



Transcription Factor VdCf2 Regulates Growth, Pathogenicity, and the Expression of a Putative Secondary Metabolism Gene Cluster in *Verticillium dahliae*

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ABSTRACT Transcription factors (TFs) bind to the promoters of target genes to regulate gene expression in response to different stimuli. The functions and regulatory mechanisms of transcription factors (TFs) in *Verticillium dahliae* are, however, still largely unclear. This study showed that a C2H2-type zinc finger TF, VdCf2 (*V. dahliae* chorion transcription factor 2), plays key roles in *V. dahliae* growth, melanin production, and virulence. Transcriptome sequencing analysis showed that VdCf2 was involved in the regulation of expression of genes encoding secreted proteins, pathogen-host interaction (PHI) homologs, TFs, and G protein-coupled receptors (GPCRs). Furthermore, VdCf2 positively regulated the expression of *VdPevD1* (*VDAG_02735*), a previously reported virulence factor. VdCf2 thus regulates the expression of several pathogenicity-related genes that also contribute to virulence in *V. dahliae*. VdCf2 also inhibited the transcription of the *Vd276-280* gene cluster and interacted with two members encoding proteins (VDAG_07276 and VDAG_07278) in the gene cluster.

IMPORTANCE Verticillium dahliae is an important soilborne phytopathogen which can ruinously attack numerous host plants and cause significant economic losses. Transcription factors (TFs) were reported to be involved in various biological processes, such as hyphal growth and virulence of pathogenic fungi. However, the functions and regulatory mechanisms of TFs in *V. dahliae* remain largely unclear. In this study, we identified a new transcription factor, VdCf2 (*V. dahliae* chorion transcription factor 2), based on previous transcriptome data, which participates in growth, melanin production, and virulence of *V. dahliae*. We provide evidence that VdCf2 regulates the expression of the pathogenicity-related gene *VdPevD1* (*VDAG_02735*) and *Vd276-280* gene cluster. VdCf2 also interacts with VDAG_07276 and VDAG_07278 in this gene cluster based on a yeast two-hybrid and bimolecular fluorescence complementation assay. These results revealed the regulatory mechanisms of a pivotal pathogenicity-related transcription factor, VdCf2 in *V. dahliae*.

KEYWORDS *Verticillium dahliae*, transcription factor, VdCf2, pathogenicity, transcriptome, gene cluster

Perticillium dahlae is a soilborne phytopathogen that causes vascular wilt disease in more than 200 plant species (1–5). The pathogen can survive in the soil by means of microsclerotia for more than 14 years (6). In response to root exudates released in the plant rhizosphere, microsclerotia germinate and produce hyphae, which then directly

Editor Ning-Yi Zhou, Shanghai Jiao Tong University Copyright © 2022 American Society for Microbiology. All Rights Reserved. Address correspondence to Xiaoping Hu, xphu@nwsuaf.edu.cn. The authors declare no conflict of interest. Received 17 August 2022 Accepted 7 October 2022 Published 7 November 2022 penetrate host roots to colonize the xylem vessels (2, 7, 8). With the progressive colonization of the xylem vessels, the host begins to show symptoms such as foliar wilting, chlorosis, plant stunting, and vascular browning (9, 10). With further disease progression, infected plants begin to senesce and wilt, and microsclerotia are formed in the necrotic tissue to serve as the primary inoculum for subsequent crops (9). The wilt disease caused by *V. dahliae* is a difficult disease to manage in commercial agriculture. A greater understanding of the mechanisms of pathogenesis and virulence in *V. dahliae* may provide knowledge for developing strategies for the management of *Verticillium* wilt.

Mechanisms of *V. dahliae* pathogenesis and virulence have been widely studied. Many genes that play important roles in pathogenicity and virulence, including those encoding secreted proteins, such as PevD1 (an Alt a 1-like protein) (11, 12), GH12 (glycoside hydro-lase 12) (13), VdSCP41 (effector) (14), and VdPEL1 (pectate lyase) (15), and genes encoding nonsecreted proteins, such as VGB (G protein β subunit) (16), VdMyo5 (myosin V protein) (17), VdNoxB/VdPIs1 (NADPH oxidases/tetraspanin) (18), Vst1 (APSES-type transcription factor [TF]) (19), and VdAtf1 (bZIP-type TF) (20), have been identified in *V. dahliae*. TFs not only play critical roles in pathogenicity and virulence in *V. dahliae*, but also could interact with other TFs (AtfA, AtfB, AtfC, AtfD) or molecular chaperones (Hsp70, Hdj1) (21, 22).

Based on the differences in the conserved DNA-binding domains, the 530 predicted TFs in the reference genome of *V. dahliae* strain VdLs.17 were classified into 42 types (23). Of these, the C2H2-type TF VdCrz1 and the MADS-box-type TF VdMcm1 are involved in fungal growth, melanin and microsclerotium formation, and virulence (24, 25). Furthermore, the Zn2Cys6-type TF VdFTF1, APSES-type TF Vst1, and bZIP-type TF VdAtf1 also play roles in virulence (19, 20, 26). When functions of specific TFs in other fungal pathogens were altered, their development and virulence to hosts were also significantly affected (27–29). In *Botrytis cinerea*, disruption of MADS-box TF Bcmads1 caused defects in growth and sclerotium production and full virulence on apple fruit (30). In *Magnaporthe oryzae*, deletion of bZIP TF MoAP1 led to defects in aerial hyphal growth and pathogenicity (31). Therefore, the disruption of several types of key TFs influences fungal development, pathogenicity, and virulence.

A total of 93 annotated C2H2-type TFs have been identified in the genome of V. dahliae strain VdLs.17 (23). However, only a few of such TFs, such as Vta2, VdCrz1, and VdMsn2, have been functionally characterized (24, 32, 33). The role of most C2H2-type TFs in virulence is largely unknown. According to our previous transcriptome data (unpublished), VdCf2 was significantly upregulated in the dormant microsclerotia, which encodes a C2H2-type chorion transcription factor in V. dahliae. And VdCf2 was found to be associated with virulence. Cf2 was first discovered in Drosophila melanogaster due to its ability to target various DNA sequences caused by its alternative splicing (34). Previous studies showed that the Drosophila TF Cf2 plays an important role in ensuring an appropriate myofibril size by regulating the transcription level of several muscle structural genes (35–37). Functions of chorion transcription factor Cf2 in phytopathogenic fungi have, however, not been studied yet. The present study showed that the VdCf2 deletion mutant impaired growth and virulence but increased melanin accumulation compared to that of the wild-type strain. Furthermore, transcriptomic analysis indicated that VdCf2 regulated the expression of several pathogenicityrelated genes, some of which also contributed to virulence in V. dahliae.

RESULTS

VdCf2 is localized to the nucleus. VdCf2 (VDAG_08721) in V. dahliae was annotated as a chorion transcription factor Cf2, and shared 34.33% identity with the Cf2 in D. melanogaster (see Fig. S1 in the supplemental material). Phylogenetic analysis showed high sequence identity with Cf2 in Verticillium homologs (Fig. 1A). It also revealed that VdCf2 was not a specifically conserved transcription factor in phytopathogenic fungi (Fig. 1A). The VdCf2 protein was predicted to possess three C2H2-type zinc finger domains in the middle and one coiled-coil region in the C terminus based on the SMART online tool (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1), as well as a nuclear



FIG 1 Sequence analysis and subcellular localization of VdCf2. (A) Dendrogram of VdCf2 and its homologues from *Verticillium longisporum*, *Verticillium nonalfalfae*, *Verticillium alfalfae*, *Colletotrichum siamense*, *Plectosphaerella cucumerina*, *M. oryzae*, *Fusarium oxysporum*, *Valsa mali*, *Puccinia striiformis*, *Ustilago maydis*, *N. crassa*, *Beauveria bassiana*, *Metarhizium anisopliae*, and *Saccharomyces cerevisiae*. The phylogenetic tree was constructed using MEGA 7.0 software based on the neighbor-joining method. The number of bootstrap replications was set as 1,000. (B) Schematic representation of the VdCf2 structure containing conserved C2H2 zinc finger domain and coiled-coil region according to the prediction from SMART as well as the nuclear localization signal predicted by cNLS Mapper. Multiple sequence alignment of the C2H2-type zinc finger domain of VdCf2 orthologs from *V. dahliae*, *Colletotrichum siamense*, *F. oxysporum*, and *N. crassa* was completed using MEGA 7.0 software and displayed using the multiple sequence comparison display online website. (C) The subcellular localization was performed in *V. dahliae* (first line, bars = 20 μ m) and tobacco (*N. benthamiana*) cells (last two lines, bars = 40 μ m). pBin-GFP was used as the control. (D) Western blot analysis of GFP and VdCf2-GFP fusion proteins in tobacco (*N. benthamiana*) cells.

localization signal based on cNLS Mapper (https://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS _Mapper_form.cgi) (Fig. 1B). Multiple sequence alignments showed that C2H2-type zinc finger domains of Cf2 orthologs from *V. dahliae, Colletotrichum siamense, Fusarium oxysporum,* and *Neurospora crassa* were highly conserved (Fig. 1B).

To investigate the subcellular localization of VdCf2 protein, green fluorescent protein (GFP)-tagged VdCf2 protein was expressed in both *V. dahliae* and *Nicotiana benthamiana* cells, and fluorescent signals were detected in the nucleus in both (Fig. 1C, Fig. S2). In contrast, florescent signals were diffused across the plant cell when free GFP was expressed (Fig. 1C). Western blots further verified that GFP and the VdCf2-GFP fusion proteins were expressed successfully in *N. benthamiana* cells (Fig. 1D). The conservation of C2H2-type zinc finger domain sequences of VdCf2 coupled with the nuclear localization of VdCf2-GFP in fungi and plant cells further suggested that *VdCf2* encodes a C2H2-type zinc finger TF because of its common function in nuclear compartmentalization.

VdCf2 is involved in growth but not in osmotic stress response. To determine the importance of *VdCf2* in *V. dahliae*, the Δ Vd*Cf2* strain was generated by homologous recombination and verified by PCR and Southern blot assays (Fig. 2A and B). To explore the role of *VdCf2* on growth, the wild-type strain XJ592 and the Δ Vd*Cf2* and Δ Vd*Cf2*/ *VdCf2* strains were cultured on potato dextrose agar (PDA), minimal medium (MM), and water agar (WA) and incubated for 14 days. Compared to strains XJ592 and



FIG 2 VdCf2 plays key roles in V. dahliae growth and melanin production. (A) The identification electrophoretogram of the $\Delta VdCf2$ and ΔVdCf2/VdCf2 strains. M, 2,000 bp marker; 1, ΔVdCf2 strain; 2, ΔVdCf2/VdCf2 strain; 3, wild-type strain; 4, negative control (sterile water). (B) Confirmation of VdCf2 deletion by Southern blot analysis. Total genomic DNA of the wild-type and Δ VdCf2 strains was digested with BamHI and subjected to Southern blot analysis. The probe in the VdCf2 gene region amplified with primer pair NF/NR was used to confirm the presence or absence of the VdCf2 gene in the wild-type and Δ VdCf2 strains. The probe in the hygromycin B resistance gene region amplified by primer pair HYG-F/HYG-R was used to verify the copy number of the hygromycin B resistance gene in the wild-type and Δ VdCf2 strains. (C) Colony morphology of the XJ592, $\Delta VdCf2$, and $\Delta VdCf2/VdCf2$ strains on PDA, MM, and WA plates. Pictures were taken from the upper side of 14-day-old cultures. (D) Colony diameter of the XJ592, $\Delta VdCf2$, and $\Delta VdCf2/VdCf2$ strains on three media. Error bars are standard errors calculated from three replicates, and different letters represent statistically significant differences (P = 0.05). The SNK test was executed among each strain in each medium. (E) VdCf2 was not required for osmotic stress response. The inhibition rate of colony growth was quantified. Strains XJ592 and Δ VdCf2 were cultured on CM plates containing osmotic stress reagents (1.2 M sorbitol, 0.8 M KCl, or 0.4 M NaCl) for 14 days. Error bars indicate standard errors calculated from three replicates, and different letters represent the statistical significance (P = 0.05). Student's t test was executed between strains in each osmotic stress reagent. (F) VdCf2 participated in oxidative stress response. The inhibition zones of the XJ592 and $\Delta VdCf2$ strains were quantified. Error bars indicate standard errors calculated from three replicates (Student's t test, P = 0.05). (G) VdCf2 curtails melanin production. The conidial suspensions of strains XJ592, $\Delta VdCf2$, and $\Delta VdCf2/VdCf2$ were cultured on PDA and MM plates for 3 days and BMM plates for 4 days. The mycelia on the BMM plates were observed under a light microscope (bars = 20 μ m).

 $\Delta VdCf2/VdCf2$, the growth rate of strain $\Delta VdCf2$ was reduced by more than 30% on all three media (Fig. 2C and D), indicating that VdCf2 is critical for the growth of V. dahliae.

