Identification of VdASP F2-interacting protein as a regulator of microsclerotial formation in Verticillium dahliae

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Summary

Verticillium dahliae, a notorious phytopathogenic fungus, causes vascular wilt diseases in many plant species. The melanized microsclerotia enable V. dahliae to survive for years in soil and are crucial for its disease cycle. In a previous study, we characterized the secretory protein VdASP F2 from V. dahliae and found that VdASP F2 deletion significantly affected the formation of microsclerotia under adverse environmental conditions. In this study, we clarified that VdASP F2 is localized to the cell wall. However, the underlying mechanism of VdASP F2 in microsclerotial formation remains unclear. Transmembrane ion channel protein VdTRP was identified as a candidate protein that interacts with VdASP F2 using pull-down assays followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, and interaction of VdASP F2 and VdTRP was confirmed by bimolecular fluorescence complementarity and coimmunoprecipitation assays. The deletion mutant was analysed to reveal that VdTRP is required for microsclerotial production, but it is not essential for stress resistance, carbon utilization and pathogenicity of V. dahliae. RNA-seq revealed some differentially expressed genes related to melanin synthesis and microsclerotial formation were significantly downregulated in the VdTRP deletion mutants. Taken together, these results indicate that VdASP F2 regulates the formation of melanized microsclerotia by interacting with VdTRP.

Introduction

Verticillium dahliae is a soil-borne plant pathogenic fungus that can cause wilting and necrosis of many important crops, which leads to significant economic losses (Maruthachalam et al., 2011). When under stress or without a suitable host, this fungus produces a melanized microsclerotia that can survive in the soil for up to 20 years until the conditions are suitable for infection (Rauyaree et al., 2005; Fradin and Thomma, 2006).

Melanized microsclerotia are crucial for the survival and propagation of V. dahliae (Fan et al., 2020). At present, an increasing number of studies have focused on genes related to melanin and microsclerotial formation. They mainly include two MAPK signalling pathways. VdMsb (transmembrane mucin) is highly conserved in the MAPK signalling pathway and is required for microsclerotial production (Tian et al., 2014). Under stress conditions, the HOG-MAPK signalling pathway, composed of the VdSsk2-VdPbs2-VdHog1 module is activated to increase stress tolerance, and VdHog1 is phosphorylated by VdSsk2 but not by VdSte11 (Wang et al., 2016a; Yu et al., 2019). This cascade is also involved in the biosynthesis of microsclerotia and melanin, with the deletion of VdHog1, VdPbs2, VdSsk2 and VdSte11 leading to delayed melanization and microsclerotial formation (Tian et al., 2016; Wang et al., 2016b). Crz1 (calcineurin-responsive zinc finger transcription factor) and VdMsn2 (C2H2 transcription factor) as downstream players of the HOG-MAPK pathway are also involved in microsclerotial formation and melanin accumulation, and are misregulated in ΔVdSsk2, ΔVdPbs2 and ΔVdHog1 mutants (Xiong et al., 2015; Tian et al., 2017; Yu et al., 2019). The VdSho1 MAPK pathway, composed of VdSho1-VdSte50-VdSte11-VdSte7, plays a key role in melanin biosynthesis in V. dahliae. VdSho1

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encodes a conserved tetraspan transmembrane protein, which is a key element of the two upstream branches of the HOG-MAPK pathway in fungi (Qi et al., 2016). VdSho1 regulates pathogenicity by activating downstream MAPK pathways. Moreover, several genes related to melanin biosynthesis are regulated by members of the VdSho1 MAPK pathway and act downstream of the pathway, including VmiK1, VdcMr1, Vdlac1, Vag1, and VdPKS1. Δvdsho1, ΔvdSte50, ΔvdSte7 and ΔvdSte11 mutants did not affect the formation of microsclerotia but caused melanin loss (Li et al., 2019). Some transcription factors are also involved in V. dahliae microsclerotia. Vta1 is required for melanin production and activates the transcription of melanin biosynthesis genes, including the polyketide synthase encoding PKS1 and lactase LAC1 (Harting et al., 2020). The VTA3 deletion strain produced 60% less microsclerotia than the wild-type V. dahliae, whereas the SOM1 deletion strain was unable to form microsclerotia (Tri-Thuc et al., 2019). The MADS-box gene VdMcm1 affects microsclerotial formation and melanin biosynthesis (Xiong et al., 2016). Other transcription factors, such as Vdpf, VdAf1, VdYap1 and VdSkn7, also regulate microsclerotial formation in V. dahliae (Luo et al., 2016; Tang et al., 2017, 2020).

Transient receptor potential channel protein superfamily (TRP superfamily) is a kind of ion channel proteins that exist widely in multicellular organisms, including TRPC, TRPV, TRPM, TRPA, TRPP, TRPML and TRPY subfamilies. Almost all of the proteins in this family contain transmembrane domains, and some TRP proteins have a conserved TRP domain. At present, the view is that TRP channel proteins mainly play a role in sensory physiology including vision, taste, smell, touch, hearing, cold, heat and stress (Palmer et al., 2005). The deletion of TRP channel protein OSM9 can lead to defects in odour and stress perception in Caenorhabditis elegans (Colbert et al., 1997). VR1, a receptor of capsaicin, is a TRPV protein. VR1 mutant weakens the response of mice to capsaicin, high temperature and pain (Caterina et al., 2000). In Drosophila melanogaster, TRPC protein exists mainly in photoreceptor cells, and its mutation will change the permeability of the channel encoded by the gene to cations (Niemyer et al., 1996; Reuss et al., 1997; Leung et al., 2000).

Verticillium dahliae serine protease cerevisin is involved in controlling spore germination, mycelium growth, microsclerotial formation and virulence. Cerevisin inactivation resulted in a decrease in the secretion of cell wall-related proteins, including the protein VdASP F2 secreted by V. dahliae (He et al., 2015). We have previously screened VdASP F2 (VDAG_04551) from a T-DNA insertion library, which is critical for V. dahliae survival in adverse environments. Phenotypic analysis has shown that VdASP F2 deficiency leads to vigorous mycelial growth, reduced branching, and delayed microsclerotial formation under limited nutrient or low-temperature treatments. The VdASP F2 mutant affected the expression of the microsclerotia formation-related genes VDH1 and VMKI (Rauyaree et al., 2005; Klimes and Dobinson, 2006; Klimes et al., 2008; Xie et al., 2017). This study aimed to identify the VdASP F2 interaction protein of V. dahliae and further explore the function of VdASP F2 interaction protein participating in the formation of V. dahliae microsclerotia.

