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 complementary 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.**

Received 24 February, 2022; revised 12 April, 2022; accepted 15 April, 2022.

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Microbial Biotechnology (2022) **15**(7), 2040–2054
doi:10.1111/1751-7915.14066

Funding information

This research was funded by the National Natural Science Foundation of China [31701734] and the Natural Science Foundation of Chongqing [cstc2021jcyj-msxmX0098, cstc2017jcyjAX0190].

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 (C_2H_2 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

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 *VMK1*, *VdCmr1*, *VdLac1*, *Vayg1*, 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* formation in 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, VdAtf1, 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, TRPN, 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 TRPV 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 (Niemeyer *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

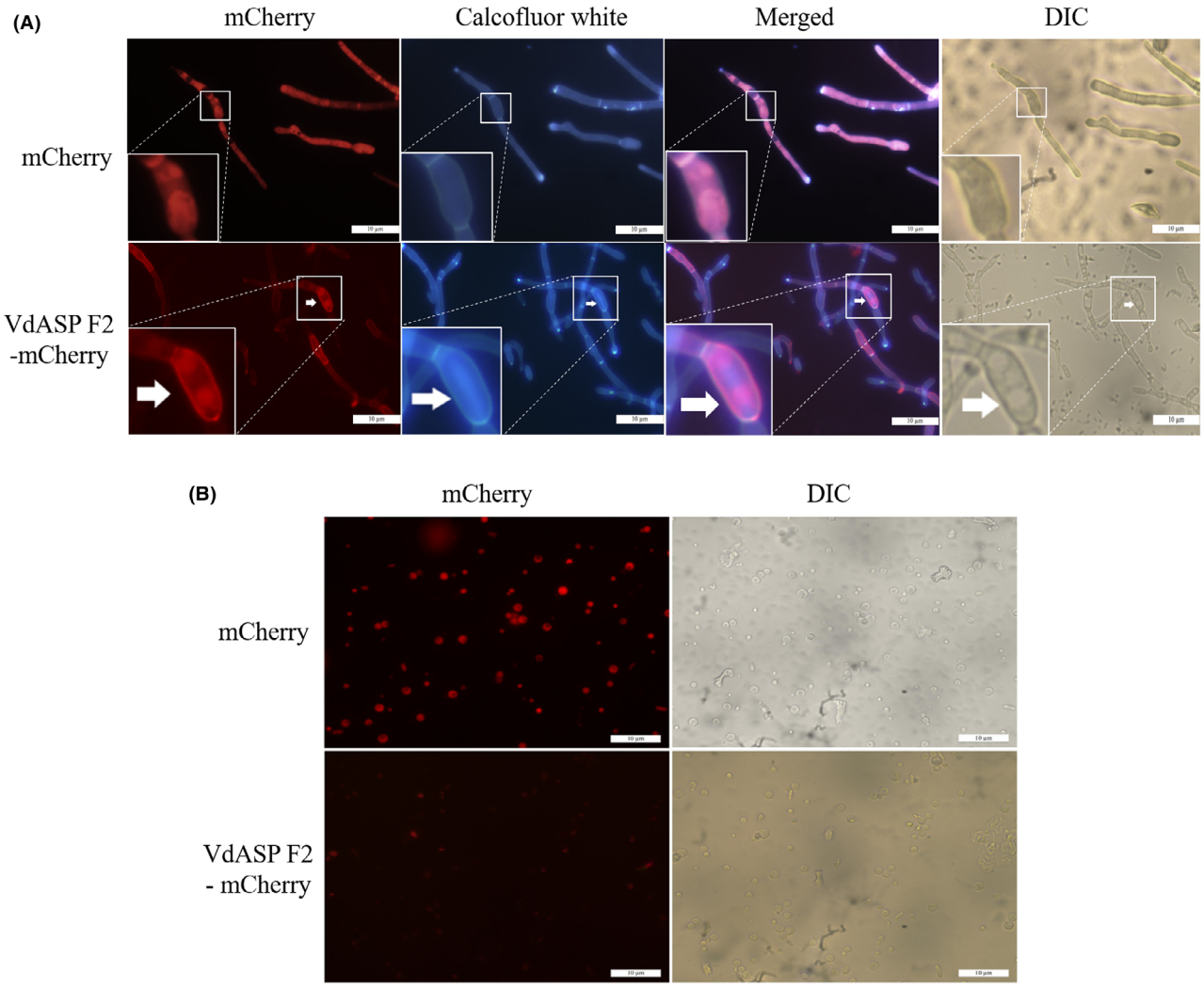


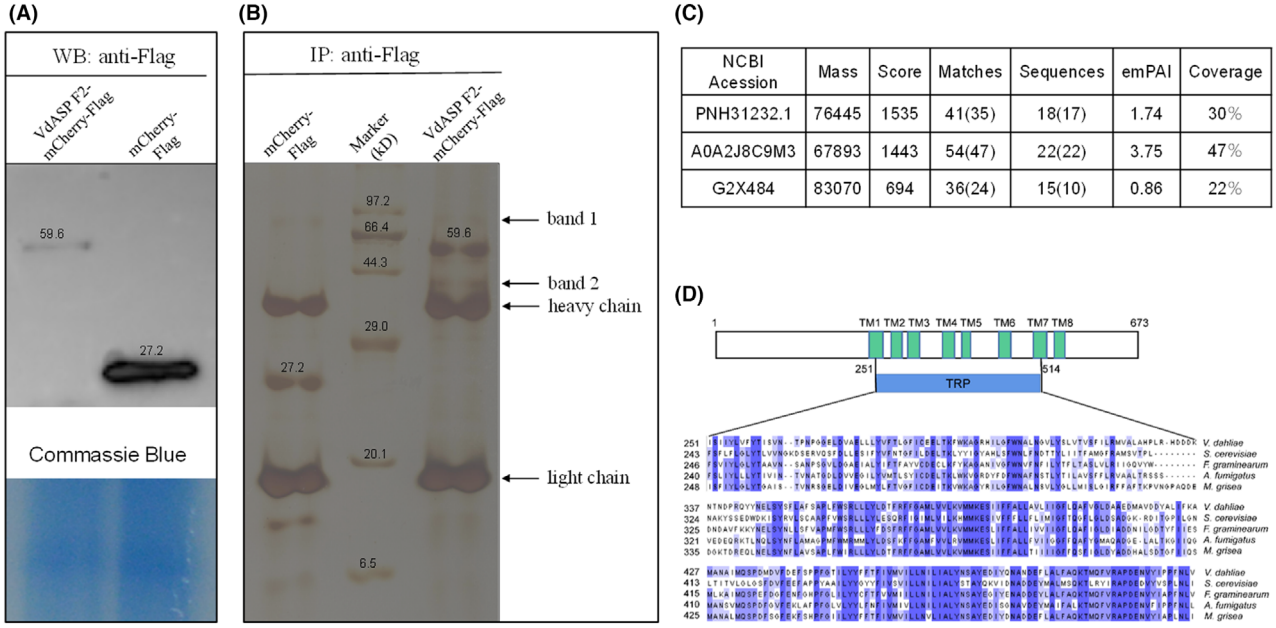
Fig. 1. Subcellular localization of VdASP F2 protein.
A. Under differential interference contrast (DIC) and fluorescence microscopy, VdASP F2-mCherry-Flag and cell wall-specific staining agent calcofluor white (CFW) exhibited similar localization.
