Cellophane surface-induced gene, VdCSIN1, regulates hyphopodium formation and pathogenesis via cAMP-mediated signalling in Verticillium dahliae

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SUMMARY

The soil-borne vascular pathogen Verticillium dahliae infects many dicotyledonous plants to cause devastating wilt diseases. During colonization, V. dahliae spores develop hyphae surrounding the roots. Only a few hyphae that adhere tightly to the root surface form hyphopodia at the infection site, which further differentiate into penetration pegs to facilitate infection. The molecular mechanisms controlling hyphopodium formation in V. dahliae remain unclear. Here, we uncovered a cellophane surface-induced gene (VdCSIN1) as a regulator of V. dahliae hyphopodium formation and pathogenesis. Deletion of VdCSIN1 compromises hyphopodium formation, hyphal development and pathogenesis. Exogenous application of cyclic adenosine monophosphate (cAMP) degradation inhibitor or disruption of the cAMP phosphodiesterase gene (VdPDEH) partially restores hyphopodium formation in the VdΔcsin1 mutant. Moreover, deletion of VdPDEH partially restores the pathogenesis of the VdΔcsin1 mutant. These findings indicate that VdCSIN1 regulates hyphopodium formation via cAMP-mediated signalling to promote host colonization by V. dahliae.

Keywords: hyphopodium, pathogenesis, surface, Verticillium

INTRODUCTION

For effective colonization, fungal pathogens have evolved sophisticated mechanisms to sense the plant surface and initiate infection-related development that promotes infection, penetration and invasive growth (Eynck et al., 2007; Vallad and Subbarao, 2008; Yadeta et al., 2011; Zhang et al., 2013; Zhao P et al., 2014). Many fungal pathogens develop infection structures, such as appressoria or hyphopodia, to penetrate plant cells. Specialized intracellular fungal structures, such as haustoria and infection hyphae, serve as machinery for the delivery of effectors, which function coordinately to modulate plant defence and physiology to promote virulence (Kamakura et al., 2002; Kou et al., 2017; Li et al., 2012; Lo Presti et al., 2015).

The model pathogen for the study of plant–fungus interactions, Magnaporthe oryzae, senses surface signals, and each spore develops a germ tube and forms an appressorium with turgor pressure to puncture through the plant cell wall (Wilson and Talbot, 2009). Plant surfaces and artificial hydrophobic surfaces can be perceived by fungal pathogens to initiate infection-related development. Magnaporthe oryzae employs G-protein-coupled receptors (GPCRs) and cyclic adenosine monophosphate-protein kinase A (cAMP-PKA)-mediated signalling pathways to regulate surface perception and appressorium formation (DeZwaan et al., 1999; Kronstad et al., 2011; Li et al., 2012). In addition, appressorium formation in M. oryzae is regulated by the conserved mitogen-activated protein kinase (MAPK) pathway (Jin et al., 2013; Li et al., 2012; Zhao X et al., 2007). Both the GPCR Pth11 (Kou et al., 2017) and the putative extracellular chitin-binding protein CBP1 (Kamakura et al., 2002) serve as receptors for the perception of surface signals to induce appressorium formation.

For the soil-borne vascular pathogen Verticillium dahliae, which infects many plants to cause Verticillium wilt diseases, a distinct colonization process has been observed (Zhao P et al., 2014; Zhao YL et al., 2014). During plant root colonization, microsclerotia of V. dahliae germinate massive quantities of hyphae surrounding the roots. A few hyphae that adhere tightly to the root surface form hyphopodia at the infection site, which further differentiate into penetration pegs to penetrate host cells (Fradin and Thomma, 2006; Schnathorst, 1981; Vallad and Subbarao, 2008; Zhao P et al., 2014; Zhao YL et al., 2014). The tetraspanin protein VdPls1 recruits the NADPH oxidase VdNoxB to regulate 

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et al., 2017). In addition, the delivery process requires the function of the vesicular trafficking factors VdSec22 and VdSyn8, and the exocyst complex subunits VdExo70 and VdSyn8 (Zhou et al., 2017). In contrast with the discovery of a few components regulating penetration peg formation and function, components regulating hyphopodium development in V. dahliae have not been identified. The molecular mechanisms underlying the regulation of hyphopodia formation in V. dahliae remain unknown.