Results

VdASP F2 protein localization to the cell wall

To clarify the subcellular localization of VdASP F2 in V. dahliae, the red fluorescent gene mCherry was fused with VdASP F2 to form a fusion expression gene, which was co-localized with the cell wall-specific stain Calcofluor White. It was found that the red fluorescence of the VdASP F2-mCherry fusion protein was co-localized in the cells around the hyphae septum with Calcofluor White, while the mCherry of the control strain was localized in the cytoplasm (Fig. 1A).

However, proteins localized around the cell and in the septa of the hyphae may also be located in the cell membrane. To rule out this possibility, we developed protoplasts of V. dahliae to observe VdASP F2-mCherry subcellular localization. The protoplast results showed that VdASP F2-mCherry was not located on the cell membrane of V. dahliae, and only a few cytoplasm regions showed little fluorescence, which may indicate proteins that had not yet been secreted from the cell, while the cytoplasm of the mCherry-expressing strain showed a strong red fluorescence signal (Fig. 1B). The above results confirmed the localization of the VdASP F2 protein in the cell wall of V. dahliae.

Identification of VdASP F2 interacting proteins

To elucidate the mechanisms by which VdASP F2 regulates the formation of V. dahliae microsclerotia under adverse conditions (Xie et al., 2017), the mCherry-Flag and VdASP F2-mCherry-Flag expressing strains were created. Total proteins were extracted and performed immunoblot analysis with anti-Flag antibody to confirm the expression of the mCherry-Flag and VdASP F2-mCherry-Flag in the transgenic strains (Fig. 2A). Next, we performed a systematic biochemical analysis to identify potential VdASP F2-interacting proteins using pull-down followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Besides the heavy chain of 50 kDa and the light chain of 25 kDa from anti-FLAG monoclonal antibodies, mCherry-Flag and VdASP F2-mCherry-Flag were detected in two samples.
respectively. Two special bands in the VdASP F2-mCherry-Flag lane were collected for the mass spectrometry analysis (Fig. 2B) and proteins identified by MS analysis are shown in Table S1. Peptide coverage of the identified proteins ranged from 0% to 47%, and representative examples from key proteins interacting with VdASP F2 are shown in Fig. 2C. This analysis identified a sequence homologous to a conserved transient receptor potential (TRP) channel domain containing protein in the V. dahliae genome, which we designated VdTRP. The protein encodes a predicted polypeptide of 673 amino acids (76 094 Da) containing eight potential transmembrane-spanning domains (TM1-TM8) and a conserved transient receptor potential (TRP) channel functional domain (Fig. 2D).

Computational analysis was performed using the STRING database (http://string-db.org) to detect known and predicted protein interactions, and some transmembrane ion channel proteins with a score > 0.7 (high confidence) were identified as interacting candidates of VdASP F2 (Table S5). Meanwhile, considering VdASP F2 localization to the cell wall, its interacting proteins may only localize to the cell membrane or extracellular space. Therefore, we choose VdTRP, which has the high MS score, as the candidate interactor of VdASP F2 for further study.

Verification of VdASP F2–VdTRP interaction
To further study the interaction between VdASP F2 and VdTRP, we performed bimolecular fluorescence...
complementation (BiFC) (Fig. 3A) and co-immunoprecipitation (Co-IP) (Fig. 3B) assays. The results of both experiments confirm the interaction of these two proteins. We further analysed protein co-localization in *V. dahliae* between VdASP F2 and VdTRP. The results of fluorescence microscopy showed that VdASP F2-mCherry-Flag and YFP-VdTRP were co-localized at the hyphal septa, whereas the control VdASP F2-mCherry-Flag and YFP were not co-localized (Fig. 3C).

**Fungal growth and susceptibility to abiotic stress**

Knockout mutants and complemented strains were generated to investigate the functions of VdTRP (Fig. S1). In *Saccharomyces cerevisiae* and *Candida albicans*, the TRP family has been reported to have a function in cell growth and Ca^{2+} homeostasis under several stresses; therefore, the sensitivity of wild-type V991 (WT), VdTRP knockout (ΔVdTRP-1/ΔVdTRP-2), and complementation strains (COM) to various stresses including KCl, H_{2}O_{2}, sorbitol, or Congo Red was tested. These strains showed strikingly reduced radial growth in the presence of the above stresses, compared to the non-stress conditions; however, compared with the wild-type V991 and complemented strains, the VdTRP deletion strains exhibited no obvious difference in vegetative hyphal growth and colony morphology (Fig. 4A).

To investigate the role of VdTRP in *V. dahliae* growth during carbon uptake or utilization of specific carbon sources, all strains were grown on Czapek–Dox medium with different carbon sources (glucose, sucrose or cellulose). These strains did not show apparent colonial morphology on the different carbon media (Fig. 4B). These results indicate that VdTRP is not involved in the abiotic stress resistance and carbon utilization mechanisms of *V. dahliae*.

**VdTRP is involved in melanized microsclerotial formation and not crucial in *V. dahliae* pathogenicity**

To further explore the role of VdTRP in the development of *V. dahliae* microsclerotia, we cultured WT, ΔVdTRP-1/ΔVdTRP-2 and COM on microsclerotia-inducing medium (MIM) and potato dextrose agar (PDA) medium. After culturing for 30 days, both the wild-type V991 and the complemented strains produced significant melanized microsclerotia, whereas no microsclerotia was observed in the VdTRP knockout mutants (Fig. 5A). It can be concluded that the deletion of VdTRP affects the formation and development of microsclerotia.

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To determine the role of VdTRP in virulence, we performed pathogenicity assays on cotton seedlings (*Gossypium hirsutum* ‘Xinluzao-13’) by root dip inoculation with conidial suspensions. All strains showed wilting symptoms 20 days after *V. dahliae* inoculation (Fig. 5B). Thus, VdTRP is not necessary for the pathogenicity of *V. dahliae*.

**RNA-Seq analysis**

To investigate the regulatory mechanisms of VdTRP in microsclerotial formation, transcriptomic analysis was conducted between the VdTRP deletion mutants and wild-type V991 strains. Both fungal strains were first inoculated on a cellophane membrane overlaid on potato dextrose agar (PDA) medium for 5 days. Then, the cellophane membrane was transferred onto MIM medium and allowed to inoculate for 3 days, which induced the expression of microsclerotia-related genes. RNA samples were extracted for RNA sequencing (RNA-seq) analysis.

