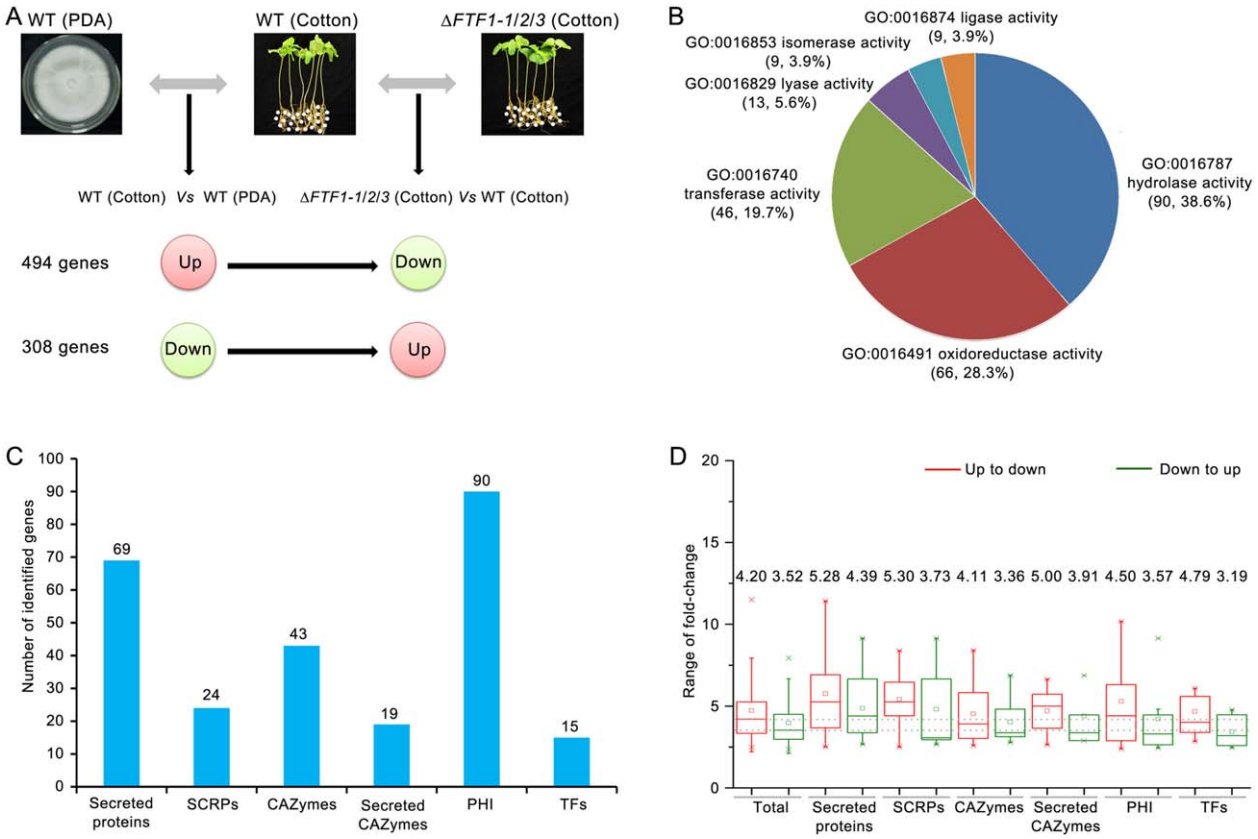

Fig. 6 VdFTF1 modulates the expression of putative pathogenesis-related genes during cotton infection. (A) Transcription (RNA-sequencing, RNA-seq) data for *VdFTF1*-targeted gene-deletion strains during infection of cotton roots compared with the wild-type (WT) strain Vd991. The roots of 2-week-old seedlings of susceptible cotton (*Gossypium hirsutum* L. 'Junmian No. 1') were treated with a high-concentration conidial suspension (2×10^9 conidia/mL), and the samples were collected 48 h after treatment. The fungus was cultured on potato dextrose agar (PDA) as a control. The *Verticillium dahliae* gene expression for the WT (Vd991) during cotton infection was compared with the control: 'WT (Cotton) vs. WT (PDA)'; gene expression associated with *VdFTF1* was determined by comparing the expression data for the *VdFTF1*-targeted gene-deletion strains during cotton infection with the WT during cotton infection: 'Δ*FTF1–1/2*/3 (Cotton) vs. WT (cotton)'; $|\log_2 \text{Ratio}| \geq 1.0$, false discovery rate (FDR) < 0.001 and $P < 0.001$. Genes regulated by *VdFTF1* were identified based on an inverted expression pattern (up to down or down to up). (B) Gene ontology (GO) annotation indicates that *VdFTF1* regulates genes in the catalytic class (GO:0003824). The numbers in parentheses represent the number of genes and ratio compared with the catalytic class. (C) Bioinformatics analysis of potential pathogenicity-related genes regulated by VdFTF1. SCRPs, small cysteine-rich proteins; CAZymes, carbohydrate-active enzymes; secreted CAZymes, CAZymes further predicted as secreted proteins; PHI, pathogen–host interaction-related proteins; TFs, transcription factors. (D) Fold-change range of the pathogenicity-related genes regulated by VdFTF1. The range of the fold-change represents the comparison of WT strain infection on cotton with normal growth on PDA medium, as revealed by the box and whisker plot. The value above the box is the median value of the indicated gene catalogue statistic. In each box plot, the central point represents the median, the rectangle provides the interval between the 25% and 75% percentiles, the whisker indicates the range and the coloured cross (×) represents an extreme outlier.

regulates the gene expression of multiple pathogenicity-related genes during cotton infection.