In this study, we identified VdCSIN1 as a regulator controlling V. dahliae hyphopodium development. VdCSIN1 is significantly induced by the artificial surface cellophane. The mutant fungus carrying a targeted deletion of VdCSIN1 (VdΔcsin1) exhibits reduced virulence in cotton plants relative to the wild-type (WT) strain. Deletion of VdCSIN1 compromises hyphopodium formation, consistent with the eliminated hyphopodium-specific VdNoxB localization in hyphae. Exogenous application of 3-isobutyl-1-methylxanthine (IBMX), which is a chemical that inhibits cAMP degradation, partially restores hyphopodium formation in the VdΔcsin1 mutant. Moreover, the defects of the VdΔcsin1 mutant in both hyphopodium formation and pathogenesis are partially restored by deletion of the cAMP phosphodiesterase gene (VdPDEH). The results reveal VdCSIN1 as a novel component regulating hyphopodium formation via cAMP-mediated signalling to promote V. dahliae pathogenesis.

RESULTS

Identification of cellophane surface-induced gene, VdCSIN1, which contributes to V. dahliae virulence in cotton plants

To identify V. dahliae components involved in the regulation of initial recognition and surface perception, a number of genes induced by the artificial surface cellophane were individually deleted in the V. dahliae V592 strain and subjected to virulence assessment in its host plants. Verticillium dahliae strains were grown on minimal medium (MM) covered with or without a cellophane layer for 24 h. Gene expression analyses were carried out to examine the expression of candidate root-induced genes (Zhang et al., 2017), and revealed a cellophane surface- induced gene (VdCSIN1, VDAG_05652) which was specifically induced by the cellophane surface (Fig. 1A). A mutant carrying a targeted deletion of VdCSIN1 (Fig. 1B,C), VdΔcsin1, exhibited severely compromised microsclerotia development relative to the WT V592 strain when cultured on potato dextrose agar (PDA) medium (Fig. 1D). The growth rate and spore germination of the VdΔcsin1 mutant remained the same as those of the WT strain (Fig. 1E), whereas the conidiogenesis of the VdΔcsin1 mutant was lower than that of the WT strain (Fig. 1E). The introduction of green fluorescent protein (GFP)-tagged VdCSIN1 into the VdΔcsin1 mutant (VdΔcsin1/VdCSIN1-GFP) restored the formation of melanized microsclerotia (Fig. 1D), confirming the targeted deletion of VdCSIN1 and complementation of VdCSIN1 function in the VdΔcsin1 mutant (Fig. 1D). The expression of the VdCSIN1-GFP protein in the VdΔcsin1/VdCSIN1-GFP strain was detected by anti-GFP immunoblot (Fig. 1F).

As microsclerotia formation is usually linked to V. dahliae virulence (Gao et al., 2010; Rauyaree et al., 2005; Tzima et al., 2011), the reduced formation of microsclerotia in the VdΔcsin1 mutant suggests a role of VdCSIN1 in pathogenesis. We compared the pathogenesis of the V592, VdΔcsin1 mutant and VdΔcsin1/VdCSIN1-GFP strains in host plants. The VdΔcsin1 mutant exhibited much weaker disease symptoms than did WT V592 and the VdΔcsin1/VdCSIN1-GFP complementation strain in upland cotton plants (Fig. 2A). Disease index analyses indicated reduced virulence of the VdΔcsin1 mutant relative to WT V592 and VdΔcsin1/VdCSIN1-GFP in upland cotton (Fig. 2B). The results indicate that VdCSIN1 contributes to V. dahliae virulence in host plants.