To explore whether *VdCf2* plays a role in the osmotic stress response, strains XJ592 and Δ Vd*Cf2* were cultured on complete medium (CM) containing 1.2 M sorbitol, 0.8 M KCl, or 0.4 M NaCl and incubated for 14 days. The growth inhibition of strain Δ Vd*Cf2* was indistinguishable from that of XJ592 (Fig. 2E), indicating that *VdCf2* did not respond to the osmotic stress induced by sorbitol, KCl, or NaCl. To evaluate the role of *VdCf2* in oxidative stress, the inhibition zone was measured on the CM containing H₂O₂. The result showed that strain Δ Vd*Cf2* exhibited smaller inhibition zones than the wild-type strain (Fig. 2F), suggesting that *VdCf2* is negatively involved in regulation of the oxidative stress response.

VdCf2 regulates melanin production. In general, melanin protects pathogens from environmental stresses and is beneficial to the long-term survival of fungi under unfavorable environmental conditions (38–41). Compared to the wild-type XJ592 and Δ Vd*Cf2/VdCf2* strains, strain Δ Vd*Cf2* accumulated more melanin on PDA, MM, and WA



FIG 3 *VdCf2* was required for full virulence on cotton. (A) *VdCf2* was not involved in cellophane membrane penetration of *V. dahliae*. Strains XJ592, Δ VdCf2, and Δ VdCf2/VdCf2 were cultured on top of cellophane membrane overlaid onto MM plates for 3, 4, or 5 days. The cellophane membranes were removed, and all strains were cultured for additional 3 days. (B) *Verticillium* wilt symptoms on susceptible cotton plants (JM11) inoculated with the wild-type, Δ VdCf2, and Δ VdCf2/VdCf2 strains at 35 days postinoculation. (C) The vascular discoloration in cotton plants inoculated with the wild-type, Δ VdCf2, and Δ VdCf2/VdCf2 strains. (D) Disease index of cotton plants inoculated with the wild-type, Δ VdCf2, and Δ VdCf2/VdCf2 strains. Error bars represent standard errors calculated from three replicates, and asterisks represent the statistical significance (SNK test, P = 0.05).

media, suggesting that VdCf2 regulates melanin production (Fig. 2C and G). In addition, strain Δ VdCf2 cultured on modified basal agar medium (BMM) produced melanized microsclerotia 4 days after plating, whereas the wild-type XJ592 and Δ VdCf2/VdCf2 strains produced comparable microsclerotia without melanin deposition following a similar incubation time (Fig. 2G). These results suggest that VdCf2 is involved in melanin homeostasis but not microsclerotium formation.

VdCf2 participated in pathogenicity of *V. dahliae* **on cotton.** To assess the potential roles of *VdCf2* during initial colonization of *V. dahliae*, the penetration ability of strains XJ592, Δ Vd*Cf2*, and Δ Vd*Cf2/VdCf2* were examined by inoculation on MM plates covered with a cellophane membrane. When the cellophane membrane was removed at 4 or 5 days postinoculation, hyphae of strains XJ592 and Δ Vd*Cf2/VdCf2* had penetrated the cellophane membrane and had grown into the medium (Fig. 3A). Similarly, strain Δ Vd*Cf2* maintained the ability to penetrate cellophane membrane (Fig. 3A). This result indicates that *VdCf2* is not required for penetration by *V. dahliae*.

To evaluate the role of VdCf2 in virulence, seedlings of susceptible cotton cultivar JM11 were inoculated with conidial suspensions of strain XJ592, Δ VdCf2, and Δ VdCf2/

VdCf2 and were assessed. The plants inoculated with strains XJ592 and Δ Vd*Cf2/VdCf2* developed chlorosis and defoliation at 35 days postinoculation (Fig. 3B). In contrast, the plants inoculated with strain Δ Vd*Cf2* showed only mild foliar chlorosis and defoliation (Fig. 3B). Furthermore, the xylem in the plants inoculated with strain XJ592 or Δ Vd*Cf2/VdCf2* were brown, whereas the vascular discoloration was incipient in the plants inoculated with strain Δ Vd*Cf2* (Fig. 3C). In addition, the disease index was significantly reduced in the plants inoculated with strain Δ Vd*Cf2* (Fig. 3C). In addition, the disease index was significantly reduced in the plants inoculated with strain Δ Vd*Cf2* strains at 5, 6, 7, and 8 weeks after inoculation (Fig. 3D). These results suggest that *VdCf2* plays a role in *V. dahliae* pathogenicity and virulence.

VdCf2 regulates the transcription of potential pathogenicity-related genes. To study the regulatory functions of VdCf2, the transcriptomes between strains XJ592 and Δ VdCf2 were compared. Mycelia of strains XJ592 and Δ VdCf2 cultured in CDM were used for RNA sequencing (RNA-seq). A total of 275 differentially expressed genes (DEGs) were identified: 154 upregulated and 121 downregulated genes (Fig. 4A, Table S1; P < 0.05 with a log₂ fold change of ≥ 1.0) in the wild-type compared to the $\Delta VdCf2$ strain. KEGG pathway enrichment analysis showed that the upregulated genes were mainly enriched in carbohydrate and amino acid metabolism (Fig. 4B). Gene Ontology (GO) enrichment analysis revealed that the majority of DEGs were related to the genes encoding extracellular proteins and integral components of the membrane (Fig. 4C, Table S2). Among DEGs, there were 174 secreted proteins, comprising 62 classical secreted proteins and 112 nonclassical secreted proteins, 76 pathogen-host interaction (PHI) homolog proteins, including 33 verified to be associated with pathogenicity (reduced virulence or loss of pathogenicity in their knockout mutants), 6 TFs, and 5 G protein-coupled receptors (GPCRs) (Fig. 5A, Table S1). Seven DEGs (VDAG_02735, VDAG_06200, VDAG_02289, VDAG_10037, VDAG_09577, VDAG_07255, VDAG_09018) were randomly chosen to quantify the gene expression level with a reverse transcriptase quantitative (RT-qPCR) assay to verify the reliability of this transcriptome data. Results showed that their expressions were all upregulated in the XJ592 strain, consistent with the RNA-seq analysis (Fig. S3).

VdCf2 regulated the expression of many genes encoding secreted proteins based on the comparative transcriptome analysis, including two previously reported secreted proteins, VdPevD1 (*VDAG_02735*) and VdPEL1 (*VDAG_06155*), which were proved to both be positive regulators in the pathogenicity of *V. dahliae* (15, 42) (Table S1). RNAseq analysis showed that VdCf2 positively and negatively regulated the expression levels of *VdPevD1* and *VdPEL1*, respectively (Table S1). However, after the fungal sample was treated with cotton root extracts for 2 days, the expression of *VdPevD1* was upregulated in the wild type, but the expression of *VdPEL1* did not differ significantly (Fig. 5B). To investigate whether VdCf2 regulates the expression of *VdPevD1* by directly binding to its promoter, electrophoretic mobility shift assay (EMSA) was performed with purified glutathione *S*-transferase (GST)-tagged VdCf2 protein and the promoter of *VdPevD1*. When VdCf2 protein was coincubated with the *VdPevD1* promoter, a shift band was observed (Fig. 5C and D), indicating an interaction between them. These results suggest that VdCf2 directly regulates the expression of *VdPevD1* in mycelia and the sample treated with cotton root extracts.

To further investigate whether the C2H2-type zinc finger domains of VdCf2 directly bind the promoter region of *VdPevD1*, EMSA was performed with the truncated VdCf2 protein (VdCf2-F1 or VdCf2-F2) and the promoter of *VdPevD1*. Both VdCf2-F1 and VdCf2-F2 proteins could bind to the promoter region of *VdPevD1* (Fig. 5C and D), suggesting that VdCf2 has a function in binding to the promoter region of *VdPevD1* via its C2H2-type zinc finger domain.

VdCf2 regulates the expression of several PHI genes. In this transcriptome analysis between the wild-type and Δ VdCf2 strains, VdCf2 regulates 76 PHI genes, including 33 associated with pathogenicity (Table 1). To identify whether these PHI genes associated with pathogenicity respond to cotton root extracts, 10 genes with the highest expression level in all PHI genes which positively regulated pathogenicity were



FIG 4 KEGG and GO enrichment analysis of differentially expressed genes in the transcriptome analysis. (A) Sample preparation of RNA-seq and the number of differentially expressed genes between the wild-type strain and $\Delta VdCf2$ strain. Three fungus blocks (6-mm diameter) were inoculated into the Czapek-Dox medium, and then the mycelia were collected at 8 days postinoculation. WT represents the XJ592 strain and Mut represents the $\Delta VdCf2$ strain. The gene expression was analyzed in the mycelia of the wild-type XJ592 strain compared to that of strain $\Delta VdCf2$. Differentially expressed genes were defined by a log₂ fold change of \geq 1.0 and *P* value of <0.05. Three repeats were performed in this transcriptome sequencing assay. (B) KEGG pathway enrichment analysis of upregulated differentially expressed genes in the precentage of genes represents the proportion of upregulated differentially expressed genes in this pathway. (C) GO enrichment analysis of all differentially expressed genes in RNA-seq analysis in the mycelia of strain XJ592 compared to that of strain $\Delta VdCf2$.

selected for quantitative analysis in fungal samples treated with cotton root extracts for 2 days. These genes include eight upregulated (*VDAG_08876, VDAG_08028, VDAG_06031, VDAG_07610, VDAG_02535, VDAG_08888, VDAG_07296, VDAG_04828*) and two downregulated (*VDAG_04714, VDAG_07838*) differential expression genes. Quantitative results showed that the expression level of most PHI genes was significantly induced in strain Δ VdCf2 but not significantly different in strain XJ592 when the fungal sample was treated with cotton root extracts (Fig. 6). These



FIG 5 VdCf2 regulated the expression of potential pathogenicity-related genes. (A) Number of SP_Cs, SP_NCs, PHIs, TFs, and GPCRs in differentially expressed genes. SP_Cs, classical secretory proteins; SP_NCs, nonclassical secretory proteins; PHIs, pathogen-host interaction-related proteins; TFs, transcription factors; GPCRs, G protein-coupled receptors. (B) The expression level of *VDAG_02735* and *VDAG_06155* was determined by an RT-qPCR experiment. Conidial suspensions (10⁷ conidia/mL) of the wild-type and Δ VdCf2 strains were cultured on the Czapek-Dox medium for 5 days before being treated with cotton root extracts for 2 days. The *β*-tubulin gene (*VDAG_10074*) was used as an internal reference. The expression level of each gene in the wild-type strain in the control was standardized as 1. Error bars represent standard errors calculated from three replicates, and different letters indicate the statistical significance of Tukey's test at *a P* value of 0.05. (C) Schematic diagrams of VdCf2-F1 and VdCf2-F2 used in the EMSA. (D) VdCf2, VdCf2-F1, and VdCf2-F2 proteins bound to the promoter of the *VDAG_02735* gene according to the EMSA.

results indicate that *VdCf2* inhibits the expression of several PHI genes in fungal samples treated with cotton root extracts for 2 days. However, *VdCf2* promoted the expression of a few PHI genes in the control, indicating that *VdCf2* played different regulatory roles in fungal samples treated without and with cotton root extracts.

VdCf2 influences the expression of other TFs. RNA-seq analysis indicated that VdCf2 affected expression of six TFs, including three upregulated (VDAG_01280, VDAG_10037, VDAG 09577) and three downregulated (VDAG 02169, VDAG 00592, VDAG 05292) genes (Fig. 7A, Table S1). VDAG_00592 and VDAG_05292, with the highest expression in all differentially expressed transcription factor genes, encoded meiosis-specific transcription factor NDT80-like protein and transcription factor vib-1, respectively. MoNdt80 played functions in the pathogenesis of M. oryzae (43), and transcription factor vib-1 in N. crassa participated in plant cell wall degradation (44). To further identify whether these differentially expressed TFs respond to cotton root extracts, the expression level of two TFs (VDAG 00592 and VDAG 05292) was quantified in fungal samples treated with cotton root extracts for 2 days. The expression level of VDAG_00592 and VDAG_05292 genes was significantly inhibited in both strains XJ592 and Δ VdCf2 when the fungal sample was treated with cotton root extracts (Fig. 7B). The extent of inhibition was, however, much greater in strain XJ592 than in the $\Delta VdCf2$ strain (Fig. 7B). In the mycelia and the fungal sample treated with cotton root extracts for 2 days, the expression level of the VDAG_00592 gene was significantly inhibited in strain XJ592 compared to the Δ VdCf2 strain, indicating that VdCf2 curtails the expression of the VDAG_00592 gene in both states (Fig. 7B).