VdFTF1 controls the expression of genes potentially encoding secreted proteins involved in plant cell wall degradation

Gene expression profile comparisons between the *VdFTF1*-deletion strains and wild-type strain Vd991 suggested that VdFTF1 probably controls the expression level of secreted proteins that participate in pathogenesis (Fig. 6D; Table S2). Among the proteins regulated by VdFTF1 were the previously characterized effectors VdNLP1 and VdIsc1 (Liu *et al.*, 2014; Santhanam *et al.*, 2013). Expression of the cytotoxic necrosis- and ethylene-inducing-like proteins (VdNLP1 and VEDA_07751, respectively), as well as the virulence factor isochorismatase-like protein (VEDA_05301, which belongs to the same family as VdIsc1), displayed lower levels in the *VdFTF1* deletion mutant relative to the wild-type during cotton infection (Table S2).

Interestingly, 19 genes that were regulated by VdFTF1-encoded secreted proteins contained CAZyme modules, which generally function in plant cell wall degradation, including

modules with auxiliary (AA2, AA3 and AA9), glycoside hydrolase (GH3, GH5 and GH28) and polysaccharide lyase (PL1) activities (Fig. 7A). Among these genes, 15 were up-regulated in the wild-type strain during infection of cotton, but down-regulated in *VdFTF1* deletion mutants under the same condition (Fig. 7A). The *in vitro* assay revealed several VdFTF1-regulated genes that were positively activated during growth on carboxymethylcellulose (CMC) sodium or pectin-containing medium and that were suppressed after deletion of *VdFTF1* (Fig. 7B). This finding suggests that secreted CAZymes regulated by VdFTF1 play important roles in plant cell wall degradation during infection of cotton.

The transcription pattern of putative cell wall-degrading enzyme genes under VdFTF1 regulation is consistent with a role in virulence

To provide further evidence that secreted CAZyme genes regulated by VdFTF1 might participate in pathogenesis, the expression patterns of these genes were surveyed in the conidial suspension treated with cotton roots two days after inoculation. qRT-PCR analysis revealed that at least nine genes were positively regulated in the conidial suspension of the wild-type strain collected from inoculated cotton roots, including genes in the AA family (VEDA_07232, VEDA_05900, VEDA_03732 and VEDA_05775), carbohydrate esterase family (VEDA_09412), GH family (VEDA_04217 and VEDA_09568) and PL1 family (VEDA_05925 and VEDA_09651) (Fig. 8A). Expression of these genes was suppressed in the *VdFTF1*-deletion strain compared with the wild-type strain, and the transcription level was recovered after re-introduction of *VdFTF1* into the deletion strain (Fig. 8A).

Functional disruption of putative secreted CAZyme VEDA_09651, regulated by VdFTF1, results in decreased virulence on cotton

The notion that VdFTF1 regulates the secretion of CAZymes that participate in pathogenicity was supported by experiments with the gene VEDA_09651. VEDA_09651, which was identified as being regulated by VdFTF1 (Table S2), is predicted to encode a protein containing a PL1 domain, which generally participates in pectin degradation (Fig. 7A). In the wild-type, VEDA_09651 expression was up-regulated in CMC sodium medium, in pectin-containing medium and in the conidial suspension collected from inoculated cotton roots (Figs 7B and 8A). In the wild-type, VEDA_09651 expression was significantly up-regulated *in planta* at 2 or 3 days after inoculation (Fig. 8B). Targeted gene-deletion experiments confirmed that VEDA_09651 was required for full virulence on cotton, as disease symptoms in plants inoculated with mutants lacking this gene were less severe compared with plants inoculated with the wild-type strain 3 weeks after inoculation (Fig. 8C). Fungal biomass accumulation of VEDA_09651 deletion mutants *in planta* detected by qPCR was less than 40% of the wild-type treatment (Fig. 8D), supporting the hypothesis that VdFTF1 plays an important role in the regulation of secreted CAZymes, which are required for full virulence in cotton.

DISCUSSION

In this study, we identified a T-DNA insertion mutant that exhibited significantly reduced virulence towards cotton. This mutant carried a T-DNA-tagged gene, *VdFTF1*, encoding a protein containing a Fungal_trans domain. Transient expression in onion epidermal cells indicated that the VdFTF1 protein was localized exclusively to the nucleus. Verticillium wilt symptoms on cotton plants inoculated with targeted gene-deletion mutants of *VdFTF1* were significantly reduced, and the virulence was restored after the re-introduction of *VdFTF1*. A comparison of wild-type and targeted gene-deletion strain global transcription patterns revealed that VdFTF1 regulated secreted protein genes at the transcript level, especially proteins involved in plant cell wall degradation, a portion of which were required for full virulence on cotton. The results illustrate that the *V. dahliae* TF *VdFTF1* affects the pathogenesis of cotton by regulating enzymes that participate in plant cell wall degradation.

The Fungal_trans domain (Interpro ID, IPR007219; Pfam ID, PF04082) is found in a number of fungal TFs that regulate a variety of cellular and metabolic processes. In general, proteins containing Fungal_trans are accompanied by a Zn(2)-C6 fungal-type DNA-binding domain (Interpro ID, IPR007219; more than 32 000 proteins) that mediates DNA binding (Cazelle *et al.*, 1998; Cho *et al.*, 2012; Chung *et al.*, 2013; Johnston, 1987). In *V. dahliae*, the gene *Vdpf* also encodes a protein containing a Zn(2)-C6 fungal-type DNA-binding domain and a Fungal_trans domain (Luo *et al.*, 2016). VdFTF1 contains a single Fungal_trans domain, but lacks the known DNA-binding domain (Fig. 2B). An investigation of the domain architectures in the Interpro database revealed that proteins encoding a single Fungal_trans domain are widespread in fungi (more than 16 000 proteins) (Jones *et al.*, 2014). The *V. dahliae* Vd991 genome, in particular, encodes at least 55 proteins that contain a single Fungal_trans domain (data not shown). Very few reports have assessed the function of proteins carrying only the Fungal_trans domain, excluding CefR, which participates in the regulation of secondary metabolism in *Acremonium chrysogenum* (Teijeira *et al.*, 2011). This type of single Fungal_trans domain-containing protein appears to lack a DNA-binding domain that acts as a coactivator, and thus it probably regulates gene expression independently of other transcription complexes or TFs that contain a DNA-binding domain (Näär *et al.*, 2001). However, we cannot exclude the possibility that the flanking sequence of the Fungal_trans domain could be involved in DNA binding.