VdCSIN1 is required for hyphopodium formation in V. dahliae

The rapid induction by the cellophane surface and the virulence function of VdCSIN1 prompted us to examine whether it plays a role in the process of initial colonization. We thus cultured the WT V592, VdΔcsin1 and VdΔcsin1/VdCSIN1-GFP strains on medium covered with a cellophane membrane which was used for hyphopodium induction in V. dahliae (Zhao YL et al., 2016). At 3 days after incubation, for WT V592 and VdΔcsin1/VdCSIN1-GFP strains, the penetration of fungal hyphae through the cellophane membrane and growth on the medium when the cellophane membrane was removed were observed (Fig. 3A). However, the VdΔcsin1 mutant hyphae did not penetrate the cellophane membrane (Fig. 3A). We then observed the hyphae of the above strains under microscopy. In contrast with the WT V592 strain, which develops a number of hyphopodia (Fig. 3B,C), almost no hyphopodium formation was observed for the VdΔcsin1 mutant (Fig. 3B,C). To visualize VdCSIN1 protein localization, the V592/VdCSIN1-GFP strain was detected by anti-GFP immunoblot (Fig. 3D), which is consistent with its role in the regulation of hyphopodium formation.

To further confirm this, the hyphopodium-specific expressing gene VdNoxB, encoding the NADPH oxidase catalytic subunit (Zhao YL et al., 2016), was fused to GFP and introduced into WT V592 and the VdΔcsin1 mutant to construct V592/VdNoxB-GFP and VdΔcsin1/VdNoxB-GFP strains. Consistently, GFP fluorescence was observed in the hyphopodia of the V592/VdNoxB-GFP strain (Fig. 4A). However, deletion of VdCSIN1 eliminates hyphopodium-specific VdNoxB localization in the VdΔcsin1/VdNoxB-GFP strain (Fig. 4A), confirming the requirement of VdCSIN1 for hyphopodium formation. Interestingly, unlike the WT V592
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A

Relative Normalized Expression

Without cellophane

With cellophane

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B

1KB
Promoter
Genomic DNA

BstBI
Hygromycin B phosphotransferase
BamHI

C

5kb
2kb
anti-GFP IB

V592 VdΔcsin1

130kDa
100kDa

CBB stain

D

E

V592 VdΔcsin1 VdΔcsin1/VdCSIN1-GFP

Strain Germination (%) Conidiation (10⁷ spores/plate) Growth rate (mm/day)

V592 97.60±0.87 50.73±1.09 3.68±0.07
VdΔcsin1 95.67±1.08 21.47±0.46 3.36±0.03
strain, for which hyphal growth curved forwards in the disengaged state, the VdΔcsin1 mutant exhibited straight and orderly arranged hyphal growth (Fig. 4A). These results suggest that VdCSIN1 plays a role in sensing the cellophane surface for the initiation of hyphopodium formation.

To further examine colonization in plants, roots of cotton plants were inoculated with the GFP-labelled V592 WT strain (V592-GFP) or VdΔcsin1 mutant (VdΔcsin1-GFP) for visualization. The cross-sections of vascular bundles isolated from V592-GFP- and VdΔcsin1-GFP-infected plants were visualized by confocal laser scanning microscopy (CLSM). The results showed that the invasive hyphae of V592-GFP reached the vascular cylinder to colonize the xylem vessels (Fig. 4B). In contrast, the green fluorescent signal was observed surrounding the peripheral region of the cross-section, but rarely in the vascular cylinder and xylem vessels, in plants inoculated with VdΔcsin1-GFP (Fig. 4B), indicating that the penetration of the root and colonization of the vascular bundles of plants by the VdΔcsin1 mutant were compromised. These results further verify that VdCSIN1 plays a role in sensing the hydroscopic root epidermis to initiate hyphopodium formation during root infection.

cAMP-mediated signalling contributes to VdCSIN1-mediated hyphopodium formation

cAMP-mediated signalling plays a crucial role in the regulation of appressorium formation in M. oryzae. The exogenous application of IBMX, a cAMP degradation inhibitor, partially restored appressorium formation in the M. oryzae Δpth1 and Δcbp1 mutants (Kamakura et al., 2002; Kou et al., 2017), indicating the role of cAMP-mediated signalling in appressorium formation of M. oryzae.

We therefore examined whether cAMP is involved in the regulation of hyphopodium development in V. dahliae by the exogenous application of IBMX in the medium. We found that hyphopodium formation was partially restored in the VdΔcsin1 mutant (Fig. 5A,B), although hyphal growth maintained its orderly arranged morphology. This result suggests the involvement of cAMP in the VdCSIN1-mediated regulation of hyphopodium formation in V. dahliae.