B. VdASP F2-mCherry-Flag localization in protoplasts after cell wall removal. There was almost no fluorescence in the cytoplasm of VdASP F2-mCherry-Flag expression strains, whereas the control had strong red fluorescence.

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



NCBI Acession	Mass	Score	Matches	Sequences	emPAI	Coverage
PNH31232.1	76445	1535	41(35)	18(17)	1.74	30%
A0A2J8C9M3	67893	1443	54(47)	22(22)	3.75	47%
G2X484	83070	694	36(24)	15(10)	0.86	22%

Fig. 2. Pull-down/MS assays indicate that VdASP F2 interacts with VdTRP.
A. Western blotting analysis confirmed that the expression of VdASP F2-mCherry-Flag and mCherry-Flag in *Verticillium dahliae*.
B. Proteins were extracted and purified with anti-FLAG M2 agarose beads. Equal protein samples were separated by 10% SDS-PAGE and stained with silver staining. Band 1 and 2 indicate the band cut from the gel and subjected to MS-based sequencing.
C. Representative examples from key proteins interacting with VdASP F2 are shown.
D. Predicted transmembrane domains and conserved TRP functional domain are marked. Sequence alignments of the TRP domain of related proteins from *V. dahliae* (PNH31232.1), *Saccharomyces cerevisiae* (AJU04758.1), *Fusarium graminearum* (PCD28072.1), *Aspergillus fumigates* (KAF4254639.1) and *Magnaporthe grisea* (XP_030976064.1).