TFs control the rate of transcription of genetic information from DNA to messenger RNA by binding to a specific DNA sequence (Karin, 1990), and thus nuclear localization is necessary

A

Gene ID	CAZymes	WT-WTI	MT1I-WTI	MT2I-WTI	MT3I-WTI
VEDA_07232	AA2	3.57	-2.42	-2.98	-2.77
VEDA_07933	AA2	1.03	-1.67	-1.30	-2.33
VEDA_05900	AA3	3.59	-1.73	-1.87	-1.06
VEDA_07021	AA3	1.54	-2.89	-2.24	-1.19
VEDA_03732	AA8	1.59	-2.44	-2.85	-1.66
VEDA_08578	AA9	2.49	-3.56	-3.56	-2.60
VEDA_05776	AA9, CBM1	3.52	-1.74	-2.63	-1.13
VEDA_04578	CE10	-1.63	1.75	2.42	1.03
VEDA_07242	CE10	3.62	-1.63	-1.75	-1.50
VEDA_09412	CE8	2.71	-1.56	-2.23	-1.02
VEDA_04217	GH18	4.42	-2.42	-3.20	-1.00
VEDA_01417	GH28	-1.51	1.42	1.33	1.36
VEDA_09607	GH28	-2.78	1.44	2.38	1.21
VEDA_09608	GH28	-4.22	2.41	3.05	2.52
VEDA_09568	GH3	1.75	-1.73	-1.96	-2.05
VEDA_04966	GH5	1.27	-1.42	-1.21	-1.48
VEDA_02441	GH55	1.06	-2.37	-3.09	-2.22
VEDA_05925	PL1	4.35	-1.78	-3.14	-1.41
VEDA_09651	PL1	1.99	-2.08	-3.77	-3.17

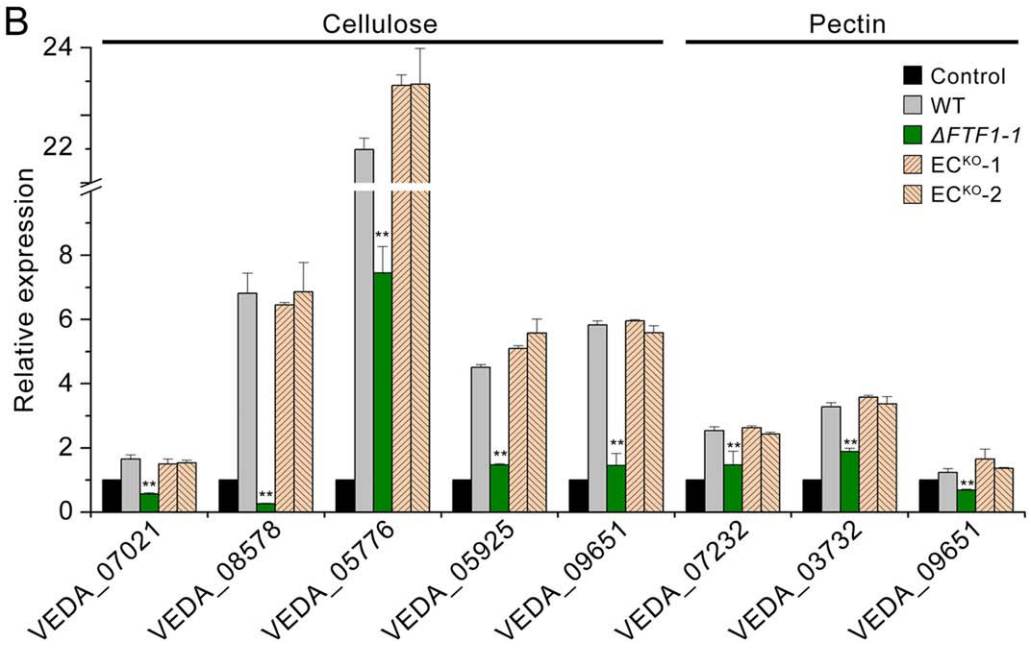

Fig. 7 Expression analysis of genes encoding secreted carbohydrate-active enzymes (CAZymes) that are regulated by VdFTF1. (A) Expression pattern of genes encoding secreted CAZymes by RNA-sequencing (RNA-seq) analysis. WTI-WT represents the differentially expressed genes of the wild-type strain during cotton infection compared with the fungus cultured on potato dextrose agar (PDA) medium. MT1I/MT2I/MT3I-WTI represents the differentially expressed genes of the *VdFTF1*-targeted gene-deletion strain during cotton infection compared with the wild-type strain during cotton infection. Coloured blocks from red to green represent up-regulated (five-fold change) to down-regulated (minus five-fold change) genes; the fold change is the original value of the $\log_{10}|\text{Ratio}|$, false discovery rate (FDR) < 0.001 and $P < 0.001$. (B) VdFTF1 regulates the expression of secreted CAZyme genes in cellulose and pectin-containing medium. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to measure gene expression levels in wild-type *Verticillium dahliae* cultured on normal PDA medium, and the wild-type *V. dahliae* (WT), *VdFTF1*-deletion mutant (Δ*FTF1–1*) and two complemented strains (EC^{Ko}-1 and EC^{Ko}-2) grown for 3 days in PDA medium substituting sucrose with carboxymethylcellulose sodium or pectin. The housekeeping gene *β-tubulin* (VDAG_10074) was used as an endogenous control. Error bars represent the standard deviation of three replicate experiments, and a double asterisk indicates the statistical significance ($P \leq 0.01$) of the gene transcription level in the gene-deletion strain compared with WT using an unpaired Student's *t*-test.