Statistical analysis revealed a total of 2462 DEGs (P-value < 0.01) between the VdTRP deletion mutants and wild-type strains, among which 1385 genes were upregulated and 1077 genes were downregulated. To characterize the functional processes of DEGs, these genes were analysed for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Among the downregulated genes in the VdTRP deletion mutants, genes involved in ‘transition metal ion binding’, ‘integral component of membrane,’ and ‘intrinsic component of membrane’ were significantly enriched. These GO terms are closely associated with the subcellular localization and molecular functions of VdTRP. Moreover, genes involved in response to stimulus were significantly downregulated, which further suggests a correlation between VdTRP and stimulus response (Fig. 6A).

KEGG cluster analysis showed that a high percentage of genes were involved in the terpenoid and polyketide metabolism pathway. Most filamentous fungi synthesize melanin via a polyketide pathway, such as the dihydroxynaphthalene (DHN) melanin biosynthesis pathway of *V. dahliae* (Upadhyay et al., 2016). The loss of melanized microsclerotial formation in the VdTRP deletion mutants were consistent with downregulated expression of genes of the terpenoid and polyketide metabolism pathway (Fig. 6B). Similarly, a large number of genes associated with transmembrane transporter were also highly upregulated in the VdTRP deletion mutants (P < 0.01) (Fig. S2).

**DEGs analysis of melanin and microsclerotial development**

Although the microsclerotia are typically darkly pigmented, the processes of melanin production and microsclerotial development in *V. dahliae* can be uncoupled. For example, the absence of Vta1 (VDAG_00192) allows microsclerotial production to occur, but the product is colourless and not melanized (Harting et al., 2020). The DHN-melanin biosynthetic cluster of genes including acetyl-CoA carboxylase (VDAG_03674) (Xiong et al., 2014), PKS encoding gene (VDAG_00184) (Wang et al., 2016a), VdPKS1 (VDAG_00190) (Wang et al., 2019), hydroxynaphthalene reductase BRN1 (VDAG_03665) (Perpetua et al., 1996; Tsuji et al., 2003), BRN2 (VDAG_00183) (Tsuji et al., 2003), Scytalone dehydratase SCD1 (VDAG_03393) (Kubo et al., 1996) and laccase VdLac1 (VDAG_00189) (Tsuji et al.,...
have been characterized in *V. dahliae*. These genes, except *VdLac1*, were found to be downregulated in the *VdTRP* deletion mutants using RNA-seq data analysis, in contrast to that in the wild-type strains.

In addition, some DEGs have been reported to be associated with microsclerotial development. Among them, the small GTPase *RAC1* (*VDAG_02474*) (Tian et al., 2015), transcription factor *Vdpf* (*VDAG_08521*) (Luo et al., 2016), glucan 1,3-beta-glucosidase (*VDAG_07185*) (Duressa et al., 2013), the glucose-repressive *GRG1* gene homologue (*VDAG_01467*) (Duressa et al., 2013) and a cell death-associated gene encoding an IDI-3 homolog (*VDAG_00261*) (Duressa et al., 2013; Wang et al., 2018a) were all downregulated in the *VdTRP* deletion mutants. The MAP kinase-mediated signalling pathway indirectly regulates pathogenicity and microsclerotial formation in *V. dahliae* (Luo et al., 2014). MAPK-related gene *VdAc* (*VDAG_04508*) (Luo et al., 2016) was also downregulated in the *VdTRP* deletion mutants. The endochitinase *VDECH* (*VDAG_08741*) has been reported to be downregulated in microsclerotia-producing cultures (Duressa et al., 2013), suggesting inhibition during microsclerotial development (Cheng et al., 2017). The gene was upregulated in the *VdTRP* deletion mutants, corroborating previous findings in *VdCmr1* deletion mutants (Wang et al., 2018a) (Fig. 7A). To confirm the accuracy of the transcriptome analysis results, we examined the mRNA
levels of these genes by quantitative reverse transcription, and the results are consistent with transcriptomic analysis (Fig. 7B).

Discussion

ASP F2 was first identified as a major allergen from *Aspergillus fumigatus*. In our previous study, VdASP F2 regulated the survival of *V. dahliae* mainly by sensing adverse environmental factors and transmitting them to cells to affect the formation of microsclerotia (Xie et al., 2017). Analogous to this, some ASP F2 orthologous proteins are involved in cellular responses to environmental stresses. ASP F2-like protein (PRA1) from *C. albicans* was differentially expressed in response to changes in the pH of the culture medium (Sentandreu et al., 1998). *S. cerevisiae* Zps1 is a homologous protein of ASP F2, and its expression is increased in response to zinc.
Many studies have demonstrated that ASP F2-like proteins are secreted into the extracellular space (Sentandreu et al., 1998; Xie et al., 2017). Here, we unambiguously elucidated the subcellular location of the cell wall of VdASP F2. Although V. dahliae VdASP F2 has been found to be an essential regulator of melanized microsclerotial formation under adverse environmental conditions (Xie et al., 2017), its mechanisms remain elusive. A transmembrane ion channel protein designated as VdTRP was identified via pull-down and mass spectrometry. Considering that the subcellular localization of VdASP F2 and VdTRP, it is very difficult to verify the protein-interaction relationship using the yeast two-hybrid system; therefore, we further demonstrated the interaction between VdASP F2 and VdTRP using the BiFC and Co-IP assays and confirmed its co-localization in the hyphal septa. Interestingly, further computational analysis was performed using the STRING database (http://string-db.org) to detect known and predicted protein interactions, and some identified interacting candidates of VdASP F2 with a score >0.7 (high confidence) also belong to the transmembrane ion channel proteins (Table S5).

According to conservative domain prediction, VdTRP contains a conserved TRP domain and belongs to the TRP channel protein superfamily (Huber et al., 1996). Yvc1 in S. cerevisiae is the earliest and most widely studied TRP protein in fungi (Palmer et al., 2001). According to the literature, Yvc1 in S. cerevisiae and C. albicans play an important role in oxidative stress and stress tolerance by regulating intracellular Ca\(^{2+}\) concentration (Chang et al., 2010; Ruta et al., 2014; Peng et al., 2020). In this study, we found that VdTRP deletion mutants cannot produce microsclerotia, even when conditions are favourable for microsclerotial development. These findings demonstrate the essential role of TRP-domain proteins in fungi in response to environmental stress.

Pra1, an orthologous protein of VdASP F2, exhibited zinc binding properties in vitro and structural modelling indicated physical interaction between Pra1 and Zrt1 (encoding a cell membrane transporters) to form a zinc scavenging system (Citiulo et al., 2012; Wilson et al., 2012). The conservation of this zinc scavenging system throughout the fungal kingdom, the orthologous gene pair, aspf2/zrfC, is also identified in A. fumigatus (Amich et al., 2010). VdTRP belongs to ion channel proteins...
and its interaction with VdASP F2 could serve the analogous function of the Pra1/Zrt1 in *V. dahliae*. Furthermore, PRA1/ZRT1 and aspf2/zrfC both are regulated by environmental pH and zinc availability (Citiulo et al., 2012). Therefore, we speculated that VdASP F2/VdTRP affect the microsclerotia formation because they can detect adverse environmental conditions such as limited nutrient conditions or low temperatures.