TABLE 1 The 33 DEGs of PHI reduced virulence or loss of pathogenicity in the transcriptome
data

	Phenotype of mutant		
Gene ID	in other pathogens	Pathogen species	Reference
VDAG_08721	Reduced virulence	Fusarium graminearum	72
VDAG_08876	Reduced virulence	Claviceps purpurea	73
VDAG_08028	Reduced virulence	Zymoseptoria tritici	74
VDAG_06031	Reduced virulence	M. oryzae	75
VDAG_07610	Reduced virulence	M. oryzae	76
VDAG_02535	Reduced virulence	Nectria haematococca	No data
VDAG_08888	Reduced virulence	M. oryzae	76
VDAG_07296	Reduced virulence	B. bassiana	77
VDAG_04828	Reduced virulence	Candida albicans	78
VDAG_03487	Reduced virulence	C. albicans	78
VDAG_10408	Reduced virulence	Aspergillus fumigatus	79
VDAG_09443	Loss of pathogenicity	M. oryzae	80
VDAG_06032	Reduced virulence	B. cinerea	81
VDAG_02241	Reduced virulence	M. oryzae	82
VDAG_02899	Reduced virulence	M. oryzae	75
VDAG_08498	Reduced virulence	M. oryzae	83
VDAG_07251	Reduced virulence	Ustilago maydis	84
VDAG_07393	Reduced virulence	M. oryzae	82
VDAG_08741	Reduced virulence	Trichoderma virens	85
VDAG_09710	Reduced virulence	M. oryzae	76
VDAG_04416	Reduced virulence	T. virens	85
VDAG_07018	Reduced virulence	Candida glabrata	86
VDAG_07929	Reduced virulence	M. oryzae	75
VDAG_09581	Reduced virulence	Grosmannia clavigera	87
VDAG_09343	Reduced virulence	C. albicans	78
VDAG_10092	Loss of pathogenicity	M. oryzae	88
VDAG_04729	Loss of pathogenicity	Erwinia amylovora	89
VDAG_07983	Reduced virulence	Histoplasma capsulatum	90
VDAG_04718	Reduced virulence	Colletotrichum coccodes	91
VDAG_06155	Reduced virulence	N. haematococca	92
VDAG_07195	Reduced virulence	F. graminearum	93
VDAG_04714	Reduced virulence	Cryptococcus neoformans	94
VDAG_07838	Reduced virulence	M. oryzae	95

VdCf2 regulates the expression of the Vd276-280 gene cluster. A previous study showed that the GATA-type transcription factor Csm1 inhibited the expression of the putative *STC1* (sesquiterpene cyclase) gene cluster in *Fusarium fujikuroi* (45). This transcriptomic data showed that VdCf2 inhibited the expression of the Vd276-280 gene cluster comprising VDAG_07276 (cystinosin-like protein), VDAG_07277 (hypothetical protein), VDAG_07278 (hypothetical protein), VDAG_07279 (indoleamine 2,3-dioxygenase 1), and VDAG_07280 (alpha-galactosidase B like protein) (Fig. 8A, Fig. S4, Table 2). This gene



FIG 6 VdCf2 regulated the expression of several PHI genes. Sample preparation and data analysis are consistent with Fig. 5B. Error bars represent standard errors calculated from three replicates, and different letters indicate the statistical significance by Tukey's test at a *P* value of 0.05.



FIG 7 VdCf2 regulated the expression of other TFs. (A) The value of the \log_2 fold change of genes encoding TFs regulated by VdCf2 according to transcriptome data. (B) The relative expression level of *VDAG_00592* and *VDAG_05292* genes in the wild-type and Δ VdCf2 strains. Conidial suspensions (10⁷ conidia/mL) were cultured in the Czapek-Dox medium for 5 days before being treated with cotton root extracts for 2 days. The β -tubulin gene (*VDAG_10074*) was used as the internal reference. The expression level of each gene in the wild-type strain in the control was standardized as 1. Error bars represent standard errors calculated from three replicates, and different letters indicate the statistical significance of Tukey's test at a *P* value of 0.05.

cluster belongs to a portion of VDAG_07259 to VDAG_07280, predicted to be a secondary metabolism gene cluster (25, 46). To further identify whether the Vd276-280 gene cluster responds to cotton root extracts, the expression level of each gene in the Vd276-280 gene cluster was quantified in fungal samples treated with cotton root extracts for 2 days. The expression level of all genes in the Vd276-280 gene cluster was significantly inhibited in wild-type XJ592 and Δ VdCf2 strains in fungal samples treated with cotton root extracts for 2 days (Fig. 8B). In addition, the expression level of all genes in this gene cluster was also significantly inhibited in the wild-type XJ592 strain compared to those in the Δ VdCf2 strain (Fig. 8B). This suggests that this gene cluster responds to cotton root extracts and that VdCf2 regulates the expression of this gene cluster when the fungal sample was treated with cotton root extracts.

VdCf2 directly interacted with VDAG_07276 and VDAG_07278. To identify proteins whose functions are related to VdCf2, the interaction targets of VdCf2 were screened based on a yeast two-hybrid (Y2H) cDNA library. We identified several proteins that potentially interact with VdCf2. The interaction was verified with a one-toone Y2H assay. The yeast cells containing BD-VdCf2 and AD-VDAG_07276 constructs were able to grow on an synthetic dropout (SD)-Trp-Leu-His plate supplemented with 40 mg/L X- α -Gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside) and turned blue (Fig. 9A). These results indicate that VdCf2 directly interacts with VDAG_07276 in the yeast two-hybrid system. To further identify whether other genes in this gene cluster interact with VdCf2, AD-VDAG_07277, AD-VDAG_07278, AD-VDAG_07279, and AD-VDAG_07280 constructs were transformed together with BD-VdCf2 into Y2H yeast competent cells, respectively. The yeast cells containing BD-VdCf2 and AD-VDAG_07278 constructs were able



Control

FIG 8 VdCf2 regulated the expression of a putative secondary metabolism gene cluster. (A) Visualization of RNA-seq coverage of this putative secondary metabolism gene cluster in the wild-type and Δ VdCf2 strains. The blue lines indicate read coverage. (B) Gene expression level of the *Vd276-280* gene cluster. The conidial suspensions (10⁷ conidia/mL) were cultured in the Czapek-Dox medium for 5 days before being treated with cotton root extracts for 2 days. The β -tubulin gene (*VDAG_10074*) was used as the internal reference. The expression level of each gene in the wild-type strain in the control was standardized as 1. Error bars represent standard errors calculated from three replicates, and different letters indicate the statistical significance of Tukey's

2 dpi

VDAG_07278

Control

VDAG_07279

2 dpi

Control

2 dpi

VDAG_07280

to grow on an SD-Trp-Leu-His plate, suggesting an interaction between VdCf2 and VDAG_07278 (Fig. 9A).

Control

VDAG_07277

2 dpi

2 dpi

Control

VDAG_07276

To further determine the interaction relationship between VdCf2 and VDAG_07276 or VDAG_07278, nYFP-VdCf2 and cYFP-07276, nYFP-VdCf2 and cYFP-07278 were coexpressed in the epidermal cells of *N. benthamiana* leaves for observation of yellow fluorescent protein (YFP) signals. The tobacco leaves coinfiltrated with nYFP and cYFP-07276, nYFP and cYFP-07278, and nYFP-VdCf2 and cYFP were used as negative controls. The result of bimolecular fluorescence complementation (BiFC) showed that YFP signals were observed in the cell nucleus of *N. benthamiana* coinfiltrated with nYFP-VdCf2 and cYFP-07276 and with nYFP-VdCf2 and cYFP-07278, whereas no yellow fluorescence signals were observed in negative controls, indicating that there is an interaction relationship between VdCf2 and VDAG_07276, as well as between VdCf2 and VDAG_07278 (Fig. 9B).

DISCUSSION

test at a P value of 0.05.

VdCf2 belongs to TFs of the C2H2 type, which is the second largest type of TFs, following the Zn2Cys6 type in *V. dahliae* (23). Only a few TFs of the C2H2 type have been characterized, and the mechanisms of most C2H2-type TFs involved in pathogenicity remain undetermined (24, 32, 33). In this study, VdCf2 was found to promote

TABLE 2 The top 20 upregulated and 20 downregulated differentially expressed genes in	
the transcriptome data	

		Log ₂ fold	
Gene ID	Regulation	change	Annotated gene function
VDAG_07673	Up	Infinite	Gst19
VDAG_04512	Up	5.27	Uncharacterized protein
VDAG_05264	Up	4.36	Hypothetical protein
VDAG_09533	Up	3.29	Uncharacterized protein
VDAG_08876	Up	2.88	Uncharacterized protein
VDAG_08028	Up	2.64	NADH-cytochrome b5 reductase
VDAG_09486	Up	2.61	Uncharacterized protein
VDAG_03485	Up	2.55	Aflatoxin biosynthesis ketoreductase nor-1
VDAG_03386	Up	2.49	Uncharacterized protein
VDAG_09527	Up	2.41	Hydrolase
VDAG_09526	Up	2.41	FAD binding domain-containing protein
VDAG_06200	Up	2.39	Uncharacterized protein
VDAG_03676	Up	2.36	Uncharacterized protein
VDAG_06031	Up	2.35	Uncharacterized protein
VDAG_00722	Up	2.29	Uncharacterized protein
VDAG_09251	Up	2.26	Oxalate decarboxylase oxdC
VDAG_09524	Up	2.23	Hypothetical protein
VDAG_00625	Up	2.21	Sphingoid long-chain base transporter RSB1
VDAG_09018	Up	2.20	Elongation factor G2
VDAG_01819	Up	2.16	Glutamate decarboxylase
VDAG_03806	Down	-2.59	Uncharacterized protein
VDAG_00794	Down	-2.65	Uncharacterized protein
VDAG_08603	Down	-2.67	Uncharacterized protein
VDAG_04714	Down	-2.73	Histidine kinase G7
VDAG_07279	Down	-3.08	Indoleamine 2,3-dioxygenase 1
VDAG_05839	Down	-3.09	Uncharacterized protein
VDAG_01292	Down	-3.25	Uncharacterized protein
VDAG_02862	Down	-3.25	Interferon-induced GTP-binding protein Mx
VDAG_06156	Down	-3.35	Uncharacterized protein
VDAG_05535	Down	-3.35	Uncharacterized protein
VDAG_06272	Down	-3.70	6-Hydroxy-D-nicotine oxidase
VDAG_05104	Down	-3.85	Uncharacterized protein
VDAG_07595	Down	-4.11	Alpha-L-fucosidase
VDAG_08513	Down	-4.26	Epoxide hydrolase
VDAG_08707	Down	-4.31	Uncharacterized protein
VDAG_07838	Down	-4.33	Cerato-ulmin
VDAG_07277	Down	-6.29	Hypothetical protein
VDAG_07280	Down	-7.58	Alpha-galactosidase B like protein
VDAG_07276	Down	-8.23	Cystinosin-like protein
VDAG_07278	Down	-8.83	Hypothetical protein

pathogenicity to cotton probably by influencing fungus growth and the expression level of secreted protein-encoding genes.

In this study, the growth of strain $\Delta VdCf2$ was reduced on PDA, MM, and WA media. Transcriptomic analysis showed that five GPCRs were significantly downregulated in the $\Delta VdCf2$ strain—carbon/amino acid receptor (*VDAG_05120*), RGS_domain GPCR (*VDAG_08194*), and 3 PTH11-related GPCRs (*VDAG_07929*, *VDAG_02899* and *VDAG_06031*). GPCRs are mainly responsible for transmitting extracellular signals to the intracellular region to trigger several signaling cascade reactions that are important for fungal development, including the cyclic AMP and mitogen-activated protein kinase pathways (47, 48). The decreased expression of genes encoding GPCRs in strain $\Delta VdCf2$ may thus be responsible for the reduced mycelial growth observed on the different media.

The interplay between virulence and melanin production is complex (32, 49). Strains with reduced melanin production often show reduced virulence (33, 49, 50). In this study, however, even though melanin production increased in the Δ VdCf2 strain, its virulence on cotton seedlings was reduced. The role of VdCf2 in melanin production and virulence was consistent with that of VTA2, which encodes a transcription activator



FIG 9 VdCf2 interacted with VDAG_07276 and VDAG_07278. (A) VdCf2 interacted with VDAG_07276 and VDAG_07278 in a yeast two-hybrid system. For analysis of interaction, the full-length cDNA of *VdCf2* was inserted into the pGBKT7 vector to generate the bait construct, and the full-length CDS of *VDAG_07276* and *VDAG_07278* was inserted into the pGADT7 vector to generate the prey construct. The Y2HGold yeast cells containing the bait construct and pGADT7 vector were used to detect self-activation. The Y2HGold yeast cells containing the bait and prey constructs were used to detect interaction. The Y2HGold yeast cells with 10-fold serial dilutions (10° , 10^{-1} , and 10^{-2}) were simultaneously cultured on SD plates –LW (lacking Leu and Trp), –LWH (lacking Leu, Trp, and His), and –LWH (lacking Leu, Trp, and His) supplemented with 40 mg/L X- α -Gal. The Y2HGold yeast cells containing the pGBKT7-Ta vectors were regarded as the negative control. (B) VdCf2 interacted with VDAG_07276 and VDAG_07278 as determined by a bimolecular fluorescence complementation assay. The epidermal cells VdCf2 interacted with nYFP and cYFP-07276, nYFP and cYFP-07278, and nYFP-VdCf2 and cYFP were negative controls. Scale bars = 20 μ m.

of adhesion (32). It is possible that *VdCf2* and *VTA2* act as intermediaries to couple virulence and melanin production. Transcription factor VdCmr1, located in a secondary metabolism cluster of genes, is a key regulator of the melanin biosynthesis pathway (51). The influence of *VdCf2* and *VTA2* on melanin formation may be related to VdCmr1.