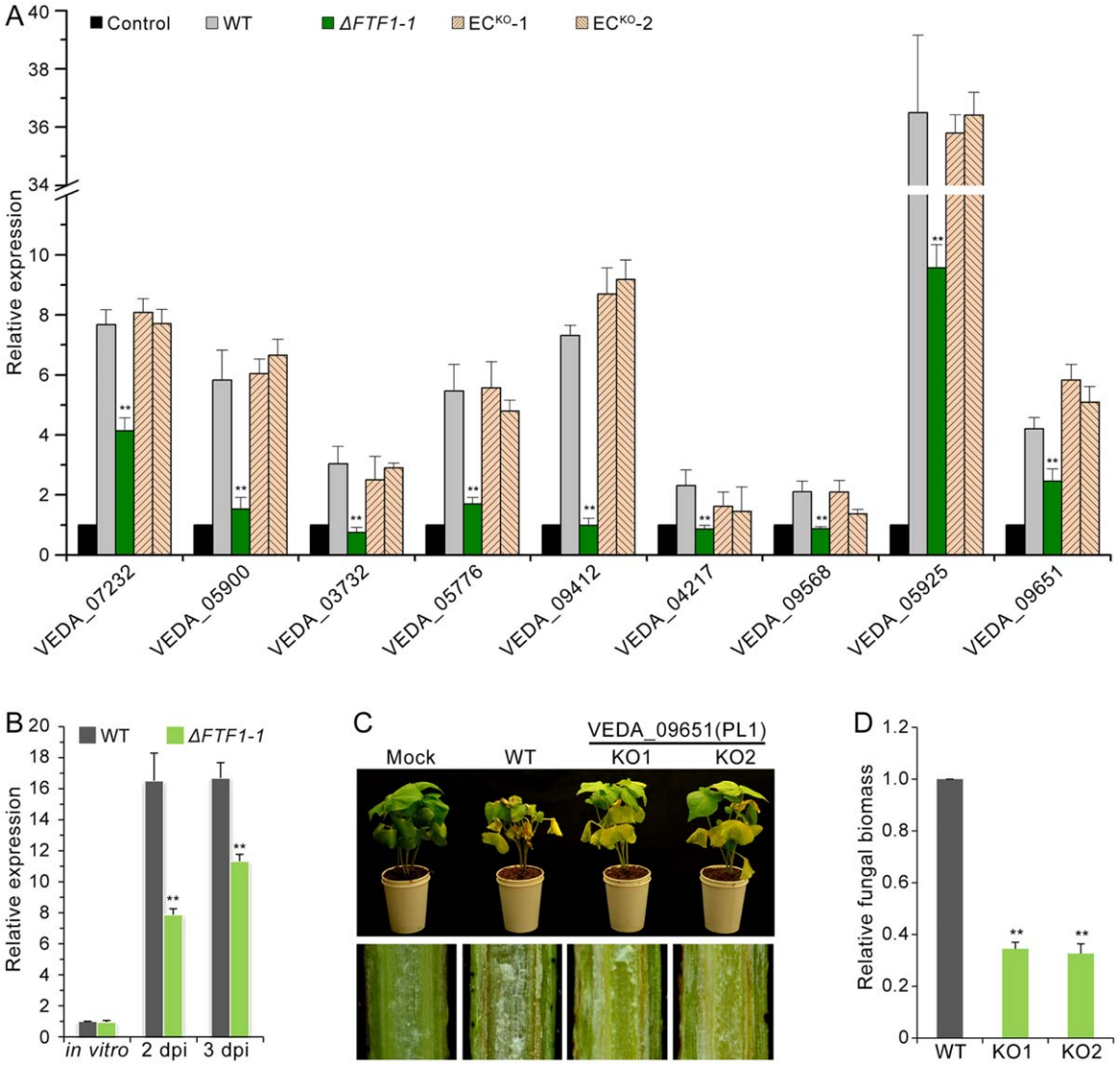

Fig. 8 Identification of the pathogenicity function of VdFTF1-regulated genes involved in pectin and cellulose degradation. (A) Analysis of the expression of pectin and cellulose degradation-associated genes regulated by VdTF1 during *Verticillium dahliae* infection of cotton roots. The infection stage conidial samples were collected 2 days after the cotton roots were treated with a suspension of 10^6 conidia/mL, including the wild-type (WT) strain, *VdFTF1*-deletion mutant (Δ*FTF1–1*) and the two complemented strains (EC^{Ko}-1 and EC^{Ko}-2). The WT strain conidia grown on potato dextrose agar (PDA) served as the control. The housekeeping gene *β-tubulin* (VDAG_10074) was used as an endogenous control. Error bars indicate the standard error, and a double asterisk indicates the statistical significance ($P \leq 0.01$) of the gene transcription level in the gene-deletion strain compared with WT using an unpaired Student's *t*-test. (B) Analysis of the gene expression of VEDA_09651 in the WT strain and the *VdFTF1*-deletion mutant (Δ*FTF1–1*) during cotton infection. The infected samples of cotton roots were collected at 2 or 3 days after inoculation with the WT and the *VdFTF1*-deletion strain, and the conidial suspension collected from inoculated cotton roots was used as the *in vitro* assay. The housekeeping gene *β-tubulin* (VDAG_10074) was used as an endogenous control. Error bars indicate the standard error, and a double asterisk indicates the statistical significance ($P \leq 0.01$) of the gene transcription level in the gene-deletion strain compared with WT using an unpaired Student's *t*-test. (C) Phenotypes of cotton seedlings and vascular discoloration in plants inoculated with the gene-deletion strains of *VEDA_09651* regulated by VdFTF1. Two-week-old seedlings of susceptible cotton, *Gossypium hirsutum* L. 'Junmian No. 1', were inoculated with sterile water (Mock), WT *V. dahliae* (WT) and the two independent gene-deletion strains of *VEDA_09651* (KO1 and KO2). The Verticillium wilt phenotype of wilting and vascular discoloration were investigated at 3 weeks after inoculation. (D) Quantitative polymerase chain reaction (qPCR) quantification of fungal biomass development of the indicated gene-deletion strains on cotton. Error bars represent the standard error, and a double asterisk indicates the statistical significance ($P \leq 0.01$) of the gene-deletion strains (KO1 and KO2) compared with WT using an unpaired Student's *t*-test.