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_2O_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.

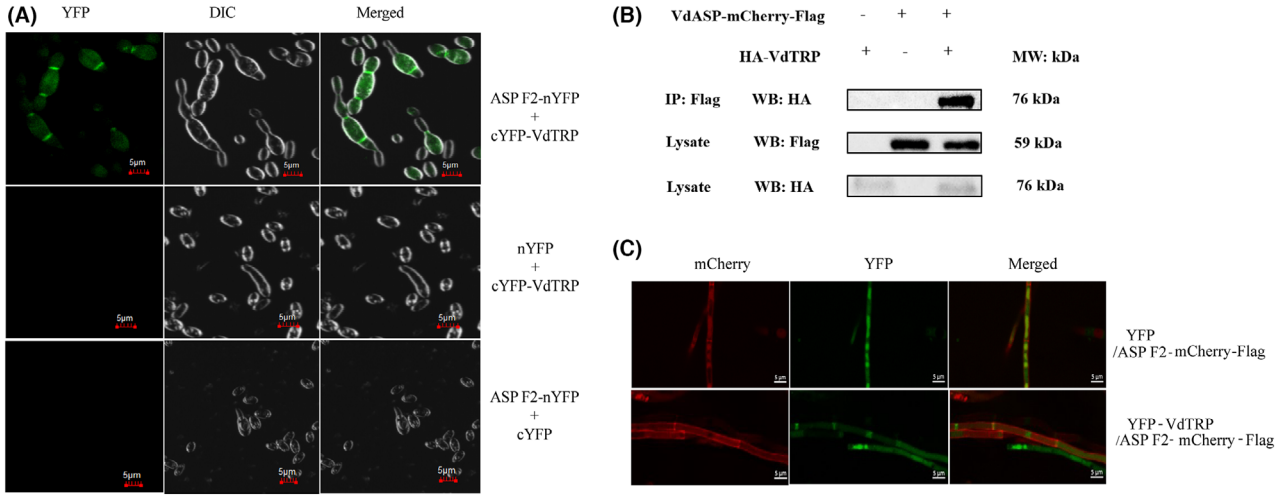


Fig. 3. (a) BiFC assay. VdASP F2-nYFP and cYFP-VdTRP were transformed into *Verticillium dahliae*, and the fluorescence was visualized by confocal microscopy. Control nYFP + cYFP-VdTRP (VdASP F2-nYFP + cYFP) were treated with the similar method. DIC, differential interference contrast. (b) CoIP assay. IP, immunoprecipitation. WB, Western blot. (c) Subcellular co-localization of recombinant VdASP F2-mCherry-Flag with YFP-VdTRP. Under fluorescence microscope, YFP and mCherry co-located in the hyphal septa.

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 Encyclopaedia 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.*,