Secreted proteins are vital for successful colonization of host plants by fungal pathogens (14, 52, 53). A fungal_trans domain-containing TF (VdFTF1) affects pathogenicity mainly by regulating the expression of those genes encoding secreted proteins (26). Many secreted proteins were differentially expressed in the wild-type strain from the Δ VdCf2 strain, including two virulence-related proteins, VdPevD1 and VdPEL1 (15, 42). Further RT-qPCR analysis verified that VdCf2 promoted the expression of *VdPevD1* in the sample treated with cotton root extracts. *VdPevD1* promotes pathogenicity by inhibiting antifungal activities of pathogenesis-related protein 5 (PR5)-like GhPR5 (42). VdCf2 probably promotes pathogenicity by enhancing the transcription of *VdPevD1* to overcome antifungal activities of host plants via GhPR5 (42). VdPEL1 belongs to the pectate lyase family, which also facilitates pathogenicity in *V. dahliae* (15). However, the expression of *VdPEL1* in the wild-type strain was not induced in the sample treated with cotton root extracts, *VdPEL1* did not participate in this process.

VdFTF1 regulates the expression of genes encoding TFs during *V. dahliae* infection of cotton (26). Som1 and Vta3 controlled the expression of numerous TFs encoding genes when *V. dahliae* was cultured on a modified simulated xylem medium (SXM) (54). The present study indicated that VdCf2 regulated the expression of six putative TF-encoding genes, one of which was *VDAG_00592*. *VDAG_00592* belonging to NDT80/PhoG TF was downregulated in the wild-type strain. In *Candida parapsilosis*, NDT80/PhoG acts as a repressor of virulence attributes (55). However, an Ndt80/PhoG domain-containing TF MoNDT80 in *M. oryzae* positively participated in pathogenicity to host rice (43). Whether NDT80/PhoG in *V. dahliae* also has a role in pathogenicity needs further investigation.

In the present study, *VdCf2* repressed the expression of a putative secondary metabolite gene cluster. Similarly, the APSES TF Vst1 regulates the expression of seven secondary metabolism gene clusters at the early stages of microsclerotium biogenesis in *V. dahliae* (19). In this study, VdCf2 inhibits the expression of the *Vd276-280* gene

cluster, and in yeast two-hybrid and BiFC assays, VdCf2 interacted physically with VDAG_07276 and VDAG_07278, two members of *Vd276-280* gene cluster. Thus, it is possible that VdCf2 regulates the expression of *VDAG_07276* and *VDAG_07278* by interacting with them.

In summary, VdCf2 is an important regulator of growth, melanin production, and virulence in *V. dahliae*. Transcriptomic analysis showed that VdCf2 curtailed the expression of virulence-related *VdPevD1* (*VDAG_02735*) by directly binding its promoter region, which might explain the reduced virulence of strain Δ VdCf2. VdCf2 also regulates the expression of the *Vd276-280* gene cluster and directly interacts with its two members. Further study is under way to research the function of this gene cluster.

MATERIALS AND METHODS

Fungal strains and culture conditions. A V. dahliae strain (XJ592) isolated from cotton in XinJiang province, China, was used as the wild-type strain in this study. V. dahlae conidial suspensions (1 μ L) of approximately 10⁷ conidia/mL were placed on potato dextrose agar (PDA) medium, minimal medium (MM) (2 g/L of dextrose, 0.5 g/L of MgSO₄·7 H₂O, 0.5 g/L of KCl, 2 g/L of NaNO₃, 0.01 g/L of FeSO₄·7H₂O, 2.6 mg/L of NH₄Fe(SO₄)₂ 12 H₂O, 0.5 mg/L of CuSO₄ 5 H₂O, 1 g/L of KH₂PO₄, 0.01 g/L of ZnSO₄ 7 H₂O, 0.1 mg/L of Na₂MoO₄: 2 H₂O, 0.01 g/L of citrate, 0.1 mg/L of MnSO₄·H₂O, 0.1 g/L of H₃BO₃, 20 g/L of agar) and water agar (WA) and incubated at 25°C for 2 weeks to evaluate colony growth by measuring the diameter. For conidial production and collection, the wild-type, $\Delta VdCf2$, and $\Delta VdCf2/VdCf2$ strains were cultured in liquid Czapek-Dox medium (CDM) for 5 days before the culture was harvested and filtered through Miracloth (Millipore, Billerica, MA, USA). For the osmotic stress response assay, conidial suspensions of 1 μL (10⁷ conidia/mL) of each strain were placed in the center of plates containing complete medium (CM) with the addition of 1.2 M sorbitol, 0.8 M KCl, or 0.4 M NaCl and incubated at 25°C for 2 weeks. For the oxidative stress response assay, conidial suspensions (10⁷ conidia/mL) of the wild-type and $\Delta VdCf2$ strains were coated on CM plates with a piece of circular filter paper (6-mm diameter) containing 1 μ L 30% H₂O₂ and cultured for 48 h. Conidial suspensions from each strain were spread onto the BMM medium (56) overlaid on a piece of cellulose membrane and cultured for 4 days before microsclerotia were assessed and photographed. Conidial suspensions of 1 μ L (10⁷ conidia/mL) were cultured on MM covered with a piece of cellulose membrane for 3, 4, or 5 days before the cellulose membrane was removed to analyze the penetration ability. The colonies were photographed 3 days after the cellulose membranes' removal. Cotton plants with two true leaves (Gossypium hirsutum cv. JIMIAN 11) were used for the virulence assay. Details of this assay are provided below.

Generation of gene deletion and complementation mutants. The upstream and downstream flanking sequences of *VdCf2* were amplified from XJ592 genomic DNA with primer pairs Cf2-AF/Cf2-AR and Cf2-BF/Cf2-BR, respectively. The hygromycin B resistance fragment was amplified from pA-HYG OSCAR vector with primer pair HYG-QF/HYG-QR. These three fragments were fused with the primer pair Cf2-AF/Cf2-BR via fusion PCR to acquire a gene deletion cassette. The fusion fragment was cloned into the pOSCAR vector digested with the restriction enzymes HindIII and EccRI to generate the knockout plasmid. The resulting vector was transformed into the wild-type strain to generate the $\Delta VdCf2$ strain. The $\Delta VdCf2$ was amplified with the primer pair Cf2-CF/Cf2-CR from XJ592 genomic DNA and then cloned into the pOSCAR-C vector digested with the restriction endonuclease Xhol. The resulting construct was then transformed into the $\Delta VdCf2$ strain.

Southern blotting was performed with DIG High Prime DNA labeling and detection starter kit I according to the manufacturer's protocol (Roche, Switzerland). The genomic DNA of the XJ592 and Δ VdC*f2* strains was digested by the restriction endonuclease BamHI. The probe for *VdCf2* gene detection was PCR amplified with the primer pair NF/NR, and the probe for hygromycin B resistance gene detection was PCR amplified with the primer pair HYG-F/HYG-R. The primers used in this study are listed in Table 3.

RNA isolation and RT-qPCR assays. For quantitative analysis of potential pathogenicity-related genes, the wild-type and Δ VdCf2 strains were cultured in the CDM for 5 days before treatment with cotton root extracts for another 2 days. Total RNA was extracted using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cDNA was synthesized with the PrimeScript RT reaction system (TaKaRa, Tokyo, Japan). RT-qPCR was performed with UltraSYBR mixture (CWBIO, Beijing, China) using a LightCycler 480 II device (Roche, Basel, Switzerland). The β -tubulin gene (*VDAG_10074*) was used as an internal reference (57). The relative expression level of all genes was calculated with the 2^{- $\Delta\Delta$ CT} method (58). Three replicates were used in all experiments. The primers used are listed in Table 3.

Virulence assays. Susceptible cotton cultivar JM11 plants with two true leaves were used in virulence assays with the quantitative root dip inoculation method (59). Conidia of the XJ592, Δ VdCf2, and Δ VdCf2/VdCf2 strains were collected by filtration and centrifugation and then resuspended in sterile water to a concentration of approximately 10⁷ conidia/mL. Five to eight cotton plants were planted in a pot, which was placed within a petri dish cover and cultured at 25°C under a 16/8-h light-dark photoperiod in a greenhouse. Three pots of cotton plants were prepared for each strain. Conidial suspensions of 35 mL were added into the petri dish cover to inoculate the cotton roots. The control group was dipped in sterile water. Each pot of all treatments was randomly assigned to specific locations in the