for their function. Generally, TFs that are active in the nucleus contain a nuclear localization signal (NLS) that directs them to the nucleus. NLSs are classified into one of at least three different classes, including a single short stretch of basic amino acids, two stretches of basic amino acids and the presence of charged/polar residues interspersed with non-polar residues. All three classes of NLS have been shown to be recognized by the importin-α/β heterodimer as the first step of nuclear transport (Chan and Jans, 2002). In VdFTF1, no clear NLS was predicted by WoLF-PSORT (Horton *et al.*, 2007), although the NLS in VdFTF1 contained charged/polar residues interspersed with non-polar residue sequences. The accumulation of VdFTF1 in the nucleus was observed via transient expression in onion epidermal cells (Fig. 3), indicating that VdFTF1 had entered the nucleus. Furthermore, RNA-seq analysis revealed that VdFTF1 affects global transcription patterns (Table S2). Together, these results suggest that VdFTF1, which contains only a Fungal_trans domain, is a TF in *V. dahliae*.

TFs are vital for cellular processes via the regulation of gene expression. In fungi, a number of TFs that regulate important processes, such as pathogenicity and virulence, vegetative growth, conidial production, colony morphology and secondary metabolism, have been characterized, and some have been found to be involved in the regulation of multiple processes (Chen *et al.*, 2015; Cho *et al.*, 2012; Luo *et al.*, 2016; Okmen *et al.*, 2014; Wang *et al.*, 2011; Zhao *et al.*, 2011). In *V. dahliae*, TFs such as VdSge1 also exhibit pleiotropic effects, including the requirement for radial growth, conidial production and pathogenicity (Santhanam and Thomma, 2013). Vdpf regulates melanized microsclerotia formation, conidial production and pathogenicity (Luo *et al.*, 2016), and Vst1 controls melanin biosynthesis, morphogenesis and secondary metabolism (Sarmiento-Villamil *et al.*, 2016). In contrast, the phenotypes of all *VdFTF1*-deletion strains were similar to that of the wild-type strain during cultivation on PDA medium, and they also exhibited similar conidial morphology and production (Fig. 3). It is not known whether VdFTF1 controls melanin biosynthesis because the wild-type strain Vd991 does not produce melanin. In addition, the transcript levels of key melanin biosynthesis-related genes, transcriptional regulator (Pig1/Cmr1 homologue VDAG_00194, orthologue is VEDA_01968 in Vd991) and tetrahydroxynaphthalene reductase (T3HR of VDAG_00183 and T4HR of VDAG_03665, orthologues are VEDA_01977 and VEDA_06970 in Vd991, respectively) (Fan *et al.*, 2016), were not affected by the loss of VdFTF1 in the current study. These results suggest that VdFTF1 is very likely to be associated exclusively with pathogenesis, but is dispensable for fungal development.

In *Verticillium* spp., the exoproteome and its role in plant cell wall degradation have gained much attention (Bidochka *et al.*, 1999; Cooper and Wood, 1973; Dobinson *et al.*, 1997, 2004; Huang and Mahoney, 1999; Wang and Keen, 1970). A comparative genomics study has reported that the *V. dahliae* genome encodes a diverse arsenal of CAZymes, particularly pectinases and cellulases, to adapt the components of the plant cell wall (Klosterman *et al.*, 2011), which mainly consists of cellulose microfibrils embedded in a matrix of hemicelluloses, pectic polysaccharides and glycoproteins (Carpita and Gibeaut, 1993). Characterization of the *V. dahliae* exoproteome revealed that at least 52 proteins participate in pectin and cellulose degradation pathways (Chen *et al.*, 2016), further confirming that the *V. dahliae* exoproteome plays a crucial role in the development of Verticillium wilt via the pathogenic mechanisms of plant cell wall degradation. *Verticillium* spp. employ many carbohydrate enzymes that function synergistically (Chen *et al.*, 2016; Fradin and Thomma, 2006; Klosterman *et al.*, 2011). However, few regulators have been reported to participate in pathogenesis by regulating the gene expression of plant cell wall degradation enzymes, excluding the sucrose non-fermenting protein kinase *VdSNF1* (Tzima *et al.*, 2011). In this study, we found that VdFTF1 has the ability to regulate the expression of carbohydrate enzyme genes (Fig. 6C), nine of which have been identified in the exproteome to play a crucial role in the development of wilting symptoms (Chen *et al.*, 2016). For example, target gene deletion of PL1, involved in pectin degradation, resulted in a significant reduction in virulence towards cotton (Fig. 8).