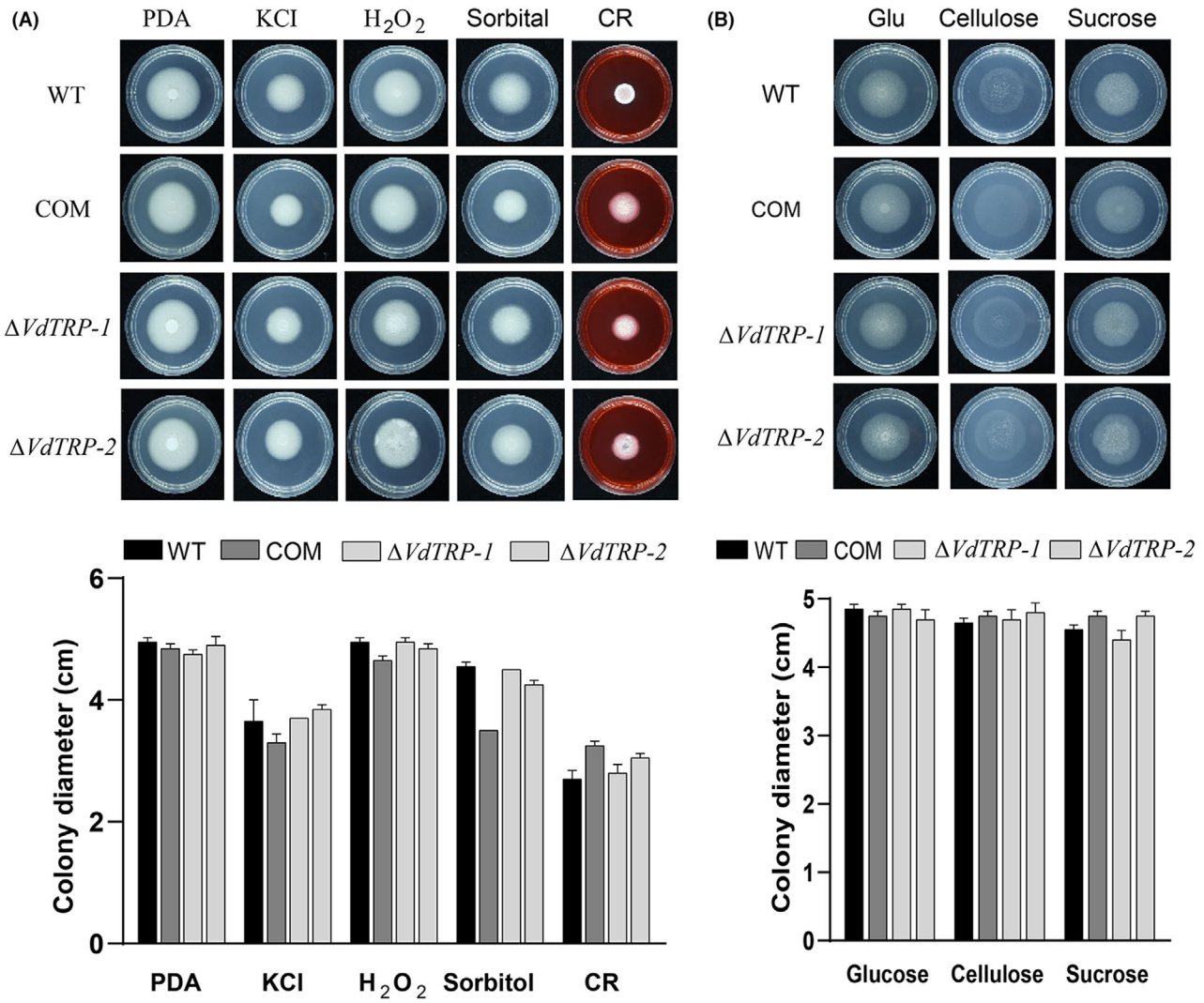


Fig. 4. Mycelial growth of wild-type V991 (WT), *VdTRP* deletion mutants (*ΔVdTRP-1/ΔVdTRP-2*) and complemented strains (COM).
A. The colony morphology of the WT, *ΔVdTRP-1/ΔVdTRP-2,* and COM strains grown on PDA medium or PDA supplemented KCl (0.7 M), H_2O_2 (1 mM), sorbitol (1 M), or Congo Red (400 μg/mL). Photographs were taken at 7 dpi.
B. These strains were cultivated on Czapek–Dox medium (CDM) with 1.5% glucose, 1.5% sucrose or 0.5% cellulose as the sole carbon source at 26°C after 8 days. The colony diameter was quantified. Error bars represent standard deviations of three replicates.