Our previous study suggested that the fungal transcription factor Vdpf mutant was melanized microsclerotia-deficient, and the expression of melanin biosynthesis-related genes (*BRN1*, *BRN2* and *SCD1*) was undetectable in the Vdpf mutant. Vdpf can also bind to the promoters of VdAc and VdPKAC1 to regulate microsclerotial formation in *V. dahliae* (Luo et al., 2016). The downregulated expression of these genes was consistent with the microsclerotia-deficient phenotype in the VdTRP mutants. Although many genes involved in the DHN melanin biosynthesis pathway were significantly downregulated, the expression of VdLac1 was not significantly different in our study. Similarly, VdLac1 has also been shown to be unnecessary for melanin production (Wang et al., 2018a). In addition, secreted carbohydrate-active enzymes (CAZys) function in the remodelling of fungal cell walls during microsclerotial maturation and were detected to be differentially expressed in our study (Duressa et al., 2018b). It was speculated that these genes may contribute to cell wall modifications that accompany morphological transitions in microsclerotial development (Fig. 7). Transcription factors Som1 and Vta3 regulate the adhesion, development, virulence and microsclerotia formation of *V. dahliae* by regulating common and unique target genes (Tri-Thuc et al., 2019). Transcription factor VdCmr1 also regulates expression of melanin biosynthetic genes (Wang et al., 2018a). Although some genes related to melanin synthesis and microsclerotial formation were regulated by Som1/Vta3 or VdCmr1, these transcription factors themselves did not show significant differential expression in our RNA-seq data.

![Fig. 7. Transcriptional profile of melanin- and microsclerotia biosynthesis-related genes in the VdTRP deletion mutants and its wild-type strains.](image)

A. Heatmap showing the expression pattern of melanin and microsclerotia biosynthesis-related genes on solid MIM medium. The heatmap was generated using fragments per kilobase of transcript sequence per million base pairs sequenced values normalized by Z-score.

B. Real-time quantitative PCR analysis of melanin- and microsclerotia biosynthesis-related genes in the VdTRP deletion mutants and its wild-type strains. Values represent mean ± SE of three independent biological replications. * and ** represent significant differences from roots of the same cultivar at *P* < 0.05, *P* < 0.01, respectively, based on Student’s *t*-test.
It may be attributed to that Som1/Vta3 and VdCmr1 regulate the microsclerotia formation through different signalling pathways or their differences were not significant in our RNA-seq data analysis.

The molecular control of microsclerotial development in *V. dahliae* has not been fully elucidated. Our results show that, by interacting with the transmembrane protein VdTRP, the cell wall protein VdASP F2 plays a crucial role in microsclerotial development. We also suggest that the deletion of VdTRP in *V. dahliae* results in differential expression of known microsclerotia-related genes between mutants and wild-type strains, which ultimately manifests in microsclerotia-formation impairments. These findings have contributed to our understanding of the importance of transmission of a signal from extracellular to intracellular under adverse environmental conditions, which conserved the developmental state of *V. dahliae* from normal growth to survival-structure-microsclerotial formation (Fig. 8).

### Experimental procedures

**Plant materials and strains**

*Gossypium hirsutum* was grown at 25°C under a 16:8 h light–dark cycle. *V. dahliae* strain V991 (preserved in our laboratory) was cultured on PDA plates at 25°C. *E. coli* strain DH5α and *Agrobacterium tumefaciens* strain AGL1 (Weidi Biotechnology, Shanghai, China) were used in this study. All vectors were transformed into *V. dahliae* V991 via Agrobacterium tumefaciens-mediated transformation as described previously (Xie *et al.*, 2017), and transformants were selected on PDA containing the appropriate antibiotics.

### Construction and transformation

To visualize the subcellular localization of VdASP F2, the full-length *VdASP F2* coding sequence without the stop codon was amplified from cDNA of wild-type V991 using primers VdASPLo-f/r. The amplification product was ligated into the SpeI/SpeI sites of the pSur-mCherry-Flag vector (unpublished) to generate the pSur-VdASP F2-mCherry-Flag vector. The pSur-mCherry-flag and the pSur-VdASP F2-mCherry-Flag vectors were transformed into wild-type *V. dahliae*. Transformants were selected with cefotaxime at 500 µg ml⁻¹ and hygromycin B at 50 µg ml⁻¹.

To generate vectors for the BiFC assay, C- and N-terminal fragments of YFP were amplified with primers nYFP-5f/r and cYFP-5f/r. The fragments of nYFP and cYFP were ligated into the SpeI/EcoRI-digested pSur vector and PacI/SpeI-digested pG418 vector to generate pSur-
nYFP and pG418-cYFP respectively. The VdASP F2 coding sequence without the stop codon and the VdTRP coding sequence with the stop codon were amplified using the primers VdASPBi-f/r and VdTRPBi-f/r. The fragments were ligated into PacI/SpeI-digested pSur-nYFP and SpeI/EcoRI-digested pG418-cYFP to generate pSur-VdASP F2::nYFP and pG418-cYFP::VdTRP respectively. pSur-VdASP F2::nYFP/pG418-cYFP::VdTRP, pSur-nYFP/pG418-cYFP::VdTRP, or pSur-VdASP F2::nYFP/pG418-cYFP was co-transformed into V991, and transformants were selected with cefotaxime at 50 µg ml⁻¹ and G418 at 25 µg ml⁻¹. For the CoIP assay, HA-VdTRP amplified with primers HA f and VdTRP r was ligated into pG418 vector, and pG418-HA-VdTRP was transformed into the VdASP F2-mCherry-Flag expression or wide-type strains. To obtain pG418-YFP and pG418-YFP::VdTRP vectors, YFP was amplified with primers YFPLo-f/r1 and YFPLo-f/r2 and ligated into PacI/EcoRI-linearized pG418 and PacI/EcoRI-linearized pG418-VdTRP vector respectively. pG418-YFP and pG418-YFP::VdTRP were transformed into the VdASP F2-mCherry-Flag expression strain. Transformants were selected with cefotaxime at 500 µg ml⁻¹, hygromycin B at 50 µg ml⁻¹ and G418 at 25 µg ml⁻¹. For the CoIP assay, HA-VdTRP amplified with primers HA f and VdTRP r was ligated into pG418 vector, and pG418-HA-VdTRP was transformed into the VdASP F2-mCherry-Flag expression or wide-type strains. To obtain pG418-YFP and pG418-YFP::VdTRP vectors, YFP was amplified with primers YFPLo-f/r1 and YFPLo-f/r2 and ligated into PacI/EcoRI-linearized pG418 and PacI/EcoRI-linearized pG418-VdTRP vector respectively. pG418-YFP and pG418-YFP::VdTRP were transformed into the VdASP F2-mCherry-Flag expression strain. Transformants were selected with cefotaxime at 500 µg ml⁻¹, hygromycin B at 50 µg ml⁻¹ and G418 at 25 µg ml⁻¹.