The CAMP phosphodiesterase gene (PDEH) functions to degrade the phosphodiester bond in CAMP, thereby down-regulating cAMP levels (Baillie and Houlsay, 2005; Beard et al., 2000; Francis et al., 2011). To further confirm the involvement of CAMP in VdCSIN1-mediated hyphopodium development, PDEH homologous sequences were searched for in the V. dahliae genome using the database for VdLs.17 (Klosterman et al., 2011). Two PDEH genes were found and designated as VdPDEH1 (VDAG_03573) and VdPDEH2 (VDAG_05759), respectively. VdPDEH1 and VdPDEH2 were each deleted in V592 or the VdΔcsin1 mutant strain to construct VdΔpdeh1, VdΔpdeh2, VdΔcsin1Δpdeh1...
and VdΔcsin1/Δpdeh2 strains (Fig. S1A–D, see Supporting Information). Partially restored hyphopodium formation was observed in both the VdΔcsin1/Δpdeh1 and VdΔcsin1/Δpdeh2 mutant strains compared with the VdΔcsin1 mutant (Fig. 5C,D).

This effect was eliminated on complementation of VdPDEH1 or VdPDEH2 in the VdΔcsin1/Δpdeh1 and VdΔcsin1/Δpdeh2 mutants (Fig. 5C,D). Consistently, vegetative hyphal growth morphology (Fig. 5C) and penetration activity were partially restored.
in both the VdΔcsin1Δpdeh1 and VdΔcsin1Δpdeh2 mutants compared with the VdΔcsin1 mutant (Fig. 5E).

To further verify the contribution of cAMP in VdCSIN1-mediated hyphopodium formation, the cAMP level was examined in V592, VdΔcsin1, VdΔcsin1Δpdeh1 and VdΔcsin1Δpdeh2 strains. Compared with V592, the VdΔcsin1 mutant accumulated less cAMP (Fig. 5F); deletion of VdpDEH1 or VdpDEH2 partially elevated cAMP accumulation in the VdΔcsin1 mutant (Fig. 5F). As expected, complementation of VdpDEH1 or VdpDEH2 in the VdΔcsin1Δpdeh1 and VdΔcsin1Δpdeh2 mutants restored the down-regulation of cAMP accumulation (Fig. 5F).

Moreover, disease symptoms and disease index analyses indicated much greater pathogenesis of the VdΔcsin1Δpdeh1 and VdΔcsin1Δpdeh2 mutants relative to that of the VdΔcsin1 mutant in cotton infection (Fig. 6). Taken together, our results demonstrate that, consistent with the results from the exogenous application of IBMX, deletion of either VdpDEH gene in the VdΔcsin1 mutant strain partially restored the capacity of fungal hyphae to sense the cellophane surface and to initiate hyphopodium development, revealing that cAMP-mediated signalling contributes to VdCSIN1-mediated hyphopodium formation and pathogenesis in V. dahliae.

Fig. 4 Deletion of VdCSIN1 eliminates hyphopodium-specific VdNoxB localization and compromises stem vascular colonization of Verticillium dahliae. (A) Deletion of VdCSIN1 eliminates hyphopodium-specific VdNoxB localization in hyphae. V592/VdNoxB-GFP and VdΔcsin1/VdNoxB-GFP strains were cultured on minimal medium (MM) overlaid with a cellophane layer for 2 days, and green fluorescent protein (GFP) fluorescence was visualized by confocal laser scanning microscopy (CLSM). (B) The VdΔcsin1 mutant exhibits reduced colonization in the stem vascular bundles of cotton plants. Cotton plants were inoculated with conidia of V592-GFP or VdΔcsin1-GFP. Cross-sections isolated from the stems of infected plants at 5 days post-inoculation were visualized by CLSM. The experiment was repeated three times with similar results. [Colour figure can be viewed at wileyonlinelibrary.com]
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DISCUSSION

In this study, we identified VdCSIN1 as a virulence factor crucial for V. dahliae pathogenesis. Further analyses uncovered VdCSIN1 as a novel component regulating hyphal development and hyphopodium formation during infection. Increasing intracellular cAMP levels, generated by the deletion of VdPDEH, partially restored the hyphopodium formation and pathogenesis of the VdΔcsin1 mutant in cotton plants, demonstrating the role of cAMP in hyphopodium development. Thus, these findings indicate that VdCSIN1 engages cAMP-mediated signalling to regulate hyphopodium formation and promote V. dahliae pathogenesis.