TABLE 3 List of the primers used in this study

CP:AP GATCCAASCTCAAGCTTCAAGCTACAGCTCAACGCAAGAGC Amplification of upstream flanking sequence of VdC2 CP:AP CAATTACAGTCGGAGGCACATCAAAGCACATCAAGT Amplification of downstream flanking sequence of VdC2 CP:BP CAATTACAGTCGGAGGCACATCAAAGCACATCAAGCACATCA Amplification of downstream flanking sequence of VdC2 CP:BP CAATTACAGTCGAGGCACATCAACGACATCAGCGCACAGATCAGTGGCGCACGAACGA	Primer name	Primer sequence $(5'-3')^a$	Application
CP-2R CATATCCCATCGGGAACCAGTMCAGCACAGAGGTCAGT CP2-BF CATATACGCTGGGGCGCAGCTCAGGAGGGAGGA CP2-BF CATATACGCTGGGGCGGGCACTACTGCAGCAGGGAGGA CP2-BF CATATACGCTGGGGCGGGCACTCACG CP3-BF CATGGTGCGGCGGCCACTCACT MFG-OP ACCGGGTGGGGCCTCACTCGTAAG CP2-BF GAAGCCACCTTCFGCAACTAGAC CP2-BF GAAGCCCCCTCGGGGCGCCCCCCCCCCCCCCCCCCCCCC	Cf2-AF ^a	GATCCAAGCTCAAGCTAAGCTTCAAGCGAGGTAGAGGAGAGG	Amplification of upstream flanking sequence of VdCf2
CP:3FR CANTAGETGGGCTGAGGCAGGCACCATCAGGAGAGGAGGA Anplification of downstream flanking sequence of VdC2 VFG:0F2 CCTCGAGCCCAGATGGAATGGAGCGGCAGGATTGGCCC Amplification of downstream flanking sequence of VdC2 VFG:0F3 CCTCGAGCCCAGATGGAATGGAGCGC Amplification of downstream flanking sequence of VdC2 CP:3FR GAGCCCACCTGTGAGCGCGAGGTCCATGGGAGC Detection of deletion mutant CP:2FR GAGCCCACCTGTGAGGGCCCCTGGGCGGGAGAGGGCCT Amplification of VdC2 gene with native promoter CP:2FR ACCCTGGGGGGCGCCCCCCCCCGGCGGGAGAGGGCCT Vector construction for subcellular localization in to bacc0 CP:3FR GAGCCCACCTGTGGGCGGGAGAGGCCT Vector construction for subcellular localization in V. dohline CP:3FR GAGCCCCGGGTGGGCGGGGGGGGGGGGGGGGGGGGGGGG	Cf2-AR	GATGCCGACCGGGAACCAGTTACAGGCACAGAGGTTCAGTT	
CP:3PR GAATTAACGCCGAATTGAATTCGTGGAACATATCG HYG-OP AACTGGTTCCGGACGCGCGCACTCATC HYG-OP AACTGGTTCCGCGGCGCGCGCACTCGTAAG CP:3PF GAACCGCCTCGTGCGACTCGTAAG CP:3PF GAACCCCCTGGTGCGCCTCGAGTCGTGATGGATGTGGTGTT CP:3PF GAACCCCCCGGTGCGCCGCGCGCGCGTGAGGGCCCTCGTGTGGATGGA	Cf2-BF	CAATATCAGTTGGGCTGCAGG GCAGCATCAAGGAAGGAGGA	Amplification of downstream flanking sequence of VdCf2
HYG-0PAACTGOTTCCCGATCGACTATATCAAmplification of hygromycin-resistant geneHYG-0RCCTCGAAGCCAACTGATATGCAGGCAGCDetection of deletion mutantCP:2NFGAAGCCACCTGATCGACAAGCTCGATGGACTGCGATGTGGTGTAmplification of VdC2 gene with native promoterCP:2CFTGTCATGGCTGGAGGAGGATCCATGGCATGGAGTGGAGGGGCTAmplification of VdC2 gene with native promoterCP:2CFTGTCATGGCTGGAGGAGGATCCATGGCCTGCGACAAGGGGCTVector construction for subcellular localization in tobaccoCP;BininACCCCGGGGGTGGAGGGATCCATGGCCTGGGGGAGAGGGCTVector construction for subcellular localization in V. dahlaeCP;BiniACCCCTGGGGGAGGAGTCCATGGCCTGGGGGAGGGGCTAmplification of VdC2 gene probe in Southern blotCP;BiniGAAGCCACCTGCTGGGGGAGAGGGCTAmplification of VdC2 gene probe in Southern blotCP;BiniGAAGCCACCTCGTGGGCGGAGGGGCTGGGGGGGGGGGGG	Cf2-BR	GAATTAACGCCGAATTGAATTCGTGGAACATATTCGCCGTCAG	
HYG-QR CCTCCAGCCCACTCGATTGAATTGAAGGAGC Detection of deletion mutant CP:NF GAGCCACCTTCGGACCTGAAG CP:AF GAGCCACCTTCGGACTGAAGC Detection of deletion mutant CP:AF ATTAAGCCCGACTGACTGATGAAGC CP:AF ATTAAGCCCGACTGACTGATGAAGC CP:AF ATTAAGCCCGACTGACTGAAGC CP:BFIF ATTCCCCGCGGTGAGGCCCCCTCCCCTCCCTTCGTGATGGATG	HYG-QF	AACTGGTTCCCGGTCGGCATCTACTC	Amplification of hygromycin-resistant gene
CP.NFGAGCCACCTTCTGACACATAGCDetection of deletion mutantCP.2FNGAAGCCTGGATGCTGAGACTGCTAGAAmplification of V/2C2 gene with native promoterCP.2 FNATTAAGCCGGAGATGCATGGATGCGTGGGATGCATGGATGG	HYG-QR	CCTGCAGCCCAACTGATATTGAAGGAGC	
CP:NB GAASCCTGCGTGACTCGTAAG CP:CF TGTCACCGTGCGCGCGCAGGGCCTGTGATGAGTGGGTGT Amplification of VdC2 gene with native promoter CP:2BinF ATCCGCGAGGGTCGAGGGCGCTGCGCGGCGAGGGGCGT Vector construction for subcellular localization in tobacco CP:3PinF ACCCGCGGGGTGAGGGGCCGCGCGGGGGGGGGGGGGGGG	Cf2-NF	GAGCCACCTTCTGACACATAGC	Detection of deletion mutant
CP2-CF TGTTACTGCTGGGCTCGAGGTCCCTTGATGGATCTGGTCT AntpactGGAATGAATTGTTGGGTCGGCGGGATCCATGGCCTCGGAAAGGGGTC CP2BinF ACCCCGGGGGTCGACGGATCCATGGCCTCGGAAAGGGGCCT Vector construction for subcellular localization in tobacco CP2BinF GAGCCTCTTGGAGGATCCATGGCCTCGGAAAGGGGCCT Vector construction for subcellular localization in <i>V. dahliae</i> CP3PF GAGCCTGCTGGACCGATAGC Amplification of <i>VdC2</i> gene probe in Southern blot CP3PF GAGCCTGCTGGACCGATAGC Amplification of <i>VdC2</i> gene probe in Southern blot CP3PF GAGCCACCTGGTGACCGATAGC Amplification of <i>VdC2</i> gene probe in Southern blot CP3PF GAGCCACCTGGATCGATCAGCCCCGGAAAGGGTC Vector construction for protein punification CP3PF GCCCATACAAGGCAACCAC Vector construction for protein punification CP3PF CCGCGGGGATCGATGGACCAACCACCCATGCCGCTGGAGCGGG Vector construction for protein punification VdC2P-11 CCGCGGGGATCGATGGACCAACCACCCATGCTCGATGCGC Vector construction for protein punification VdC2P-11 CCGCGGGGATCGATGGAGCAACCACCCCCGATGGCCCGAGGGGG Vector construction for brotein punification VdC2P-11 CCGCGGGGTCGAAGGCGGACCACCACCCCCCGATTGCCCCACCACCAGGCGGTCAACGCGCGGAGGGGGGAGGGGTC Vector construction for brotein-encoding VdC2P-11 CCGCGGGGTCCAAGCGGGCGGGAGCGGGCGGGGGGGCGGGGGCGGGAGGGGGG	Cf2-NR	GAAGCCTGCGTGACTCGTAAG	
CP-CRATTACCCCGANTTCANTCCTCCCTCCTTCCTTCCTTCATTCATCP2pBinFACCCCCCGGCTCCACCGACTCATCCCTCTGCCCTCGAAGGGCCTVector construction for subcellular localization in tobaccoCP2pBinFACCCCTCGGCGACTCCACCCCCGCGCGCGAGGGGCCTVector construction for subcellular localization in V. dahlaceCP2nFGCAGCTCTGCCACCATACCAAmplification of VdC2 gene probe in Southern blotCP2nFGCAGACCGGGACTCGACCCGCACCACCACAmplification of VdC2 gene probe in Southern blotCP2nFGCAGACAGGACACCACCACSouthern blotCP2nFGCCGCGTGGACTCGACCCGCACCACCACSouthern blotCP2nFGCCGCGTGGACCCGCGAGTCGACCCCCGGAATCATGAGGCGCACCACCACCCCGGACTCGAGGGGTGVector construction for protein purificationCP2nFGCCGCGTGGACCGCGAGGGGACTCATGCGCCGGAAGGGTGVector construction for protein purificationCP2nFGCCGCGTGGACCGCGAGGCGCCCGCGGAGGGGTGACGCGTGAGGGGTGVector construction for protein purificationVdC2n+1CCCGCGTGGACCGCGAGGCGACCCACACCCCGCGGGAGGGGAmplification of VDAG_02735 promoter probe in EMSAVdC2n+1CCCGCGTGGCCGCGGAGGCGACCCACCValidation of the transcriptome data in RT-qPCRVdC2n+1CCGCGGTGCCGGAGGGAGGCACCCACCCACCCACCCCCCGCACCCCCGCACCCCCGGACCCCCGCGACCCCCC	Cf2-CF	TGTCTACTGCTGGCCTCGAGGTCCGTTGATGGATGTGGTGTT	Amplification of VdCf2 gene with native promoter
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CPDpFAACCTCTCAGAGGACCCCCCCCCCCCCCCCCCCCCCCCC	Cf2pBinR	TCTAGTTCATCTAGAGGATCCTGCCTTGGGCGTGAGGGCCT	
CF2pRCGACCTTCTGCGAATTCTGCCTGGACGCTGAGGGCCTCF2+NFGAGCCACCTTCTGAACATAGCAmplification of V/C/2 gene probe in Southern blotCF2+NFGAGCCACCTCGTGACACGAGGSouthern blotCF2-NFCCCGCTGGATCCCGGAATTCATGGCCTCGGGAAAGGGTCVector construction for protein purificationCF2pGexFCCGCGTGGATCCCCGGAATTCATGGCCTCGGGAAAGGGTCVector construction for protein purificationCF2pGexFCCGCGTGGATCCCCGGAATTCATGGCCTCGGGCGTGAGGGVector construction for protein purificationV4CPA-11CCGCGTGGATCCCCGGAATTCATGGCCTCGGACCCCCGTAAmplification of VDAG_02735 promoter probe in EMSAV3755prafCTCGATGACAATGACGACAmplification of the transcriptome data in RT-qPCRV2735prafCAGGCTCAAGCCAACTCATValidation of the transcriptome data in RT-qPCRV2735prafCCGACTTCAACGCGACCCAACCAValidation of the transcriptome data in RT-qPCRV2735prafCCGACTTCCTCTCCCTTAValidation of the transcriptome data in RT-qPCRV2735prafCCGCATCGCAGCCAACCAAValidation of the expression level of secret proteinV3735prafTCGCATGCTCCTCCTTGGGValidation of the expression level of secret protein-V3735prafCCGCATCGCACTCGATCGCATCACValidation of the expression level of secret protein-V3735prafCCGCACTCGCATCGCATCGCATCACValidation of the expression level of PHI protein-encodingV3735prafCCGCACTCGCATCGCATCGCACCValidation of the expression level of PHI protein-encodingV3735prafCCGCACTCGCATCGCATCGCCACCValidation of the expression level of PHI protein-encodingV3735prafCCCACTCGCATCGCATCGCCACCValidation of the expr	Cf2pF	AACCTCTAGAGGATCCGCCACCATGGCCTCGGAAAAGGGTTC	Vector construction for subcellular localization in V. dahliae
CP2-NF GAGCCCCCCTCTICTGACACATAGC Amplification of Vg/C2 gene probe in Southern blot CP2-NF GCAGACAGGCAACCAAGGCAACCA Amplification of Vg/C2 gene probe in Southern blot CP2-GENER GCTCCATACAGCCAACCAC Southern blot CP2-GENER GATGGCGCCCCCGGGATTCATGGCCTCGGCAGAAGGGGTTC Vector construction for protein purification CP2-GENER GATGGCGCCCCTCGGGATTCATGGCCTCGGGCGGAGGG Vector construction for protein purification VdCP.+1 CGCGGTGGACTCCTGAGTCGACCCACCCCGGATGGCGCCGAGGG Amplification of VD/G_02735 promoter probe in EMSA VdCP.+1 CGCGGTGGACTCTCAAGCCCC Validation of the transcriptome data in RT-qPCR VdCP.41 CAGGCTCAAGCGAACCAC Validation of the transcriptome data in RT-qPCR Vd2735-gF CCAATGCGCGACTCTAAGG Validation of the transcriptome data in RT-qPCR Vd280-qF ACATGTACCGAGAGAACCA Validation of the transcriptome data in RT-qPCR Vd280-qF CCAATCGTCACGGCACCTCC Validation of the transcriptome data in RT-qPCR Vd280-qF CCGACTTCCGCGAACCTCCCC Validation of the transcriptome data in RT-qPCR Vd280-qF CCGACTTCCGTCGGGAGCCACCCCCC Validation of the transcriptome data in RT-qPCR Vd280-qF TGCATGCGGCGCCCCCCCCCCCCCCCCCCCCCCCCCCCC	Cf2pR	GCAGCTTCTGCGAATTC TGCCTTGGGCGTGAGGGCCT	