As our understanding of the pathogenicity of *V. dahliae* increases, it appears that a variety of genes are required for virulence towards host plants, including protein kinases (Rauyaree *et al.*, 2005; Tian *et al.*, 2014; Tzima *et al.*, 2010, 2011, 2012), TFs (Luo *et al.*, 2016; Santhanam *et al.*, 2013; Sarmiento-Villamil *et al.*, 2016; Tran *et al.*, 2014; Xiong *et al.*, 2015), effectors (de Jonge *et al.*, 2012, 2013; Liu *et al.*, 2014; Santhanam *et al.*, 2013), enzymes involved in plant cell wall degradation (Chen *et al.*, 2016; Liu *et al.*, 2013; Maruthachalam *et al.*, 2011) and other functional genes (Gao *et al.*, 2010; Klimes and Dobinson, 2006; Li *et al.*, 2015; Maruthachalam *et al.*, 2011; Santhanam *et al.*, 2017; Zhang DD *et al.*, 2016; Zhang YL *et al.*, 2015, 2016). In this study, we identified a new pathogenicity and virulence-related gene encoding a Fungal_trans domain-containing TF (Figs 4 and 5). Based on the general role of TFs, it can be anticipated that TFs affect pathogenesis by regulating global transcription patterns; this claim has been validated for several pathogens (Alkan *et al.*, 2013; Cho *et al.*, 2012; Ridenour and Bluhm, 2017; Tran *et al.*, 2014; Yan *et al.*, 2011). A comparison of the global transcription patterns revealed that *VdFTF1* regulates global transcription in *V. dahliae*, thus demonstrating that a large number of genes are affected after *VdFTF1* gene deletion (Table S1). The function of VdFTF1 in pathogenesis appears to be primarily related to the regulation of genes involved in catalytic processes (233 genes), especially proteins that are associated with hydrolase and oxidoreductase activity (Fig. 6B), which are important for pathogenesis in *V. dahliae* (Sarmiento-Villamil *et al.*, 2016; Tran *et al.*,

2014; Tzima *et al.*, 2012). The importance of effectors in interactions between host plants and *V. dahliae* has been established previously (Santhanam *et al.*, 2013; Sarmiento-Villamil *et al.*, 2016; Tran *et al.*, 2014). In the current study, the expression of potential effector genes was affected in the *VdFTF1* gene-deletion strains, including 14 SCRPs (Fig. 6C). VdFTF1 regulation of effector genes is supported by observations obtained for VdNLP1 (Table S2). In addition, five genes encoding protein kinases and 15 genes encoding TFs were affected by VdFTF1 (Table S1). Because Vdpf has the ability to regulate the protein kinase VdPKAC1 and the TF VdSge1 (Luo *et al.*, 2016), the current study provides evidence for interconnections among signalling factors that regulate pathogenicity in *V. dahliae*.

In summary, we conclude that *VdFTF1*, which encodes a TF containing a Fungal_trans domain, is crucial for the global regulation of multiple virulence factors, especially plant cell wall degradation enzymes critical for the full virulence of *V. dahliae* in cotton.

EXPERIMENTAL PROCEDURES

Identification of the T-DNA-tagged gene

The T-DNA mutant (M06D06) was cultured on complete medium (CM) with hygromycin B (HygB) at 25 °C for 5 days, and the mycelium was then harvested for genomic DNA isolation. The flanking sequences of T-DNA were identified using a hiTAIL-PCR method with degenerate primers (Table S3, see Supporting Information), as described previously (Liu and Chen, 2007). All amplicons were cloned, sequenced and then aligned to the reference genome of Vd991 (data not shown) using the BLASTN program (Altschul *et al.*, 1997) to ascertain the physical location of the T-DNA in the genome. To clone the T-DNA-tagged gene in order to assess the presence of introns, a 5×10^6 conidial suspension of the *V. dahliae* wild-type strain was collected from cotton roots 24 hours after inoculation for total RNA extraction (for details, see section on 'RNA-seq and gene expression profile analysis' below). cDNA was synthesized using the FastQuant cDNA Reverse Transcriptase Kit (TianGen, Beijing, China). The T-DNA-tagged gene was cloned using RT-PCR with cDNA and PCR with genomic DNA, and the protein-coding genes were then identified using CLUSTAL X with transcript and genomic sequence information (Larkin *et al.*, 2007). All gene-specific primers designed for gene cloning are listed in Table S3. Conserved domain and functional homology searches were performed using the SMART software package (Letunic *et al.*, 2015). The *VdFTF1* homologous genes in other fungi were analysed using the BLASTP program, and sequence alignment of the Fungal_trans domain was constructed using the CLUSTAL X software package (Larkin *et al.*, 2007).

Fungal transformation

To generate *VdFTF1* deletion constructs, the flanking sequences of *VdFTF1* coding sequences were amplified from the Vd991 genomic DNA and integrated with the hygromycin cassette using fusion PCR, as described previously (Liu *et al.*, 2013). The amplified products were then cloned into the pGKO2-gateway vector (Khang *et al.*, 2005). To generate the complementation transformants, a 3925-bp *Hin*dIII/*Xba*I fragment containing the VdFTF1 coding sequence, with sequences of 1505 bp upstream and 743 bp downstream, were amplified from *V. dahliae* strain Vd991 genomic DNA and cloned into the binary vector pCOM that carried the geneticin resistance (Zhou *et al.*, 2013). ATMT of *V. dahliae* was conducted as described by Liu *et al.* (2013), and the transformants were selected on PDA (potato, 200 g/L; glucose, 20 g/L; agar, 15 g/L) supplemented with hygromycin or geneticin at 50 μg/mL for gene deletion or complementation, respectively. Homologous recombination in the deletion mutants was verified by PCR with the internal and flanking primers of *VdFTF1* (Tables S3). Targeted gene deletion of the *VdFTF1* regulated gene (VEDA_09651) was performed as described above.