Overlapping function of VdPDEH1 and VdPDEH2 genes

In V. dahliae, deletion of the G-protein β-subunit gene, VGB (Tzima et al., 2012), and PKA catalytic subunit genes, VdPKAC1 and VdPKAC2 (Tzima et al., 2010), resulted in reduced virulence, suggesting a role of GPCR and cAMP-PKA-mediated signalling in the regulation of V. dahliae infection-related development and pathogenesis.

We observed significantly reduced hyphopodium formation in the VdΔcsin1 mutant. Indeed, deletion of either VdPDEH1 or VdPDEH2, which down-regulate the cAMP level, partially restored the formation of hyphopodia, demonstrating a function of cAMP-mediated regulation in hyphopodium development. However, deletion of either VdPDEH1 or VdPDEH2 only partially restored the defect of the VdΔcsin1 mutant in hyphopodia formation, indicating an overlapping function of VdPDEH1 and VdPDEH2 in the regulation of this process. Thus, failure to fully restore the defects in hyphopodia formation in V. dahliae in both the VdΔcsin1/VdΔpdeh1 and VdΔcsin1/VdΔpdeh2 mutants may be caused by the functional redundancy of the VdPDEH1 and VdPDEH2 genes.

Possible differential receptors are engaged by different fungi to sense surface signals

In M. oryzae, two membrane protein coding genes, Pth11 and CBP1, function to perceive hydrophobic surface signals to induce appressoria formation. The M. oryzae Δpth11 and Δcbp1 mutants showed reduced appressorium formation (Kamakura et al., 2002; Kou et al., 2017). Although we identified two proteins in V. dahliae (VdPth11-1 and VdPth11-2) homologous to M. oryzae Pth11 and two proteins (VdBMP1-1 and VdBMP1-2) homologous to M. oryzae CBP1 by sequence alignment analyses, none of the V. dahliae mutants carrying the targeted deletion of VdPth11-1, VdPth11-2,
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VGCBP1-1 or VGCBP1-2 (VdΔpth11-1, VdΔpth11-2, VdΔcbp1-1 or VdΔcbp1-2) exhibited obvious defects in hyphopodium formation (Fig. S2A, B, see Supporting Information). Redundant functions between two VdPth11 genes or two VGCBP1 genes were also ruled out, because mutants carrying either the double deletion of VdPth11-1/VdPth11-2 (VdΔpth11-1/Δpth11-2) or VdCBP1-1/ VdCBP1-2 (VdΔcbp1-1/Δcbp1-2) developed normal hyphopodium formation (Fig. S2A, B). Consistently, similar to WT V592, both VdΔpth11-1/Δpth11-2 and VdΔcbp1-1/Δcbp1-2 mutants could penetrate the cellophane membrane covering the medium (Fig. S3, see Supporting Information). All of these observations indicate that neither VdPth11 nor VGCBP1 gene plays a role in hyphopodium formation, and suggest that differential receptors are engaged by *V. dahliae* to sense surface signals. Whether VdCSIN1 directly associates with or modifies *V. dahliae* components of the surface sensor complex remains to be further investigated.

**EXPERIMENTAL PROCEDURES**

**Fungal strains, plant materials and culture conditions**

The *V. dahliae* strain V592 (Gao et al., 2010) was used in this study. *Verticillium dahliae* strains were grown on PDA medium with the appropriate antibiotics at 25 °C in the dark. To collect conidia, mycelial plugs were cultured in potato dextrose broth liquid medium with shaking at 200 g at 25 °C for 3–5 days. Cotton plants (‘Xinluzao No. 16’) were used for virulence assessment in this study (Zhou et al., 2017). All primers used in this study are listed in Text S1 (see Supporting Information).

**Southern blot analysis**

Genomic DNA (20 μg) isolated from WT V592 or the indicated mutant strains was digested with the indicated restriction enzymes. Digested DNA was separated by electrophoresis on an agarose gel overnight and transferred onto a nylon membrane. Gene-specific probes were labelled using the DiG High Prime DNA Labelling Kit and the presence of corresponding DNA fragments was detected by the Detection Starter Kit I (Roche, Indianapolis, IN, USA).