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HYG-FGCAGACAGGAACGAGGACATAmplification of hygomycin-resistant gene probe inHYG-RGCTCCATACAAGCCCAACCACSouthern blot(CPgGesk7CCGCGTGGATCCCCGGGAATCATGAGCCACACGCGGVector construction for protein purification(YdC2-F1CCGCGTGGATCCCCGGAATCATGAGCCAACCCATGGCGGCAGGGGVector construction for protein purification(YdC2-F1CCGCGTGGATCCCCGGAGTCGACCCATGCTCGACCGATGGCCAmplification of VDAG_02735 promoter probe in EMSA(27355proRCTTGGATGACAATGACGACAmplification of VDAG_02735 promoter probe in EMSA(27355qrdCATGCGCGCGTGGAACGACGAValidation of the transcriptome data in RT-qPCR(2735-qfCAGGCTGAGAGGAAGCAAValidation of the transcriptome data in RT-qPCR(2289-qRGGGGTACCGTGACAACGAValidation of the transcriptome data in RT-qPCR(2289-qRGGGGTACCGTGCAACGAAGCAValidation of the transcriptome data in RT-qPCR(2289-qRGGGGTACCGTGCACAGCAAGCAValidation of the expression level of secreted protein-(2755-qFTTGGACCGCCACGAAGCAACCCValidation of the expression level of secreted protein-(2755-qFATGGAGCCCATCATGCTGGValidation of the expression level of Secreted protein-(2755-qFATGGAGCGCCATCGTGGGValidation of the expression level of Secreted protein-(2755-qFATGGAGCGCCATCGTGGGValidation of the expression level of PHI protein-encoding(2875qRATGCAGCGGGGAACGAValidation of the expression level of PHI protein-encoding(2875qRATGCAGCGGGGAAATGGTGValidation of the expression level of PHI protein-encoding(2875qRATGCAGCGGGGAAATGCGGValidation of	Cf2-NR	GAAGCCTGCGTGACTCGTAAG	
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Cr2pGexR GATCCGACTCGACTCGACTCGACTCATCGCGTCAGGG V4C12-F1 CCGCGTGGACTCGCGACTCGACCACAGTCGCCCGTA V4C12-F1 CCGCGTGACACATCGACCACAGTCGCCCGTA V4C12-F1 CCGCGTGCACATCGCACCCACCGCCGTA V4C12-F1 CCGCGTGCACATGCGCACCCGCGTG 02735-qF CTGGTGGCGTGGGGTG 02735-qF CATGCGACTCAACGTCC Validation of the transcriptome data in RT-qPCR 02735-qF CATGCGACGCGACTCAT 06200-qF TCGCATGCGGGTGGAACAGG 06200-qF TCGCATGCGGTGCTAACGTG 06200-qF TCGCATGCGGTGCTAACGG 06200-qF TCGCATGCGGTGCTAACGG 06200-qF TCGCATGCGGTGCTAACGG 06200-qF TCGCATGCGGTGCTGACGGG 07289-qF ACATGTACGCGACGAACGA 07289-qF ACATGTACGCGACGAACGA 07289-qF ACATGTACGCGACGAACGA 07289-qF ACAGGCGCGCGTGCTTGG 09018-qF TGGGCGTGCCGTGGTG 09018-qF GGGGTGCGCGCACTCACG 09018-qF GATGGACGCCACTCTCACC 09018-qF CCCACTGCGGTGGGGG 09018-qF CCCACTGCGGTGGGGG 09018-qF CCCACTGCGGTGGGGG 09018-qF CCCACTGCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	Cf2pGexF	CCGCGTGGATCCCCGGAATTC ATGGCCTCGGAAAAGGGTTC	Vector construction for protein purification
VdC12+1 CCGCGGGATCCCCGGAATTCAIGAACAACGTCGCCGATCGCCGATCGCCG VdC12+R CGCGATGACAGCGGCGCGCGCGCGCGCGCGCGCGCGCGCG	Cf2pGexR	GATGCGGCCGCTCGAGTCGAC TCATGCCTTGGGCGTGAGGG	
 VdC12-RI GATGCGGCCGACTCGAAGCCAACCCACCATGCACCCATGCTCGATCGCC Amplification of VDAG_02735 promoter probe in EMSA Q2735-qF CATGCGCAATGCAGGTG Validation of the transcriptome data in RT-qPCR Q2736-qF CCAATGCGGTGTCAAACAGG Validation of the transcriptome data in RT-qPCR Q2736-qF CCAATGCGGTGTCAAACAGG Validation of the transcriptome data in RT-qPCR Q2736-qF CCAATGCGGTGTCAAACAGG Q289-qF ACAGGTCGGTGTCTCAGGGT Q289-qF ACATGTACGGCGAAGAGAACA Q289-qF CGGTGTTCTTCGTGGGCT Q289-qF CGGTGGTGTCTCAGGGTT Q275-qF TGGTGCGTGGTGGCTGGGT Q275-qF AGGCCGCACTAAGTCACC Q275-qF AGGCGGCGCCGCGCGGCG Q289-qF AGGCGCGCTCGATCGGCTGGTGG Q275-qF AGGCCGCATCAGTCGACC Q275-qF AGGCGCGCATCAGTCGGCG Q275-qF AGGCGCGCATCAGGTGG Q289-qF AGGCGCGCATCAGGTGGC Q289-qF AGGCGCGCATCAGGTGGCG Q289-qF AGGCGCGCATCAGGTGGCG Q289-qF AGGCGCGCATCAGTGGGCG Q289-qF ATGGCGGAAAGCCAGGGGAAATGCCA Q289-qF AGGCGGGGAAAATGCAGGCG Q289-qF AGGCGGGGAAAATGCAGCA Q289-qF AGGCGGGGGAAAATGCAGCA Q289-qF AGGCGCGGGGGAAAATGCCAGCA Q289-qF AGGCGGGGGGGGGAAAGCAGA Q289-qF AGGCGGGGGGGAAAGCCA Q289-qF AGGCGGGGGGGGGAAAGCAGA Q289-qF AGGGGGGGGGGGGAAAGCCA Q289-qF AGGGGGGGGGGGAAAGCCA Q29-QF GCACATGGAGGGGAAAATGCAAGCA Q29-QF GCAAGGGGGGGGGGGAAA Q29	VdCf2-F1	CCGCGTGGATCCCCGGAATTC ATGAAGCACAAGTGCCCGTA	
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D2/35prok111Cat G1 TGGG GAGG T1GD2/35s-qRCATTGCCGCACTCAAGGGGACTCATD6200-qFTCCAATGCGTGTCAAACAGGD6200-qFTCGCATTTCCCTCTGGGTAD2289-qFACATGTACCGAGACGAAGCAD2289-qFCAGTGTCCTTCACTGGG10037-qFTGTGTGTGTCTTCACTGGG10037-qFTGTGTACGCCAGCAAACTT09577-qFTATCCGAGCCCAGGAACTT09577-qFACGACCACCATCAGTCAACC07255-qFGAGGCTCGACTACCAGTCA0918-qFGATGGACGCATCCCATCAGCT0918-qFCCCCCTTTCTCCGATGGCT06155qFATCAAGGGTGTCTCGCGGGAGGAA06155qFTCCCAGCGCAGCAAATCT06155qFACCAATCTCCGAGGCATAGCAC0828qAACGTGCGGAGCATAGCAC0808qACCCGCCTTCGGAGGCATAGCAC0808qBACCGGCGGACAAGCCACCCCAGGAAATCC0808qBACCGACCGGGAAAATCCAC0808qBCCCGCCTGCGCAGCAAACTCC0808qBCCCGCCTGCCGCACCAAGGCAC07510qFCAGCAATCAGGTCGCCGCC07610qFCAGCATTCAGTCGGCGCAA02353qFGCAAATGGAAGCCACCACC07610qFCAGCAATTGCGTGGAGGGAAA02353qFGCAAATGGAAGCCACCAC07610qFCAGCCTGGGAATGGCGAA02353qFGCAAATGGAGGCAAGCGG02353qFGCAAATGGAGGAAATCGCCAC07296qFGTGCCCGGAATGGAGGAAA07296qFGTGCCCGGAATGGAGGAAA07296qFGTGCCCGGAATGGAGGAAA07296qFGTGCCCCGAGATGGACCCTCC07296qFGTGCCCCGAGATGGACCCTCC07296qFGTGCCCCGAGATGGACCCTCC07296qFGTGCCCCGAGATGGACCCTCC07296qF	02735FamF	CTCGATGACAATGACGAC	Amplification of VDAG_02735 promoter probe in EMSA
02735-qP CATGCCGACTCGAGCGAACTCAT 02209-qF TCCAATGCGTGTCAAACAGG 02209-qF CCCAATGCGAGCGACACTCAT 02209-qF ACATGTACGCAGACGAACA 02289-qR GGGGTACCGTGACTTCTTGG 10037-qF TGCTGCTTGTGTCTCACTGGG 10037-qF TGCTGCTTGTGTCTCACTGGG 10037-qF TGCTGCGTGTGTCTCACTGGG 10037-qF ACGACCACCACCACTGGCT 09577-qF ATCCGACGCCAGCAAACTT 09577-qF ACGGCCCACCACTCAGTCACC 07255-qF ATGGCCGACATCCTCATCAC 07255-qF ATGGCCGACATCGTCACC 07255-qF ATGGCCGACATCGTCACC 09018-qR CCCCCTTTTCCGCAGCG 09018-qR CCCCCTTTTCCGAATGCT 09018-qR CCCCCTTTTCCGAGGGCGA 09018-qR CCCCCTTTTCCGAGGGCGA 09018-qR CCCCCTTTCGCGAGGGCGA 09018-qR CCCCCTTTCGCGAGGGCGA 09018-qR CCCCCTTTCGCGAGGGCGA 09018-qR CCCCCTTTCGCGAGGGCGA 09018-qR CCCCCTTTCGCGAGGGCGA 09018-qR CCCCCTTTCGCGAGGGCGA 09018-qR CCCAACGTCCGTCGAGGG 09018-qR CCCAACGTCCGTGGGGC 09018-qR CCCAACGTCCGTGGGGCGA 09018-qR CCCAACGTCCGTGGGGCGA 09018-qR CCCAACGTCCGTGGGGCGA 09018-qR CCGACGGGACATAGGTGG 09028qF TCCCCACGTGAGGGCGA 09019 09028qF CCCCACTCGGTGGGGCGA 09031qR CCGACGTCGACACTGGTG 09031qR CCGACGTCGGGGACAAATCCTTC 09031qR CCGACGTGGGGGCGAAATCCTTC 09031qR CCGACGTGGGGGGCGACA 09031qR CCGGGGGGGGGGGGGGCG 09031qR CCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	02/35proR		
02735-qR CAGGC (CAGGGCGAAC (CA) 02200-qR TCGATGCGGTGCAACAGG 02200-qR TCGCATGCGGTGCAACAGG 02289-qF ACATGTACGGCGGAACGAAGCA 02289-qF ACATGTACGGTGAATTCTTGG 10037-qF TGGTGCGTGTGCTTGGG 10037-qR TGGTACCGGCAAGCAACTT 09577-qF ACGCGCACCACAGGCCAGCCAACT 09577-qF ACGACCCACCACAGTGCACC 07255-qF AGGCGGACGCCAGCCAGCCAGCC 07255-qF AGGCGCGACGCTGTGC 09018-qF GATGGCGCGCCTCTGC 09018-qF GATGGCGCGCATCATCGCT 09018-qF CCACGTTTTGCGATGGCT 09018-qF CCACGCTCATCGACG 09018-qF CCACGCTCATCGATGGC 09018-qF CCACGCTCATCGATGGC 09018-qF CCCCGTTTTGCGAGGGC 09018-qF CCCCCGTTTTGCGACGGC 09018-qF CCCCCGTTTTGCGACGGC 09018-qF CCCCCGTTTTGCGACGGC 09018-qF CCCCACGTCACCCC 09018-qF CCCCCGTGCTCGATGGC 09018-qF CCCCCGTGCACGCC 09018-qF CCCCCGTGCACGCAGCCCC 09018-qF CCCCACGCCACTGCGC 09018-qF CCCCCGGGAAAATCGTC 09018-qF CCCCCGGGACATGGCC 09018-qF CCCCCGGGAAAATCGTC 09031qF CAAGATTCGGTGCGCACC 09031qF CAAGATTCGGTGGGGACAA 02353qF GCCCGGCAAATCGACC 096031qF CAAGATCGGGGAGAATCCTC 09033qF CCCCCGGGAAAATCGAC 096031qF CAAGATCGGGGGAGAA 02353qF GCCCGGCAAATCGACCA 096031qF CAGCATTCGGTGGCCAC 096031qF CAGCATTCGGTGGCCAC 096031qF CAGCATTCGGTGGCGCA 096031qF CAGCATTCGGTGGCAAA 02353qF GCCCGGAAAATCGACCCA 096031qF CAGCATTCGGTGGCACA 096031qF CAGCATTCGGTGGCAAA 02353qF GCCCGGAAATCGATCGGCG 098088qF CCCTCCACCCACCACG 096031qF CAGCAATCGGTCGCTC 098088qF CCCCGCACCACGGACAA 02353qF GCCCGGAAATCGTCCGCCC 096031qF CAGCAATCGGTGGCGGAAA 02353qF GCCCGGAAGTGGGCGAA 02353qF GCCCGGAAGTGGGCG 098088qF CCTCACCCCGCACCACGG 098088qF CCTCACCCCACCACAGG 09888qF CCCCCGCACCGCGCGCG 09888qF CCCCCGCACGCGGCG 09888qF CCCCCGCACGGAGGGCG 09888qF CCCCCGCACGGGAGGG 09888qF CCCCCGCACGGGAGGG 09888qF CCCCCGCACGGGGAAA 07296qF GGTCCCCGGAAGGGCG 07296qF GGTCCCCGGAAGGGCG 07296qF GGTCCCCGGAGGAGGCG 07296qF GGTCCCCGGAGGAGGCG 07296qF GGCCGCCCCGCCCCCCCCCCCCCCCCCCCCCCCCCCC	02/35-qF		Validation of the transcriptome data in RT-qPCR
002200-qR TGCGATTTTCCCTTGGGTA 02289-qF ACATGTAGCAGAGGAAGCA 02289-qF GGGGTACCGTGATTTTGG 10037-qF TGCTGCGTTGTCTTCACTGGG 10037-qR TGGTACCGCTCATGGCTTT 09577-qF TATCCGCAGCCAGCAACT 09577-qF ACGACGCCAGCAACT 09577-qF ACGACGCACGCAGCAACT 09018-qF GATGGACGCCACTCTGTG 09018-qF GATGGACGCCCATCATCGACT 09018-qF CCCCCTTTCTCGAGTGC 09018-qF CCCCCTTTCTCGAGCG 09018-qF CCCCCTTTCTCGACGCG 09018-qF CCCCCTTTCTCGACGG 09018-qF CCCCCTTTCTCGACGG 09018-qF CCCCCTTTCTCGACGG 09018-qF CCCCCTTTCTCGACGG 09018-qF CCCCCTTTCTCGACGG 09018-qF CCCCCTTTCTCGACGG 09018-qF CCCCCTTTCTCGACGG 09018-qF CCCCCGGTCTCGGCG 09018-qF CCCCCGGACATGTCA 09018-qF CCCCCGGACATGTCA 09018-qF CCCCCCTTCTCGACGGG 09018-qF CCCCCCTTCTCGCGG 09018-qF CCCCCGGACAATGCCA 09019 08276qF ACCTAGTGCCGGACAATGCAC 09019 08276qF ACCTAGTCGGACGCACTGGC 08028qF TCCCCCGGGACAATGCAC 09019 08028qF CCCCACGGACAATGCAC 09010 08028qF CCCCACGGGAAATGCAC 09010 08028qF CCCCCGGGACAATGCAC 09010 08028qF CCCCCGGGACAATGCAC 09010 09018-QF CAGCGGAAATGCAAGCCA 097010 09018-QF CAGCAGTCGGGCAAA 072050qF CCCCGGACGCAAATGCAAGCCA 07610qF CAGCGCAAATGCGAGGC 07610qF CAGCGCACTGCGTC 092535qF CCCAATGCGGCACA 07610qF CAGCACTGGATGGCAAA 072550qF CCCCGCACTGCGCC 072550qF CCCCGCACTGCGTC 08888qF CGTACAGCGCCGCAC 072550qF CCCCACTGCAGGAGAATCGGC 08888qF CGTCAAGCTCGGACAGGG 08888qF CGTCAAGCTCGGACGC 098888qF CGTCAAGCTCGGACGC 098888qF CGTCAAGCTCGGCC 098888qF CGTCAAGCTCGGACGCG 098888qF CGTCAAGCTCGGACGCG 098888qF CGTCAAGCTCGGACGGAGAATCGGC 098888qF CGTCAAGCTCGGACGCG 098888qF CGTCAAGCTCGGACGCG 098888qF CGTCAAGCTCGGACGCG 098888qF CGTCAAGCTCGGACGCGC 098888qF CGTCAAGCTCGGACGCG 098888qF CGTCAAGCTCGGACGGAAATCGGC 098888qF CGTCAAGCTCGGACGCGC 098888qF CGTCAAGCTCGGACGCGC 098888qF CGTCAAGCTCGGACGCGCCCCCCCCCCCCCCCCCCGGAGGGAG	02/35-qR		