To generate the VdTRP gene-replacement plasmid, two homologous arms were amplified with primers VdTRP 5'-5r and VdTRP 3'-3r from the genomic DNA of V. dahliae strain V991. The Hyg' cassette (selection marker) was amplified from the vector psilent1 (Hyg f/r), and the three fragments were linked to the green fluorescent protein plasmid (pGFP) using the ligation-independent cloning method (Xie et al., 2017). The VdTRP-deleted strains were screened according to our previously published protocol (Xie et al., 2017). Transformants were selected with cefotaxime at 500 µg ml⁻¹ and hygromycin B at 50 µg ml⁻¹.

To generate the VdTRP complementation vector, the entire VdTRP gene with its promoter (1500 bp) and terminator (1000 bp) sequences (VdTRPcom f/r) were cloned into the complementation plasmid pG418 and introduced into the VdTRP deletion strains. Transformants were selected with cefotaxime at 500 µg ml⁻¹, hygromycin B at 50 µg ml⁻¹ and G418 at 25 µg ml⁻¹ (Xie et al., 2017).

**Pull-down/MS assays**

First, the mCherry-Flag and VdASP F2-mCherry-Flag expressing strains were constructed. The V. dahliae strain expressing mCherry-Flag or VdASP F2-mCherry-Flag were cultured on PDA medium covered with a cellophane layer. The hyphae were collected in liquid nitrogen and suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.2% Triton X-100, 100 mM NaCl, 1 mM EDTA, 1 mM 2-ME and a cocktail of proteinase inhibitors). Samples were centrifuged three times at 13 000 g for 10 min at 4°C. The supernatants were incubated on ice with agarose beads coupled with anti-FLAG monoclonal antibodies and gently shaken for 3 h at 4°C. The beads were collected by centrifugation at 13 000 g for 10 min at 4°C and then washed five times with lysis buffer to reduce non-specific binding. The beads were mixed with 50 µl of 1× SDS-PAGE loading buffer and boiled at 95°C for 10 min. Proteins were separated by SDS-PAGE (10%), and the bands were visualized by silver staining. The strain of mCherry-Flag were used as a negative control. The specific bands were excised according to the results of silver staining and subjected to mass spectrometry at BGI Co., Ltd. (http://www.genomics.cn). Briefly, the gel pieces were incubated in a destaining solution until the colour fades. Protein bands were reduced with 10 mM DTT at 56°C for 1 h, alkylated with 55 mM iodoacetamide (IAM) for 1 h in a dark, dehydrated with acetonitrile and digested overnight in trypsin solution (0.01 µg µl⁻¹ in 25 mM ammonium bicarbonate, pH 8.9) at 37°C. After removal of the solution, the samples were extracted with 50% acetonitrile and 0.5% formic acid solution. The extracts were dried in a vacuum concentrator. The sample was resuspended with 2% acetonitrile and 0.1% formic acid and centrifuged 15 min. Supernatant was loaded onto an Acclai PepMap C18-reversed phase column (Thermo Fisher Scientific, USA) and separated with reversed phase C18 column (Agela Technologies, China). Peptides were eluted using a different concentration gradients of buffer B (98% Acetonitrile, 0.1% Formic Acid) at a flow rate of 400 nl min⁻¹. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA), setting in positive ion mode and data-dependent manner with full MS scan from 350–2000 m/z. Peptide identification was carried out on the Mascot software (Version 2.3.01, Matrix Science, UK) using the Uniprot database search algorithm and the integrated false discovery rate (FDR) analysis function.

**Microscopic observation**

To observe the subcellular location of VdASP F2, the V. dahliae strains expressing mCherry-Flag or VdASP F2-mCherry-Flag were cultured on PDA medium. The conidial and hyphal were collected and stained with the cell wall fluorescence stain calcofluor white (Sigma-Aldrich) at 1 µg ml⁻¹ final concentration. Protoplasts were prepared as previously described. Fluorescence was observed under a fluorescent microscope (DM3000, Carl Zeiss, Germany).

To observe the fluorescence of BiFC, transformants expressing VdASP F2::nYFP/ cYFP::VdTRP, nYFP/
cYFP::VdTRP, or VdASP F2::mYFP/cYFP were cultured on PDA medium. YFP fluorescence was detected using a confocal microscope (Olympus, Tokyo, Japan).

To observe the fluorescence of co-localization between VdASP F2 and VdTRP, V. dahliae strains expressing YFP/VdASP F2-mCherry-Flag or YFP::VdTRP/VdASP F2-mCherry-Flag were cultured on PDA medium. Fluorescent images of GFP and mCherry were obtained using confocal microscope (Olympus, Tokyo, Japan).

Stress response and carbon utilization assays

For abiotic stress assays, PDA plates were supplemented with KCl (0.7 M), H₂O₂ (1 mM), sorbitol (1 M) or Congo Red (400 μg ml⁻¹). PDA plates without such supplements were used as controls. Drops of spore suspensions were cultured on PDA plates with or without supplements and incubated at 28°C. To investigate the role of VdTRP in V. dahliae growth during carbon uptake or utilization of specific carbon sources, wild-type V991 strains (WT), VdTRP deletion strains (∆VdTRP-1/∆VdTRP-2) and complemented strains (COM) were grown on Czapek–Dox medium (0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄·7H₂O and 1.5% sucrose) with or without such supplements and incubated at 28°C. The colony diameters of all strains were measured after 15 days of incubation. All experiments were repeated thrice (Xie et al., 2017).

Microsclerotial formation assays

To determine whether VdTRP is required for microsclerotial formation, all strains (WT, ∆VdTRP, COM) were inoculated on the nitrocellulose membrane, which was overlaid on PDA and MIM medium (glucose, 5.0 g l⁻¹; NaNO₃, 0.2 g l⁻¹; KCl, 0.52 g l⁻¹; MgSO₄·7H₂O, 0.52 g l⁻¹; K₂HPO₄, 1.52 g l⁻¹; vitamin B₁, 3.0 μM; vitamin H, 0.1 μM; agar, 15 g l⁻¹) (Hu et al., 2013). After 7 or 30 days, hyphal and microsclerotial samples were collected for microscopic observation.