Growth and conidiogenesis assays

A conidial suspension (2×10^6 conidial/mL) of *V. dahliae* strains was prepared for phenotypic analysis. For the growth assay, 2 μL of the conidial suspension were placed in the centre of a 9-cm-diameter PDA plate prior to incubation at 25 °C. The colony diameter was measured at 2-day intervals, and the fungal growth phenotypes were investigated 14 days after incubation. To evaluate the production of conidia by different mutants, agar plugs were collected from the edge of the fungal colony 9 days after incubation using a 5-mm-diameter cork borer, and the quantity of conidia was determined using a haemocytometer after the agar plugs had been shaken in 1 mL of sterile water. Three biological experiments were conducted in triplicate for each genotype. The shapes (minor and major axes) of the *V. dahliae* conidia were measured using a microscope, with three biological experiments consisting of 16 conidia per genotype.

Pathogenicity assays

Pathogenicity assays were performed on cotton seedlings using the root-dipping method, as described previously (Zhang DD *et al.*, 2016). Briefly, susceptible cotton plants (*G. hirsutum* cv. 'Junmian No. 1') were grown and maintained in a glasshouse at 28 °C under a 14-h light/10-h dark photoperiod until the development of the first euphylla. Seedlings were gently uprooted, washed and dipped into 15 mL of 5×10^6 conidia/mL suspensions prepared in sterile water for 5 min, and then transplanted. Six pots of cotton seedlings with three replicates were prepared for each transformant. Seedlings were then grown and maintained at 28 °C under a 14-h light/10-h dark photoperiod, and the disease symptoms were assessed 3 weeks after inoculation. For fungal biomass quantification, the roots of three plants were harvested at 21 days after inoculation and ground to a powder for genomic DNA extraction. The fungal biomass was determined by qPCR as described previously (Santhanam *et al.*, 2013). *Verticillium* elongation factor 1-α (*EF-1α*) was used to quantify fungal colonization, and the cotton *18S* gene served as an endogenous plant control.

RNA-seq and gene expression profile analysis

The wild-type strain Vd991 and three Δ*VdFTF1–1/2/3* mutants were cultured in a shaking incubator in CM at 25 °C for 5 days. The mycelium was filtered out with four layers of gauze, and the conidia were collected by centrifugation at 6000 ***g*** at 25 °C for 20 min. The conidia were resuspended in sterile distilled water and adjusted to 2×10^9 conidia/mL. Thirty two-week-old cotton seedlings were immersed in 100 mL of

conidial suspension for 5 min, carefully removed to ensure that the roots were covered with a sufficient number of conidia, and transferred into a sterilized glass box to maintain the humidity for 2 days. The inoculated plants were washed with 50 mL of sterile distilled water to collect the conidia covering the roots 1 day after inoculation, and activated infection samples were obtained from the conidial eluent via centrifugation. The conidial suspension washed from a PDA plate was harvested as a control sample. Total RNA was isolated for RNA-seq with an Illumina Hiseq 2000 (San Diego, CA, USA) according to standard Illumina protocols. The SOAP-aligner/SOAP2.0 software package (Li *et al.*, 2009) was used to map the reads to the reference sequence of Vd991 (genome unpublished), with less than two mismatches allowed in the alignment. The uniquely mapped read counts were normalized and the expression levels were calculated using the reads per kilobase per million mapped reads (RPKM) method (Mortazavi *et al.*, 2008). A strict algorithm was used to identify significantly differentially expressed genes between the control and wild-type, and between the wild-type and Δ*VdFTF1–1/2/3* mutants. The FDR was set to 0.001 to determine the threshold $P < 0.001$ in multiple tests, and the absolute value of $|\log_2\text{Ratio}| \geq 1.0$ (Audic and Claverie, 1997).

Bioinformatics analysis of VdFTF1-regulated genes

Of the VdFTF1-regulated genes, secreted proteins were predicted as described previously (Chen *et al.*, 2016; Klosterman *et al.*, 2011). Proteins that were less than 400 amino acids long and contained up to four cysteine residues were designated as SCRPs. The annotation of putative CAZymes was performed using the Hidden Markov Model (HMM)-based routine of the Carbohydrate-Active-EnZymes database (Cantarel *et al.*, 2009). CAZymes involved in plant cell wall degradation were collected according to the classification methods described in previous publications (Battaglia *et al.*, 2011; Goodwin *et al.*, 2011). Homologues of known pathogenicity-related genes were predicted using the PHI database (Version 3.6, http://www.phi-base.org/) (Winnenburg *et al.*, 2008). Putative TFs were identified based on InterPro annotations. Protein kinases were predicted by running HMM searches locally with Kinomer (Version 1.0) (Martin *et al.*, 2009).

Gene expression analysis under varying conditions

Differential expression of VdFTF1-regulated genes under different conditions was assessed using six different treatments: (1) basic Czapek-Dox agar plates containing 30 g/L sucrose; (2) Czapek-Dox plates containing 10 g/L CMC sodium (Cat# C9481, Sigma-Aldrich); (3) Czapek-Dox plates containing 10 g/L pectin (Cat# P8471, Sigma-Aldrich); (4) 24 h of growth on cotton roots after root-dipping; (5) 2 or 3 days of growth on cotton roots after root-dipping; and (6) PDA. Four isolates were used in the experiments: the wild-type strain (WT), a *VdFTF1*-deletion mutant (Δ*FTF1–1*) and two complemented strains (EC^{Ko}-1 and EC^{Ko}-2). Strains were grown for 2 days on Czapek-Dox treatments. The collection of conidia covering the cotton roots 24 h after root-dipping was performed as described above for RNA-seq. For the remaining two treatments, conidia were collected from cotton roots at 2 or 3 days post-inoculation after root-dipping, and a conidial suspension was washed from a PDA plate and harvested as a control.