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02299-qP ACATGTACGLAGACGAAGCA 2289-qR GGGTACCGTACTCTTGG 10037-qF TCGTCGTTGTCTTCACGGG 10037-qR TGTACCGCTCATGGCTTT 09577-qR ACGACCACCGACGCAACCT 09577-qR ACGACCCACCATCATCACC 07255-qR AGGGCCGCACTACGTCACC 09018-qF GATGGACGCCATCATCGACT 09018-qR CCCCCTTTCTCGGATGGTG 09018-qR CCCCCTTTCTCGAATGCT 06155qF ATCAAGGGTGTCTTCGGACGG Validation of the expression level of secreted protein- 06155qF ATCAAGGGTGTCTCGAGAGG 08076qF ACCAACGTTCTGGTGGCC 080826qF ACCAACGTTCGGTAGCCGA 08028qR ACGGGGGCACTACTGATG 08028qR ACCGACGGGAAAATCCTTC 06031qF CAAGACGTCTCGGTAGCCAC 07610qF GGATGGGAGGCCACC 07610qF GGATGGGAGGCAAGCCA 07610qF GGATGGGAGGCAAGCCA 07610qF GGATGGGAGGCAAGCCA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAAG 07610qF GGATGGGAGGAAAGCCA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAAGACCCA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGGCAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGGGGGAAA 07610qF GGATGGGGGGGCAAA 07610qF GGATGGGGGGGAAA 07610qF GGATGGGAGGAAA 07610qF GGATGGGGGAGAAA 07610qF GGATGGGGGAGAAA 07600qF GGATGGGGGGGGAAA 07600qF GGGCGGGGGGGAAA 07600qF GGGCGGAGGGGGAAA 07600qF GGGGGGGGGAAA 07600qF GGGGGGGGGAAA 07600qF GGGGGGGGGAAA 07600qF GGGGGGGGGAAA 07600qF GGGGGGGGGGGGGAAA 07600qF GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	06200-qK		
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09018-qRCCCCCTTTTCCCGAATGCT06155qFATCAAGGGTGTCTTCGACGGValidation of the expression level of secreted protein-06155qRCCAAACGTTCTCGATGGTGCencoding genes in RT-qPCR08876qFACCATATTGCTCGAGGCGAValidation of the expression level of PHI protein-encoding08876qRATGTCGCGGAGCATAGTCACgenes in RT-qPCR08028qFTCCCCACGTCACACTGATTGgenes in RT-qPCR08028qRACCGACCGGGAAAATCCTTCgenes in RT-qPCR06031qFCAAGATCTCGGTCCTCGCTC06031qR06031qFCAAGATGGAAAATGCAAGCCA07610qFCAGCAATTCAGTTCGCGCAC07610qFCAGCAATTCAGTTCGCGCAC07610qFCGAATACGACGCTCCGTTC02535qF02535qFGCCAAATACGACGCTCCGTTC02535qF02535qFGCCGACTTGCTAGTGACTT08888qR027296qFGTGTCCCCGGAATGAGGCG08888qR07296qRGTCCACGCAAGGCCG07296qR07296qRCTGCGCATCGATCGGCT04828qF07296qRGTCAAGCTCCAGATCGGC04828qFCGTAAGCTCCAGCTCGGCT04828qFCGTCAAGCTCCAGATCGCC04828qRCTGCAGCAACGCGATCGCAT04714qFAGCAAGACTCAGCTCGCAT04714qFGGAAGGACTCAGCTCACATT0738aFGACCGCCAAGTCTACATCCC	09018-qF	GATGGACGCCATCATCGACT	
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06155qRCCAAACGTTCTCGATGGTGCencoding genes in RT-qPCR08876qFACCATATTGCTCGTAGGCGAValidation of the expression level of PHI protein-encoding08876qRATGTCGCGGAGCATAGTCACgenes in RT-qPCR08028qFTCCCCACGTCACACTGATTGgenes in RT-qPCR08028qRACCGACCGGAAAATCCTTCgenes in RT-qPCR0801qRCAAGATCTCGGTCCTCGCTCgenes in RT-qPCR06031qRGCACTGGAAAATGCAAGCCAgenes in RT-qPCR07610qFCAGCAATTCAGTTCCGCCACgenes in RT-qPCR07510qFCAGCAATTCAGTGCGCACAgenes in RT-qPCR07510qRTGGATTCGGTGGATGGCAAAgenes in RT-qPCR02535qFGCAATAACGACGTCCGTTCgenes in RT-qPCR02535qFGCAAATACGACGCTCCGTTCgenes in RT-qPCR02535qRGCCCGACTTGGTAGTGACTTgenes in RT-qPCR08888qRCGTAAAACGCAGATGAGCGGgenes in RT-qPCR07296qFGTGTCCCCGGAATGAGGAAAgenes in RT-qPCR07296qRGCTCCAGGAGAAATCGGCgenes in RT-qPCR04828qRCTGACGCGAATCAGGCCGgenes in RT-qPCR04828qRCTGGCGCATCCAGATCGCCgenes in RT-qPCR04714qFAGCAAGAACTCAGCTCGCATgenes in RT-qPCR04714qRGGAAGGCTCAACTGTGTAgenes in RT-qPCR07838qFGACCGCCAAGTCTACACTCCgenes in RT-qPCR07838qFGACCGCCAAGTCTACATCCCgenes in RT-qPCR	06155qF	ATCAAGGGTGTCTTCGACGG	Validation of the expression level of secreted protein-
08876qFACCATATTGCTCGTAGGCGAValidation of the expression level of PHI protein-encoding08876qRATGTCGCGGAGCATAGTCACgenes in RT-qPCR08028qFTCCCCACGTCACACTGATTG08028qRACCGGACGAAAATCCTTC06031qFCAAGATCTCGGTCCTCGCTC06031qRGCACTGGAAAATGCAAGCCA07610qFCAGCAATTCAGTTCCGCCAC07610qRTGGATTCGGTGGATGGCAAA02535qFGCAAATACGACGCTCCGTTC02535qRGCCCGACTTGCTAGGGATGGCAAA02535qRCCCTCACCTCCAGCACGG07296qFGTGTCCCCGGAATGAGGCAAA07296qRGCTCCAGATGAGAAGCCG07296qRCCGTACGGAAGACCCGC04828qFCCGTAAGCTCCGATCTTGC04828qFCCGTACGGAAGAATCTGGC04828qFCGGAACGCGATCGTGTA04714qFAGCAAGAACTCAGCTGGCAT04714qRGGAAGGCGTCCAAGTCGCAT07838qFGACCGCCAAGTCAACTCCC	06155qR	CCAAACGTTCTCGATGGTGC	encoding genes in RT-qPCR
08876qRATGTCGCGGAGCATAGTCACgenes in RT-qPCR08028qFTCCCCACGTCACACTGATTG08028qRACCGACCGGGAAAATCCTTC06031qFCAAGATCTCGGTCCTCGCTC06031qRGCACTGGAAAATGCAAGCCA06031qRGCACTGGAAAATGCAAGCCA07610qFCAGCAATTCAGTTCCGCCAC07610qRTGGATTCGGTGGATGGCAAA02535qFGCCAAATACGACGCTCCGTTC02535qRGCCCGACTTGCTAGTGACTT08888qFCACTCTCACCTCCAGCAAGG07296qFGTGTCCCCGGAATGAGGAAA07296qRGCTCCAAGTCCCAGTCCTC04828qFCCGTACGGAGAAATCTGGC04828qRCTGGGCGATCCGGTTT04828qRCTGGCGCATCCGGC04714qFAGCAAGACTCAGCTGCAT04714qRGGAAGGCGTCCAACTGTGTA0738qFGACCGCCAAGTCACTCC	08876qF	ACCATATTGCTCGTAGGCGA	Validation of the expression level of PHI protein-encoding
08028qFTCCCCACGTCACACTGATTG08028qRACCGACCGGGAAAATCCTTC06031qFCAAGATCTCGGTCCTCGCTC06031qRGCACTGGAAAATGCAAGCCA07610qFCAGCAATTCAGTTCCGCCAC07610qRTGGATTCGGTGGATGGCAAA02535qFGCAAATACGACGCTCCGTTC02535qRGCCCGACTTGCTAGTGACTT08888qFCACTCCACCTCCAGCAAGG08888qRCGTAAAACGCAGATGAGCCG07296qFGTGCCCCGGAATGAGGAAA07296qRGCTCAAGCTCCAGTCGGC04828qRCGTAAGAGCTCCAGTCGGC04828qRCTGACGGAAATCTGGC04714qFAGCAAGAACTCAGCTCGAT04714qRGAAAGCGCTCCAACTGTGTA0738aqFGACCGCCAAGTCTACATCCC	08876qR	ATGTCGCGGAGCATAGTCAC	genes in RT-qPCR
08028qRACCGACCGGGAAAATCCTTC06031qFCAAGATCTCGGTCCTCGCTC06031qRGCACTGGAAAATGCAAGCCA07610qFCAGCAATTCAGTTCCGCCAC07610qRTGGATGGATGGCAAA02535qFGCAAATACGACGCTCCGTTC02535qRGCCCGACTTGCTAGTGACTT08888qFCACTCCACCTCCAGCAAGG08888qRCGTAAAACGCAGAGGAGAAA07296qFGTGTCCCCGGAATGAGGAAA07296qRGCTCCAGGAGGAGAAA04828qRCCGTAAGCTCCAGTCCCTC04828qRCTGGCGCATCCGATCTTTG04714qFAGCAAGAACTCAGCTGGCAT04714qRGAAGGCGTCCAACTGTGTA0738qFGACCGCCAAGTCTACATCCCC	08028qF	TCCCCACGTCACACTGATTG	
06031qF CAAGATCTCGGTCCTCGCTC 06031qR GCACTGGAAAATGCAAGCCA 07610qF CAGCAATTCAGTTCCGCCAC 07610qR TGGATTCGGTGGATGGCAAA 02535qF GCAAATACGACGCTCCGTTC 02535qR GCCCGACTTGCTAGTGACTT 08888qF CACTCTCACCTCCAGCAAGG 07296qF GTGTCCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGTCCGC 04828qF CCGTACGGAGAGAATCTGGC 04828qF CCGTACGGAGAGAATCTGGC 04828qF CGGAAGAACTCAGCTCGAT 04828qF GGGAGAAATCTGGC 04828qF GGGCGCATCCGATCTTTTG 04714qF AGCAAGAACTCAGCTGGCAT 04714qF GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	08028qR	ACCGACCGGGAAAATCCTTC	
06031qR GCACTGGAAAATGCAAGCCA 07610qF CAGCAATTCAGTTCCGCCAC 07610qR TGGATTCGGTGGATGGCAAA 02535qF GCAAATACGACGCTCCGTTC 02535qR GCCCGACTTGCTAGTGACTT 08888qF CACTCTCACCTCCAGCAAGG 07296qF GTGTCCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGTCCCTC 04828qF CCGTAAGACTCCAGATCGGC 04828qF CCGTACGGAGAGAAATCTGGC 04828qR CTGGCGCATCCGATCTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACACTCCC	06031qF	CAAGATCTCGGTCCTCGCTC	
07610qF CAGCAATTCAGTTCCGCCAC 07610qR TGGATTCGGTGGATGGCAAA 02535qF GCAAATACGACGCTCCGTTC 02535qR GCCCGACTTGCTAGTGACTT 08888qF CACTCTCACCTCCAGCAAGG 07296qF GTGTCCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGATGAGCCG 04828qF CCGTAAGACTCCAGATCCCTC 04828qF CCGTACGGAGAGAAATCTGGC 04828qR CTGGCGCATCCGATCTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	06031qR	GCACTGGAAAATGCAAGCCA	
07610qR TGGATTCGGTGGATGGCAAA 02535qF GCAAATACGACGCTCCGTTC 02535qR GCCCGACTTGCTAGTGACTT 0888qF CACTCTCACCTCCAGCAAGG 0888qR CGTAAAACGCAGATGAGCCG 07296qF GTGTCCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGATCGCC 04828qF CCGTACGGAGAGAATCTGGC 04828qR CTGGCGCATCCGATCTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	07610qF	CAGCAATTCAGTTCCGCCAC	
02535qF GCAAATACGACGCTCCGTTC 02535qR GCCCGACTTGCTAGTGACTT 0888qF CACTCTCACCTCCAGCAAGG 0888qR CGTAAAACGCAGATGAGCCG 07296qF GTGTCCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGATCGCC 04828qF CCGTACGGAGAGAAATCTGGC 04828qR CTGGCGCATCCGATCTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	07610qR	TGGATTCGGTGGATGGCAAA	
02535qR GCCCGACTTGCTAGTGACTT 0888qF CACTCTCACCTCCAGCAAGG 0888qR CGTAAAACGCAGATGAGCCG 07296qF GTGTCCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGATCCCTC 04828qF CCGTACGGAGAGAAATCTGGC 04828qR CTGGCGCATCCGATCTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	02535qF	GCAAATACGACGCTCCGTTC	
08888qF CACICICACCICCAGCAAGG 08888qR CGTAAAACGCAGATGAGCCG 07296qF GTGTCCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGATCCCTC 04828qF CCGTACGGAGAGAAATCTGGC 04828qR CTGGCGCATCCGATCTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	02535qR	GCCCGACTTGCTAGTGACTT	
08888qR CGTAAAACGCAGATGAGCCG 07296qF GTGTCCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGATCCCTC 04828qF CCGTACGGAGAGAAATCTGGC 04828qR CTGGCGCATCCGATCTTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	08888qF	CACTCTCACCTCCAGCAAGG	
07296qF GTGTCCCGGAATGAGGAAA 07296qR GCTCAAGCTCCAGATCCCTC 04828qF CCGTACGGAGAGAATCTGGC 04828qR CTGGCGCATCCGATCTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	08888qR		
07250qn GCTCAGGTCCAGATCCTC 04828qF CCGTACGGAGAGAATCTGGC 04828qR CTGGCGCATCCGATCTTTG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	0/296qF		
04828qFCCGTACGGAGAGATCTGGC04828qRCTGGCGCATCCGATCTTTG04714qFAGCAAGAACTCAGCTCGCAT04714qRGGAAGGCGTCCAACTGTGTA07838qFGACCGCCAAGTCTACATCCC	0/296qK		
04620qn CroccCATCCOATCITIG 04714qF AGCAAGAACTCAGCTCGCAT 04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	048289F		
04714qR GGAAGGCGTCCAACTGTGTA 07838qF GACCGCCAAGTCTACATCCC	040209K		
07838qF GACCGCCAAGTCTACATCCC	04714aP		
	078384E		
	07838aP		
00592nE ATCGACGTGTTTCACCCACA Validation of the expression level of transcription factors in	00592aF	ATCGACGTGTTTCACCCACA	Validation of the expression level of transcription factors in
00592qR TACGTGTTACAGCGGTTGCT RT-aPCR	00592qR	TACGTGTTACAGCGGTTGCT	RT-qPCR