Pathogenicity assays

To evaluate the role of VdTRP in the pathogenicity of V. dahliae, four-week-old G. hirsutum were inoculated with spores from WT, ∆VdTRP, COM and sterile water (mock) using the root dip inoculation method as described previously. Plants were grown in a greenhouse at 25°C under a 16:8 h light–dark cycle (Parthasarathy and Bart, 2013). Results were recorded approximately 20 days after inoculation with V. dahliae.

All pathogenicity assays were independently performed three times with three plants for each genotype.

RNA-Seq

All strains (WT and ∆VdTRP) used for transcriptome profile analyses were first inoculated on a cellophane membrane overlayed on PDA medium for 5 days, after which they were transferred onto MIM and allowed to inoculate for 3 days. Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies). Before transcriptome sequencing, the concentration, quality and integrity were determined using a NanoDrop spectrophotometer (Thermo Scientific). RNA (3 μg) was used as the input material for the RNA sample preparations. Sequencing libraries were generated according to the following steps. First, mRNA was purified from total RNA using poly T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations at elevated temperatures in an Illumina proprietary fragmentation buffer. First-strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second-strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities, and the enzymes were removed. After adenylation of the 3’ ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. To select cDNA fragments of the preferred 400–500 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments with ligated adaptor molecules on both ends were selectively enriched using the Illumina PCR Primer Cocktail in a 15 cycle PCR reaction. The products were purified (AMPure XP system) and quantified using an Agilent high sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). The sequencing library was then sequenced on the NovaSeq 6000 platform (Illumina) by Shanghai Personalbio Technology Co., Ltd. Clean reads filtered from the raw transcriptome sequence data were mapped to the reference genome of V. dahliae, and these mapped reads were used for further analyses. Gene expression profile analysis was done as described previously (Zhang et al., 2018). GO and KEGG analysis using Ttools software (Chen et al., 2020).

RT-qPCR and gene expression analysis

Total RNA was extracted from the ∆VdTRP and wild-type V991 strains. Isolated total RNA was reverse transcribed using the PrimeScript RT Reagent Kit (TaKaRa Dalian Biotechnology). RT-qPCR was performed in a Bio-Rad CFX96 Real-Time system using SYBR Premix
ExTaq II (TaKaRa Dalian Biotechnology). The PCR protocol was as follows: Cycling was carried out for 1 min at 95°C, followed by 40 cycles of 5 s at 95°C, 15 s at 58°C and 10 s at 72°C. The 18S rRNA gene was amplified as an endogenous control. The resulting data were analysed using GraphPad Prism 8 software (https://www.graphpad.com/). The relative normalized expression between ΔVdTRP and WT was determined using the comparative Ct method (2^(-ΔΔCt)).

All gene accession numbers are listed in Table S2, and the primers used in this study are listed in Table S3. The V. dahliae V991 transformants used in this study are listed in Table S4.

Bioinformatics and data analysis

Homologous sequences of VdTRP in other fungi were obtained by BLASTP and aligned using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) (Kumar et al., 2016), and the alignment results were visualized using Jalview. Transmembrane helices were predicted using TMPHMM SERVER v.2.0 (Krogh et al., 2001). Protein interaction network analysis was carried out using STRING based on the V. dahliae VdLs.17 Organism database (http://string-db.org) (Supplementary Table S5).

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Conflict of interest

The authors declare no conflict of interest.

Author Contributions

CM.G. and CJ.X. contributed to conceptualization and writing—original draft preparation; HLS. and C.C. contributed to methodology; ZJ.H. contributed to software; X.Y. and XY.Z. contributed to investigation; XY.Y. contributed to resources; CJ.X. contributed to supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The RNA-Seq sequence data set supporting the results of this study is available at NCBI with accession numbers SRR17906242, SRR17906243, SRR17906244 and SRR17906245.

References

Amich, J., Vicentefranqueira, R., Leal, F., and Calera, J.A.A. (2010) Aspergillus fumigatus survival in alkaline and extreme zinc-limiting environments relies on the induction of a zinc homeostasis system encoded by the zrfC and aspl2 genes. Eukaryot Cell 9: 424–437.

Caterina, M., Leffler, A., Malmberg, A., Martin, W., Trafton, J., Petersen-Zeitz, K., et al. (2000) Impaired nociception and pain sensation in mice lacking the capsacin receptor. Science 288: 306–313.

Chang, Y., Schlenstedt, G., Flockerzi, V., and Beck, A. (2010) Properties of the intracellular transient receptor potential (TRP) channel in yeast, Yvc1. FEBS Lett 584: 2028–2032.

Chen, C., Chen, H., Zhang, Y., Thomas, H.R., Frank, M.H., He, Y., and Xia, R. (2020) TBtools: an integrative toolkit developed for interactive analyses of big biological data. Mol Plant 13: 1194–1202.

Cheng, X.-X., Zhao, L.-H., Klosterman, S.J., Feng, H.-J., Feng, Z.-L., Wei, F., et al. (2017) The endochitinase VDECH from Verticillium dahliae inhibits spore germination and activates plant defense responses. Plant Sci 259: 12–23.

Citiulo, F., Jacobsen, I.D., Miramón, P., Schild, L., Brunke, S., Zipfel, P., et al. (2012) Candida albicans scavenges host zinc via Pra1 during endothelial invasion. PLoS Pathog 8: e1002777.

Colbert, H., Smith, T., and Bargmann, C. (1997) OSM-9, a novel protein with structural similarity to channels, is required for olfaction, mechanosensation, and olfactory adaptation in Caenorhabditis elegans. J Neurosci 17: 8259–8269.

Duressa, D., Anchietà, A., Chen, D., Klimes, A., García-Pedrajas, M., Dobinson, K., and Klosterman, S. (2013) RNA-seq analyses of gene expression in the microsclerotia of Verticillium dahliae. BMC Genom 14: 607.

Fan, R., Gong, X., Gao, L., Shang, W., Hu, X., and Xu, X. (2020) Temporal dynamics of the survival of Verticillium dahliae microsclerotia with or without melanin in soils amended with biocontrol agents. Eur J Plant Path 157: 521–531.

Fradin, E., and Thomma, B. (2006) Physiology and molecular aspects of Verticillium wilt diseases caused by V. dahliae and V. albo-atrum. Mol Plant Pathol 7: 71–86.