After RNA extraction and cDNA synthesis, qRT-PCR was performed to identify the expression levels using FastFire qPCR premix (SYBR Green, TianGen), and the relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with β-tubulin (VDAG_10074 in VdLs.17) as an internal control. Real-time PCR conditions consisted of an initial denaturation step at 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min.

Subcellular localization analysis

To examine the subcellular localization of the VdFTF1 protein, the full-length VdFTF1 cDNA without a termination codon was amplified and introduced into the 5′ side of the GFP gene of the pRTL2 vector to generate the *35S::VdTF1:GFP* construct. The *CaMV35S::GFP* construct (empty vector) was used as the control. Onion epithelial cells were obtained by peeling the outer layers of sweet onion purchased locally. Approximately 2–3-cm^2 onion slices were placed on the surface of Murashige and Skoog (MS) medium agar plates (Sigma-Aldrich, Saint Louis, MO, USA) prepared with 0.5 × MS, pH 5.7 and 1% sucrose in 9-cm Petri dishes. DNA transformation was performed using microprojectile bombardment technology with the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA). Gold particles (1.0 μm) were used as DNA microcarriers; 5.0 μL of 1.0 μg/μL plasmid DNA containing *35S::VdTF1:GFP* or *CaMV35S::GFP* constructs were precipitated onto 50 μL (3 mg) of microcarriers in the presence of 2.5 M $CaCl_2$ and 0.1 mM spermidine, as described by the manufacturers. Subsequently, for each macrocarrier, 6.0-μL aliquots (approximately 500 μg) of the microcarriers were removed, spread evenly over the central 1 cm of the macrocarrier using a pipette tip and delivered via PDS-1000/He into the onion epithelial monolayer. The bombardment parameters were as follows: 71.12 cm of Hg in less than 30 s, a target distance of 6 cm and a helium pressure of 77.34 kg/cm^2. Following bombardment, the tissue slices were allowed to recover for 48 h at 28 °C in the dark. GFP expression of chimeric proteins was visualized using a laser scanning confocal microscope (LSM T-PMT) with excitation at 488 nm and emission at 510 nm.

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SUPPORTING INFORMATION

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Fig. S1 Analysis of T-DNA-tagged gene in the M06D06 mutant by thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR). (A) Schematic view of the hiTAIL-PCR method to determine the T-DNA flanking sequence in the M06D06 mutant. LB and RB represent the left and right borders of the T-DNA region; the selective marker gene *Hpt* (encodes hygromycin phosphotransferase) under the control of the *TrpC* promoter from *Aspergillus nidulans*. RB-1, RB-2 and RB-3 are the primers in the RB region. LAD1–LAD5 represent the degenerate primers designed from the *Verticillium dahliae* genomic sequence. (B) Detection of the genomic integration site of T-DNA in the mutant. The initial PCR was amplified by the mutant genomic DNA using nested PCR with the degenerate primers and RB-1; lane RB-2 represents the first-round nested PCR product using the degenerate primers and RB-2; lane RB-3 is the second-round PCR product using the degenerate primers and RB-3. M represents the DNA ladder. (C) Sequence result of TAIL-PCR amplicons and alignment to the reference genome Vd991. Three amplicons from the third round by the primer pairs LAD2/RB-3, LAD3/RB-3 and LAD5/RB-3 were sequenced, respectively. The alignment to the reference genome was performed using Clustal X2. Nucleic acids in blue represent the right border of the T-DNA region.

Fig. S2 Targeted deletion of *VdFTF1* in *Verticillium dahliae*. (A) Schematic view of the targeted deletion of *VdFTF1* using a homologous recombination method. The two flanking sequences of *VdFTF1*, upstream-flanking (UF) and downstream-flanking (DF), were amplified by the corresponding primer pairs P1/P3 and P4/P6, respectively. The hygromycin phosphotransferase gene (*Hpt*) was amplified by the Hpt-F and Hpt-R primer pair that contain the adaptor reverse complement to P3 and P4, respectively. The products of UF, *Hpt* and DF were fused using a fusion polymerase chain reaction (PCR) and confirmed with the primer pair P2/P6. The fusion amplicon was integrated into the pGKO2-gateway vector in a BP recombinant reaction, which contains the herpes simplex virus thiamine kinase gene (*HSVtk*) that acts as a toxic compound for negative selection against ectopic transformants. LB and RB indicate the left and right borders of the T-DNA sequence, respectively. The vectors were transferred into the *Agrobacterium tumefaciens* AGL-1 strain for transformation of the wild-type (WT) strain Vd991. Targeted gene deletion of *VdFTF1* was confirmed by PCR with three primer pairs. Primers Hpt-F/Hpt-R resulted in an amplicon in *VdFTF1* deletion stains, but not in the WT. Primers T1-F/T1-R resulted in an amplicon in the WT, but not in the *VdFTF1* deletion stains. Primers T2-F/T2-R resulted in amplicons of different sizes in WT and *VdFTF1* deletion stains. (B) Agarose gel electrophoresis of PCR products for fusion. M, 1-kb DNA ladder. (C) Agarose gel electrophoresis of the fusion amplicon for construction of the targeted gene deletion. (D) Detection of the positive targeted gene-deletion strains by detection of the *Hpt* fragment. WT, wild-type strain Vd991; KO1–KO3, three independent targeted deletion mutants of *VdFTF1*. (E) Detection of the positive targeted gene-deletion strains by targeting a *VdFTF1* internal fragment. (F) Detection of the positive targeted gene-deletion strains by using a flanking primer pair of *VdFTF1*.

Table S1 Differentially expressed genes affected by the VdFTF1 gene deletion compared with the wild-type.

Table S2 List of genes regulated by VdFTF1 identified by RNA-sequencing (RNA-seq).

Table S3 Primers used in this study.