**Generation of fungal strains**

To construct the gene deletion plasmids pKOVDcsin1, pKOVDpth1-1 and pKOVDcbp1-1, the upstream and downstream flanking sequences of the corresponding genes were polymerase chain reaction (PCR) amplified from V592 genomic DNA and cloned into the pGKO-HPT vector (Wang et al., 2016). To construct the gene deletion plasmids pKOVDpeh1, pKOVDpeh2, pKOVDpth1-1-2 and pKOVDcbp1-2, the upstream and downstream flanking sequences of the corresponding genes were PCR amplified from V592 genomic DNA and cloned into the pGKO-G418 vector. The resulting plasmids were used for Agrobacterium-mediated transformation (ATMT), as described previously, to generate the deletion mutants (Wang et al., 2016). To obtain VdΔcsin1/VdΔpeh1 and VdΔcsin1/VdΔpeh2 mutant strains, pKOVDPEH1 and pKOVDPEH2, respectively, were transformed into the VdΔcsin1 mutant. To obtain VdΔpth11-1/VdΔpth11-2 and VdΔcbp1-1/VdΔcbp1-2, pKOVDPh1-1-2 and pKOVDcbp1-2 were transformed into the VdΔpth11-1 and VdΔcbp1-1 mutants, respectively. The genomic coding region of VdCSIN1 was PCR amplified from V592 genomic DNA and cloned into the pNat-Tef-GFP vector (Zhou et al., 2017) to obtain the pNat-Tef-VdCSIN1-GFP plasmid. The resulting construct was transformed into *Agrobacterium tumefaciens* strain EHA105, and used for ATMT to generate the VdΔcsin1/VdΔCSIN1-GFP and V592/VdCSIN1-GFP strains. To obtain the GFP-labelled strains, pNEO-GFP was introduced into V592 or the VdΔcsin1 mutant to obtain V592-GFP and VdΔcsin1-GFP strains, respectively. To obtain V592/VdNoxB-GFP and VdΔcsin1/VdNoxB-GFP strains, VdNoxB::GFP (Zhao YL et al., 2016) was transformed into V592 and the VdΔcsin1 mutant, respectively. To generate the PDEH complementary strains, the genomic coding regions of VdPDEH1 and VdPDEH2 were PCR amplified from V592 genomic DNA and cloned into the pSULPH-Tef-myc vector to obtain the pSULPH-Tef-VdPDEH1-myc and pSULPH-Tef-VdPDEH2-myc plasmids, respectively. The resulting constructs were transformed into *Agrobacterium tumefaciens* strain EHA105, and used for transformation into the VdΔcsin1/VdΔpeh1 and VdΔcsin1/VdΔpeh2 mutants, respectively.

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)**

*Verticillium dahliae* conidia were cultured on MM overlaid with or without a cellophane layer. The cultured strains were collected for RNA extraction at 24 h post-inoculation (hpi). Total RNA was isolated with the TRIzol reagent (Carlsbad, California, Invitrogen, USA) and used for cDNA synthesis with a SuperScript III First-Strand Synthesis System for RT-PCR (Carlsbad, California, Invitrogen, USA). Quantitative PCR was performed with a SYBR Premix Ex Taq kit (Caojin, Shiga, TaKaRa, Japan) following standard protocols. The expression level of VdCSIN1 was normalized to that of VdGAPDH.

**Fluorescence microscopy**

*Verticillium dahliae* strains were grown on MM overlaid with a cellophane layer for 2 or 3 days as indicated. The mycelial morphology and hyphopodium formation were visualized using a Leica SP8 CLSM system. The plasma membrane was stained with FM4-64 (Waltham, Massachusetts, ThermoFisher Scientific, USA) when needed.
Infection assay

The conidia of *V. dahliae* strains were collected and resuspended at a concentration of 10^7/mL and used as inocula. Cotton plants were infected by the root-dipping inoculation method (Gao et al., 2010). The disease grade was classified as follows: 0 (no symptoms), 1 (0%–25% wilted leaves), 2 (25%–50% wilted leaves), 3 (50%–75% wilted leaves) and 4 (75%–100% wilted leaves). The disease index was calculated as 100 × [sum (number of plants × disease grade)]/[(total number of plants) × (maximal disease grade)] (Xu et al., 2014).