Harting, R., Hoefer, A., Van, T., Weinhold, L.M., Barghahn, S., Schluerer, R., and Braus, G. (2020) The Vta1 transcriptional regulator is required for microsclerotia melanization in Verticillium dahliae. Fungal Biol UK 124: 490–500.

He, X., Li, X., and Li, Y. (2015) Disruption of Cerevisin via Agrobacterium tumefaciens-mediated transformation affects microsclerotia formation and virulence of Verticillium dahliae. Plant Pathol 64: 1157–1167.

Hu, X., Bai, Y., Chen, T., Hu, D., Yang, J., and Xu, X. (2013) An optimized method for in vitro production of Verticillium dahliae microsclerotia. Eur J Plant Path 136: 225–229.

Huber, A., Sander, P., Gobert, A., Bähner, M., Hermann, R., and Paulsen, R. (1996) The transient receptor potential protein (Trp), a putative store-operated Ca^2+ channel
essential for phosphoinositide-mediated photoreception, forms a signaling complex with NorpA, InaC and InaD. EMBO J 15: 7036–7045.

Klimes, A., Amyotte, S.G., Grant, S., Kang, S., and Dobinson, K.F. (2008) Microsclerotia development in Verticillium dahliae: Regulation and differential expression of the hydrophobin gene VDH1. Fungal Genet Biol 45: 1525–1532.

Klimes, A., and Dobinson, K.F. (2006) A hydrophobin gene, VDH1, is involved in microsclerotial development and spore viability in the plant pathogen Verticillium dahliae. Fungal Genet Biol 43: 283–294.

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305: 567–580.

Kubo, Y., Takano, Y., Endo, N., Yasuda, N., Tajima, S., and Furusawa, I. (1996) Cloning and structural analysis of the melanin biosynthesis gene SCD1 encoding scytalone dehydratase in Colletotrichum lagenarium. Appl Environ Microb 62: 4340–4344.

Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874.

Leung, H., Geng, C., and Pak, W. (2000) Phenotypes of trp mutants and interactions between the transient receptor potential (TRP) and TRP-like channels in Drosophila. J Neurosci 20: 6797–6803.

Li, J., Zhou, L., Yin, C., Zhang, D., Klosterman, S.J., Wang, B., et al. (2019) The Verticillium dahliae Sho1-MAPK pathway regulates melanin biosynthesis and is required for cotton infection. Environ Microbiol 21: 4852–4874.

Luo, X., Mao, H., Wei, Y., Cai, J., Xie, C., Sui, A., et al. (2016) The fungal-specific transcription factor Vdfp influences conidia production, melanized microsclerotia formation and pathogenicity in Verticillium dahliae. Mol Plant Pathol 17: 1364–1381.

Luo, X., Xie, C., Dong, J., Yang, X., and Sui, A. (2014) Interactions between Verticillium dahliae and its host: vegetative growth, pathogenicity, plant immunity. Appl Microbiol Biot 98: 6921–6932.

Maruthachalam, K., Klosterman, S.J., Kang, S., Hayes, R.J., and Subbarao, K.V. (2011) Identification of pathogenicity-related genes in the vascular wilt fungus Verticillium dahliae by Agrobacterium tumefaciens-mediated T-DNA insertional mutagenesis. Mol Biotechnol 49: 209–221.

Niemeyer, B., Suzuki, E., Scott, K., Jalink, K., and Zeker, C. (1996) The Drosophila light-activated conductance is composed of the two channels TRP and TRPL. Cell 85: 651–659.

Palmer, C., Aydar, E., and Djamgoz, M. (2005) A microbial TRP-like polycystic-kidney-disease-related ion channel gene. Biochem J 387: 211–219.

Palmer, C.P., Zhou, X.-L., Lin, J., Loukin, S.H., Kung, C., and Saimi, Y. (2001) A TRP homolog in Saccharomyces cerevisiae forms an intracellular Ca^{2+}-permeable channel in the yeast vacuolar membrane. Proc Natl Acad Sci USA 98: 7801–7805.

Parthasarathy, S., and Bart, T. (2013) Verticillium dahliae Sge1 differentially regulates expression of candidate effector genes. Mol Plant Microbe Interact 26: 249–256.

Peng, L., Yu, Q., Zhu, H., Zhu, N., Zhang, B., Wei, H., et al. (2020) The V-ATPase regulates localization of the TRP Ca^{2+}-channel Yvc1 in response to oxidative stress in Candida albicans. Int J Med Microbiol 310: 151466.

Perpetua, N., Kubo, Y., Yasuda, N., Takano, Y., and Furusawa, I. (1996) Cloning and characterization of a melanin biosynthetic THR1 reductase gene essential for appressorial penetration of Colletotrichum lagenarium. Mol Plant Microbe Interact 9: 323.

Qi, X., Zhou, S., Shang, X., and Wang, X. (2016) VdSh1 regulates growth, oxidative adaptation and virulence in Verticillium dahliae. J Phytopathol 164: 1064–1074.

Rauyaree, P., Ospina-Giraldo, M.D., Kang, S., Bhat, R.G., Subbarao, K.V., Grant, S.J., and Dobinson, K.F. (2005) Mutations in VMK1, a mitogen-activated protein kinase gene, affect microsclerotia formation and pathogenicity in Verticillium dahliae. Curr Genet 48: 109–116.

Reuss, H., Mojet, M., Chyb, S., and Hardie, R. (1997) In vivo analysis of the Drosophila light-sensitive channels, TRP and TRPL. Neuron 19: 1249–1259.

Ruta, L., Popa, V., Nicolau, I., Danet, A., Iordache, V., Neagoe, A., and Farcasanu, I. (2014) Calcium signaling mediates the response to cadmium toxicity in Saccharomyces cerevisiae cells. FEBS Lett 588: 3202–3212.

Sentandreu, M., Elorza, M., Sentandreu, R., and Fonzi, W. (1998) Cloning and characterization of PRA1, a gene encoding a novel pH-regulated antigen of Candida albicans. J Bacteriol 180: 282–289.

Tang, C., Jin, X., Klosterman, S.J., and Wang, Y. (2020) Convergent and distinctive functions of transcription factors VdYap1, VdAtf1, and VdSkn7 in the regulation of nitrosative stress resistance, microsclerotia formation, and virulence in Verticillium dahliae. Mol Plant Pathol 21: 1451–1466.

Tang, C., Xiong, D., Fang, Y., Tian, C., and Wang, Y. (2017) The two-component response regulator VdSkn7 plays key roles in microsclerotial development, stress resistance and virulence of Verticillium dahliae. Fungal Genet Biol 108: 26–35.