2001) 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

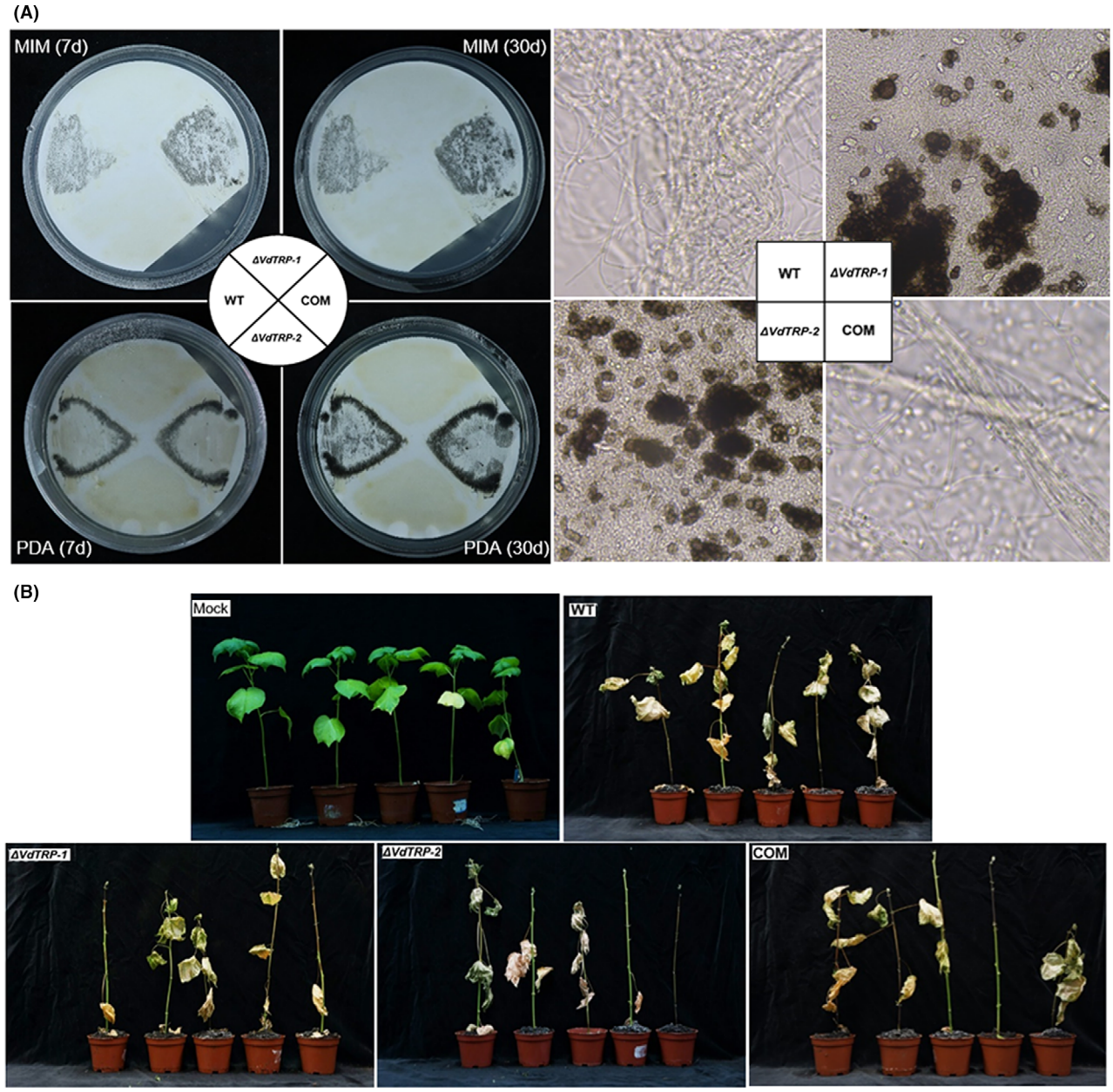


Fig. 5. (a) Strains were cultured on solid MIM medium (upper plate) and PDA medium (lower plate). Mycelia and microsclerotia were scraped off the membrane for examination under the microscope. The *VdTRP* deletion mutants reduced microsclerotial development. Photographs of the same Petri dishes were taken 7 and 30 days post-inoculation. (b) One-month-old *Gossypium hirsutum* was incubated with the conidia of wild-type (WT), *VdTRP* deletion mutants (*ΔVdTRP-1*/*ΔVdTRP-2*) and complemented strains (COM). Sterile water treatments were used as control (Mock). Photographs were taken 20 days post-inoculation, and the experiment was repeated three times