Penetration assays

Sterilized cellophane membrane (DINGGUO, Beijing, China) was overlaid onto MM. Equal amounts of conidia collected from *V. dahliae* strains as indicated were inoculated on the cellophane membrane and grown for 3 days. The cellophane membrane was then removed and further cultured for an additional 3 days.

cAMP measurements

*Verticillium dahliae* conidia with the indicated genotypes were cultured on MM overlaid with a cellophane layer for 24 h. The cultured strains were then collected and ground into a powder with liquid nitrogen, and homogenized in 0.1 M HCl after weighing. cAMP was measured using a cAMP Enzyme Immunoassay Kit, Direct (Merck KGaA, Darmstadt, Sigma-Aldrich, Germany). cAMP measurements and sample analysis were performed according to the manufacturer’s instructions (Lomovatskaya et al., 2011).

Western blot for detection of VdCSIN1-GFP

VdΔcsin1 and VdΔcsin1/VdCSIN1-GFP conidia were cultured on MM overlaid with a cellophane layer for 2 or 3 days. The cultured strains were collected for protein extraction with extraction buffer (50 mM HEPES (4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid sodium salt), pH 7.5, 150 mM KCl, 1 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5% Triton X-100, 1× proteinase inhibitor cocktail). Protein lysates were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the presence of VdCSIN1-GFP was detected by anti-GFP (Basel, Basel-Stadt, Roche, Switzerland, 11814460001) immunoblot.

Analysis of growth and developmental characteristics of the mutants

*Verticillium dahliae* strains with the indicated genotypes were cultured on PDA medium plates. The colony diameter was recorded at intervals of 3–13 days. To measure conidial production, 13-day-old fungal colonies grown on PDA plates were suspended in 100 mL of sterilized water and shaken. A suspension was placed on a haemocytometer and spores were counted under a microscope. For spore germination tests, conidia of the indicated strains were suspended on PDA medium at 10^7 spores/mL and incubated at 25 ºC. The germination rate was determined at 18 h post-incubation by counting 100 conidia for each strain.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site: Fig. S1 Generation of VdΔpdeh1, VdΔpdeh2, VdΔcsin1/VdΔpdeh1 and VdΔcsin1/VdΔpdeh2 mutants. (A, B) Schematic descriptions of the generation of VdΔpdeh1 (A) and VdΔpdeh2 (B) deletion. (C, D) Southern blot analyses indicate VdΔpdeh1 (C) and VdΔpdeh2 (D) deletion in the mutant strains. Genomic DNA samples isolated from V592, VdΔpdeh1, VdΔpdeh2, VdΔcsin1/VdΔpdeh1 and VdΔcsin1/VdΔpdeh2 mutant strains were double digested by SphI and NdeI (VdPDEH1), or EcoR I and KpnI (VdPDEH2), as indicated and subjected to Southern blot analysis. Fig. S2 VdPth11 and VdCBP1 are not required for hyphopodium formation. (A) Deletion of VdPth11 or VdCBP1 did not affect hyphopodium formation. V592, VdΔpth11-1, VdΔpth11-2, VdΔcbp1-1, VdΔcbp1-2, VdΔpth11-1/Δpth11-2 and VdΔcbp1-1/Δcbp1-2 mutants were cultured on minimal medium (MM) overlaid with a cellophane layer for 2 days. (B) Quantitative analysis of hyphopodia formed by the indicated strains. Error bars indicate standard deviation. Student’s t-test was carried out to determine the significance of the difference between indices. Fig. S3 VdPth11 and VdCBP1 are not required for the penetration of the cellophane membrane. Deletion of VdPth11 or VdCBP1 did not compromise Verticillium dahliae penetration of the cellophane membrane. V592, VdΔpth11-1, VdΔpth11-2, VdΔcbp1-1, VdΔcbp1-2, VdΔpth11-1/Δpth11-2, and VdΔcbp1-1/Δcbp1-2 mutants were grown on minimal medium (MM) overlaid with a cellophane layer for 3 days and photographed (above). The cellophane was removed and the plates were further incubated for 3 days and photographed (below). Text S1 Primers used in this study.