Tian, H., Zhou, L., Guo, W., and Wang, X. (2015) Small GTPase Rac1 and its interaction partner Clα4 regulate polarized growth and pathogenicity in Verticillium dahliae. Fungal Genet Biol 74: 21–31.

Tian, L., Wang, Y., Yu, J., Xiong, D., Zhao, H., and Tian, C. (2016) The Mitogen-activated protein kinase kinase VdPbs2 of Verticillium dahliae regulates microsclerotia formation, stress response, and plant infection. Front Microbiol 7: 1532.

Tian, L., Xu, J., Zhou, L., and Guo, W. (2014) VdMsb regulates virulence and microsclerotia production in the fungal plant pathogen Verticillium dahliae. Gene 550: 238–244.

Tian, L., Yu, J., Wang, Y., and Tian, C. (2017) The C_{3}H_{2} transcription factor VdMsn2 controls hyphal growth, microsclerotia formation, and virulence of Verticillium dahliae. Fungal Biol UK 121: 1001–1010.

Tri-Thuc, B., Harting, R., Braus-Stromeyer, S.A., Van-Tuan, T., Leonard, M., Hoefer, A., et al. (2019) Verticillium dahliae transcription factors Som1 and Vta3 control microsclerotia formation and sequential steps of plant root penetration and colonisation to induce disease. New Phytol 221: 2138–2159.
Tsuiji, G., Fujikawa, J., Ishida, H., Horino, O., and Kubo, Y. (2001) Laccase gene LAC1 of Colletotrichum lagenarium is not essential for melanin biosynthesis and pathogenicity. J Gen Plant Pathol 67: 182–190.

Tsuiji, G., Sugahara, T., Fujii, I., Mori, Y., Ebizuka, Y., Shiraiishi, T., and Kubo, Y. (2003) Evidence for involvement of two napthol reductases in the first reduction step of melanin biosynthesis pathway of Colletotrichum lagenarium. Mycol Res 107: 854–860.

Upadhyay, S., Xu, X., Lowry, D., Jackson, J.C., Roberson, R.W., and Lin, X. (2016) Subcellular compartmentalization and trafficking of the biosynthetic machinery for fungal melanin. Cell Rep 14: 2511–2518.

Wang, G., Liu, Z., Lin, R., Li, E., Mao, Z., Ling, J., et al. (2016a) Biosynthesis of antibiotic leucinostatins in biocontrol fungus Purpureocillium lilacinum and their inhibition on phytophthora revealed by genome mining. PLoS Pathog 12: e1005685.

Wang, Y., Hu, X., Fang, Y., Ancheta, A., Goldman, P.H., Hernandez, G., and Klosterman, S.J. (2018a) Transcription factor VdCmr1 is required for pigment production, protection from UV irradiation, and regulates expression of melanin biosynthetic genes in Verticillium dahliae. Microbiology 164: 685–696.

Wang, Y., Hu, X., Fang, Y., Ancheta, A., Goldman, P., Hernandez, G., and Klosterman, S.J. (2019) The polyketide synthase VdPKS1 and the transcription factor VdCmr1 are required for pigment production and UV protection in Verticillium dahliae. Phytopathology 109: 99.

Wang, Y., Tian, L., Xiong, D., Klosterman, S.J., Xiao, S., and Tian, C. (2016b) The mitogen-activated protein kinase gene, VdHog1, regulates osmotic stress response, microsclerotia formation and virulence in Verticillium dahliae. Fungal Genet Biol 88: 13–23.

Wang, Y., Weisenhorn, E., MacDiarmid, C.W., Andreini, C., Bucci, M., Taggart, J., et al. (2018b) The cellular economy of the Saccharomyces cerevisiae zinc proteome. Metallomics 10: 1755–1776.

Wilson, D., Citifulo, F., and Hube, B. (2012) Zinc exploitation by pathogenic fungi. PLoS Pathog 8: e1003034.

Xie, C., Li, Q., and Yang, X. (2017) Characterization of VdASP F2 secretory factor from Verticillium dahliae by a fast and easy gene knockout system. Mol Plant Microbe Interact 30: 444–454.

Xiong, D., Wang, Y., Ma, J., Klosterman, S., Xiao, S., and Tian, C. (2014) Deep mRNA sequencing reveals stage-specific transcriptome alterations during microsclerotia development in the smoke tree vascular wilt pathogen, Verticillium dahliae. BMC Genom 15: 324.

Xiong, D., Wang, Y., Tang, C., Fang, Y., Zou, J., and Tian, C. (2015) VdCrz1 is involved in microsclerotia formation and required for full virulence in Verticillium dahliae. Fungal Genet Biol 82: 201–212.

Xiong, D., Wang, Y., Tian, L., and Tian, C. (2016) MADS-Box transcription factor VdMcm1 regulates conidiation, microsclerotia formation, pathogenicity, and secondary metabolism of Verticillium dahliae. Front Microbiol 7: 1192.

Yu, J., Li, T., Tian, L., Tang, C., Klosterman, S.J., Tian, C., and Wang, Y. (2019) Two Verticillium dahliae MAPKKKS, VdSsk2 and VdSte11, have distinct roles in pathogenicity, microsclerotia formation, and stress adaptation. mSphere 4: e00426-19.

Zhang, W.Q., Gui, Y.J., Short, D.P.G., Li, T.G., Zhang, D.D., Zhou, L., et al. (2018) Verticillium dahliae transcription factor VdFTF1 regulates the expression of multiple secreted virulence factors and is required for full virulence in cotton. Mol Plant Pathol 19: 841–857.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. VdTRP knock-out and complementation in V. dahliae. A, 5’- and 3’-flanking region of VdTRP gene and hygromycin resistance gene (HPH®). B, GFP is not observed in null mutants but is present in ectopic insertion transformants or null mutants with ectopic insertions. C, The gene deletion event was verified using a pair of primers complementary to the region outside of the two homologous arms of the VdTRP gene-deletion cassettes. D, Verification of RNA level reveals the elimination of the VdTRP gene in gene-deletion mutants. 18S RNA served as an internal control.

Fig. S2. GO annotation and KEGG enrichment analysis for upregulated DEGs. A, GO enrichment (P < 0.01). MF, molecular function, BP, biological process, CC, cellular component. B, KEGG pathway enrichment (P < 0.01).

Table S1. The proteins interacting with VdASP F2.

Table S2. Known and putative melanin and microsclerotia biosynthesis-related genes of V. dahliae.

Table S3. Primers used in this study.

Table S4. V. dahliae strains used in this study.

Table S5. Predicted functional partners of VdASP F2 in V. dahliae.

Method S1. Protocol for gene knockout.