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

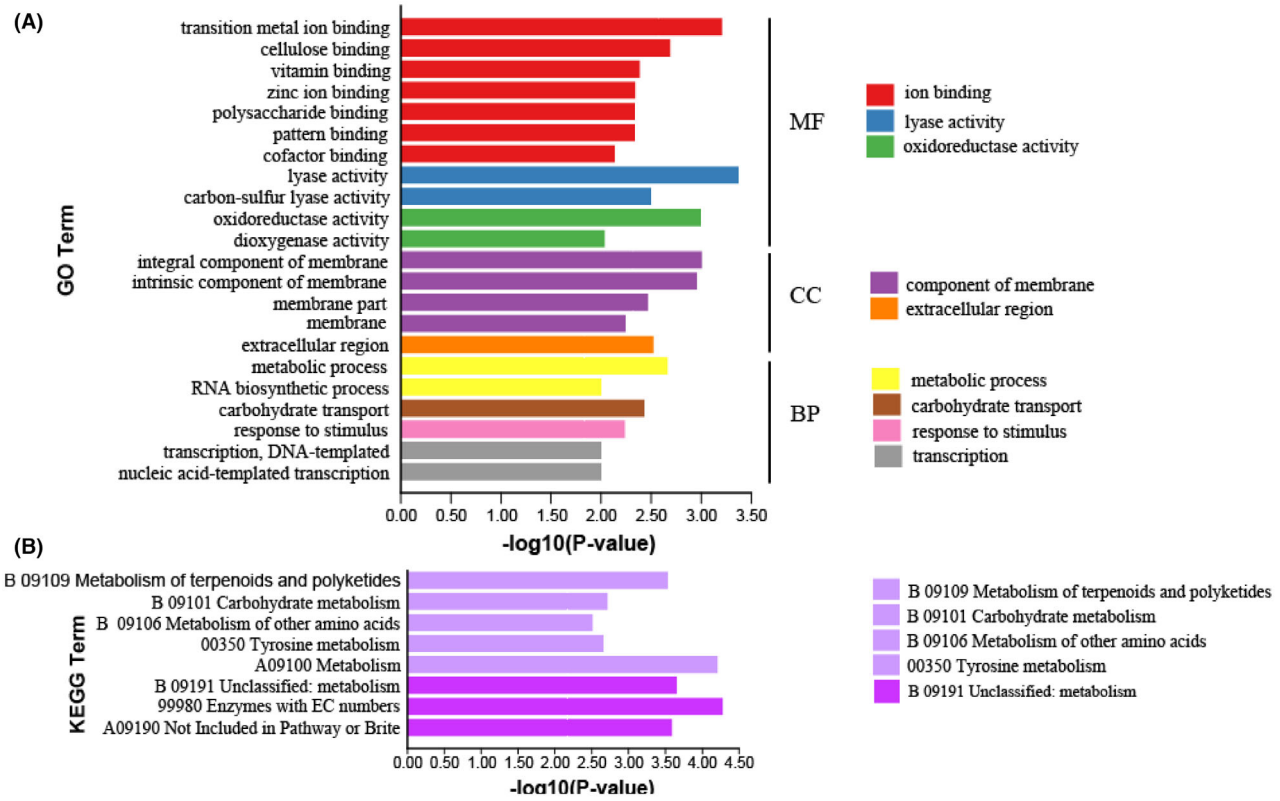


Fig. 6. GO annotation and KEGG enrichment analysis for downregulated DEGs.
A. GO enrichment ($P < 0.01$). MF, molecular function, BP, biological process, CC, cellular component.
B. KEGG pathway enrichment ($P < 0.01$).

deficiency (Wang *et al.*, 2018b). 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, a 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