TABLE 3 (Continued)

Primer name	Primer sequence (5'-3') ^a	Application
05292qF	ACACTGGTGGATGCTCTTCG	
05292qR	TGTCGAGACTGGGTAGCTGA	
07276qF	CAGGAAGGAAGAAGCCCGAT	Validation of the expression level of gene clusters in RT-
07276qR	CTTGGCCTCTTCAGGCTTGG	qPCR
07277qF	GACCACGTTATAGGCCCCTG	
07277qR	ACATAACGGTCAACAGGCGT	
07278qF	TCGTCGCGAAGCAGAGATAC	
07278qR	TTGCGCTTCGACTTGGTCTT	
07279qF	AGAGAACCCGACGATCCAGA	
07279qR	TCCCATTCGAGTACGTTGCC	
07280qF	CGTATCTGGAACGACTCGGG	
07280qR	ACTGGACAACAGCATCAGGG	
β-tubulin-F	TTTCCAGATCACCCACTCC	Internal control in RT-qPCR
β-tubulin-R	ACGACCGAGAAGGTAGCC	
CF2-BKF	CCATGGAGGCCGAATTCACC ATGGCCTCGGAAAAGGGTTC	Vector construction for yeast two-hybrid assay
CF2-BKR	CGCTGCAGGTCGACGGATCCTCATGCCTTGGGCGTGAGGG	
07276-ADF	GCCATGGAGGCCAGTGAATTC ACCATGGACACCAAG	
07276-ADR	ATGCCCACCCGGGTGGAATTCTCACAGCAAAGCAAGAGTCA	
07277-ADF	GCCATGGAGGCCAGTGAATTC ACCATGGACATCATCGCGCTCCT	
07277-ADR	ATGCCCACCCGGGTGGAATTCTCAGTCCCTGACATACTGGC	
07278-ADF	GCCATGGAGGCCAGTGAATTC ACCATGTCGTCGCGAAGCAGAGA	
07278-ADR	ATGCCCACCCGGGTGGAATTCTTACAGGAGAGCAAGCTCCA	
07279-ADF	GCCATGGAGGCCAGTGAATTC ACCATGCAGACACCCGCTCCTGC	
07279-ADR	ATGCCCACCCGGGTGGAATTC TCACGAACTCGTACCAATAT	
07280-ADF	GCCATGGAGGCCAGTGAATTC ACCATGGCCGACACTACTACGAA	
07280-ADR	ATGCCCACCCGGGTGGAATTCTTATTTGTTCTTTCCCGTGA	
Cf2-NeYFP-F	TCTGAGGAGGATCTTCCTAGGATGGCCTCGGAAAAGGGTTC	Vector construction for bimolecular fluorescence
Cf2-NeYFP-R	GGGAGGCCTGGATCGACTAGT TGCCTTGGGCGTGAGGGCCT	complementation (BiFC)
07276-CeYFP-F	CTAGTCGACTCTAGCCTCGAG ATGGACACCAAGGACCAGCAAC	
07276-CeYFP-R	ATCGTATGGGTACATCCTAGG CAGCAAAGCAAGAGTCAGAT	
07278-CeYFP-F	CTAGTCGACTCTAGCCTCGAG ATGTCGTCGCGAAGCAGAGA	
07278-CeYFP-R	ATCGTATGGGTACATCCTAGG CAGGAGAGCAAGCTCCAGAT	

^aBold letters in the primer sequences represent the recombination sequence.

greenhouse. Wilt development of cotton plants was assessed based on a five-grade infection scale: 0, no symptoms on leaves; 1, one or two cotyledon leaves showing symptoms; 2, a single true leaf showing symptoms; 3, more than two leaves showing symptoms; and 4, plant dead (50). The stems of the cotton plants were longitudinally cut and photographed.

Subcellular localization. The full-length coding DNA sequence (CDS) of *VdCf2* without the termination codon, amplified with primer pair Cf2pF/Cf2pR, was inserted into pNEO binary vector digested with the restriction endonuclease BamHI and EcoRI. The successful construct pNEO-VdCf2-GFP was transformed into *Agrobacterium tumefaciens* EHA105. The agrobacteria containing pNEO-VdCf2-GFP were cocultured with the conidial suspensions of strain Δ VdCf2 to generate the VdCf2-GFP strain. The GFP signal was visualized with a confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan).

The full-length CDS of *VdCf2* without the termination codon was amplified with the primer pair Cf2pBinF/Cf2pBinR and then cloned into the pBin vector digested with the restriction endonuclease BamHI to generate the construct pBin-VdCf2-GFP. The resulting construct was transformed into *A. tume-faciens* GV3101. The agrobacteria containing pBin-VdCf2-GFP or pBin-GFP were injected into the epidermal cells of 4-week-old *Nicotiana benthamiana* leaves. Infiltrated tobacco seedlings were grown at 25°C under a 16/8-h light-dark photoperiod in a greenhouse for another 2 to 3 days before assessment. The GFP signals in tobacco leaves were observed and photographed under a laser scanning confocal microscope (Olympus Corporation, Tokyo, Japan).

Protein extraction and Western blot analysis. Total protein was extracted from *N. benthamiana* leaves using the plant protein extraction kit (Solarbio, Beijing, China) according to the manufacturer's instructions. GFP and VdCf2-GFP fusion proteins were detected with the anti-GFP antibody (Beyotime, Shanghai, China).

Bimolecular fluorescence complementation (BiFC) assay. The full-length CDS of VDAG_07276 and VDAG_07278 was cloned into the pER8-CeYFP vector digested with the restriction endonucleases Xhol and Avrll, respectively. The full-length cDNA of VdCf2 was inserted into the pER8-NeYFP vector digested with Avrll and Spel. The resulting constructs were transformed into *A. tumefaciens* GV3101 (Tsingke, China). The agrobacteria harboring the combinations nYFP-VdCf2 and cYFP-07276, nYFP-VdCf2 and cYFP-07278, nYFP and cYFP-07276, nYFP and cYFP-07278, and nYFP-VdCf2 and cYFP together with P19 were injected into the epidermal cells of *N. benthamiana* leaves. After 2 to 3 days of infiltration, injected leaves were sampled and observed for YFP signals under a laser scanning confocal microscope (Olympus Corporation, Tokyo, Japan).

Electrophoretic mobility shift assay (EMSA). The full coding region of VdCf2 amplified with primer pair Cf2pGexF/Cf2pGexR was inserted into the pGEX-4T-1 vector digested with the restriction endonucleases EcoRI and Sall to generate the prokaryotic expression construct pGEX-VdCf2. The successful construct was transformed into the *Escherichia coli* BL21(DE3) strain. VdCf2 protein was purified via the GST-tag protein purification kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The EMSA experiment was carried out according to the EMSA binding buffer kit (Beyotime) instructions. The 6-carboxyfluorescein (FAM)-labeled probe was amplified with the primer pair 02735FamF/02735proR.

Yeast two-hybrid (Y2H) assay. The full coding region of *VdCf2* amplified with the primer pair CF2-BKF/CF2-BKR was inserted into the pGBKT7 vector digested with the restriction endonucleases EcoRI and Sall to generate the bait construct. The full-length cDNA of *VDAG_07276* amplified with primer pair 07276-ADF/07276-ADR was inserted into the pGADT7 vector digested with the restriction endonuclease EcoRI to generate the prey construct. Generation of prey constructs for other candidate proteins was similar to that for VDAG_07276. The bait and prey constructs were cotransformed into Y2HGold yeast cells. The yeast cells containing bait and prey constructs were cultured on SD base medium without Leu, Trp, and His supplemented with 40 mg/L X- α -Gal to determine their interaction. The yeast cells harboring the bait construct and pGADT7 vector were cultured on SD base medium without Leu, Trp, and His to test self-activation. The yeast cells containing the pGBKT7-53 and pGADT7-T vectors and pGBKT7-Lam and pGADT7-T vectors were regarded as the positive and negative controls, respectively.

RNA-seq analysis. Three fungal mycelial plugs (6-mm diameter) of the wild-type and $\Delta VdCf2$ strains were inoculated into 90 mL liquid CDM and cultured at 150 rpm for 8 days. Fungal mycelia were collected with Miracloth and then rapidly quick-frozen in liquid nitrogen before being delivered to OE Biotech Co., Ltd. (Shanghai, China). Total RNA was extracted with the mirVana microRNA (miRNA) isolation kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. RNA-seq was performed on the Illumina HiSeq X Ten sequencing platform, and 150-bp paired-end reads were generated.

Raw data were trimmed and filtered using Trimmomatic to remove the low-quality reads and ploy-N (unreliable sequences) (60). Clean reads were aligned to the reference genome of V. dahliae strain VdLs.17 with HISAT2, and the fragments per kilobase per million (FPKM) of each gene was estimated with Cufflinks (61-63). The DESeq (2012) R package functions estimateSizeFactors and nbinomTest identified differentially expressed genes (DEGs) in the mycelia between the wild-type and $\Delta VdCf2$ strains (64). A P value of <0.05 and fold change of >2 were set as the threshold for DEG identification. Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, which were used to annotate gene functions, were conducted by using R based on the hypergeometric distribution (65). Secreted proteins were predicted based on SignalP 5.0, TMHMM 2.0, and SecretomeP 2.0 (66-68). The prediction of PHI homologs was performed based on the PHI database (69). TFs were identified based on the Fungal Transcription Factor Database (FTFD) and HMMER online website (23). All G protein-coupled receptors (GPCRs) were identified based on a previous analysis (70). The Vd276-280 gene cluster (VDAG_07276, VDAG_07277, VDAG_07278, VDAG_07279, and VDAG_07280) is part of a secondary metabolism gene cluster from VDAG_07259 to VDAG_07280, which was identified based on Secondary Metabolite Unknown Region Finder (25, 46). Visualization of RNA-seq coverage of the Vd276-280 gene cluster in the wild-type and Δ VdCf2 strains was completed using Integrative Genomics Viewer (71).

Statistical analyses. Statistical analyses were performed using SPSS Statistics 22.0. One-way analysis of variance (ANOVA) was applied, and then the Student-Newman-Keuls (SNK) test with a *P* value of 0.05 was conducted to determine significant differences among three treatments. When more groups were compared, Tukey's test with a *P* value of 0.05 was used to analyze significant differences. The pairwise comparison was conducted using Student's *t* test with a *P* value of 0.05 based on RStudio 1.1.463.

Data availability. The RNA-seq raw reads were submitted to the NCBI SRA database under BioProject number PRJNA797877 and BioSample numbers SAMN25039392 (XJ592) and SAMN25039393 (Δ VdCf2).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only. SUPPLEMENTAL FILE 1, PDF file, 1.1 MB. SUPPLEMENTAL FILE 2, XLS file, 0.1 MB. SUPPLEMENTAL FILE 3, XLS file, 0.03 MB.

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X.H. and W.S. designed the research. T.L. and Y.C. mainly contributed to the all experiments. J.C., K.V.S., and X.X. assisted with specific experiments. T.L. and J.Q. prepared

the manuscript, and X.H., M.K.M., J.C., K.V.S., and X.X. revised the manuscript. None of the authors have conflicts of interest with this manuscript.

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