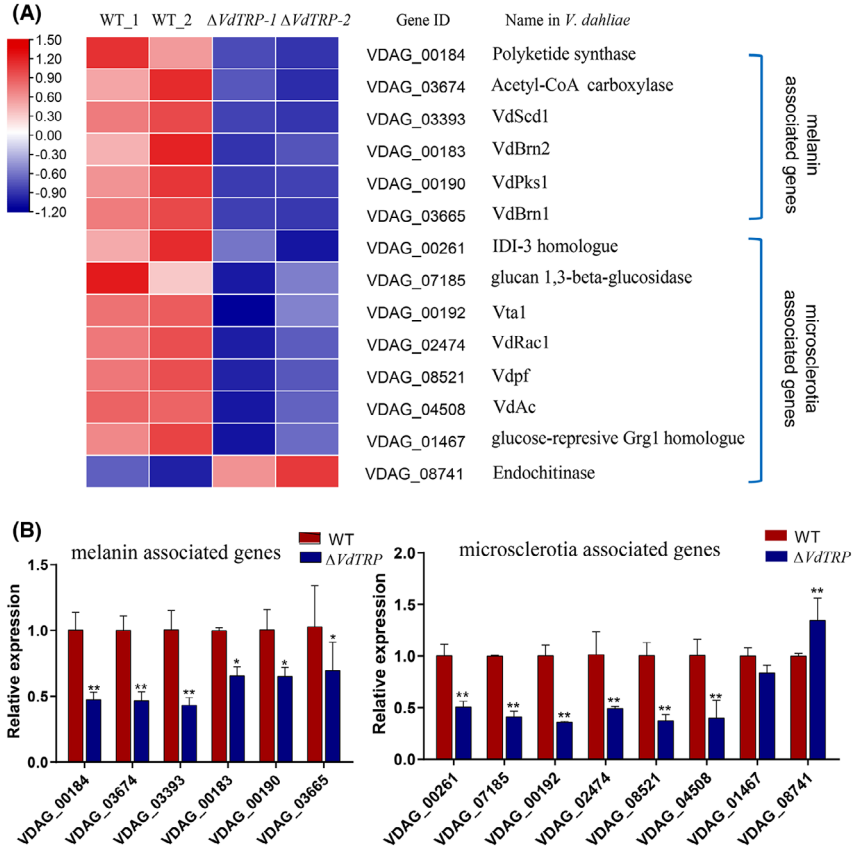


Fig. 7. Transcriptional profile of melanin- and microsclerotia biosynthesis-related genes in the *VdTRP* deletion mutants and its wild-type strains.
A. Heatmap showing the expression pattern of melanin and microsclerotia biosynthesis-related genes on solid MIM medium. The heatmap was generated using 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.

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.*, 2013; Wang *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

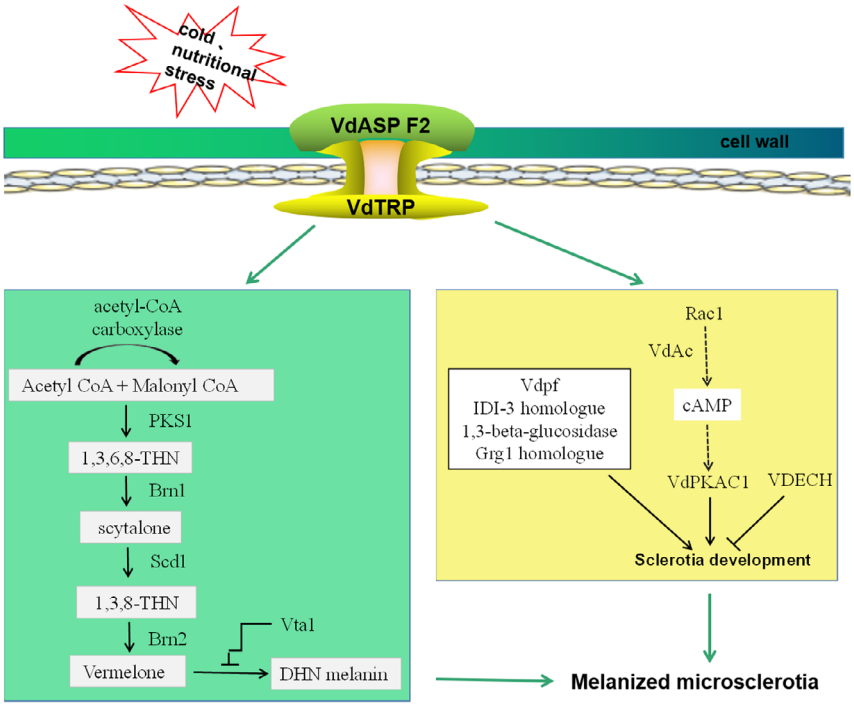


Fig. 8. Model of how VdASP F2 regulates the formation of melanized microsclerotia in *Verticillium dahliae*. Under adverse environmental conditions, cell wall protein VdASP F2 transmits external signals into cells, through interacting with membrane protein VdTRP, to activate melanin and microsclerotia biosynthesis pathway. The DHN melanogenesis pathway was drawn based on previous reports. The simplified MAPK signalling pathways was drawn according to the reports. The solid black arrows represent confirmed results, while dashed black arrows represent inference steps.

analysis. 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 DH5a and *Agrobacterium tumefaciens* strain AGL1 (Weidi Biotechnology, Shanghai, China) were used in this study. All vectors were transformed into *V. dahliae* V991 via Agrobacterium tumefacien-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 *Pac*I/*Spe*I 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-f/r and cYFP-f/r. The fragments of nYFP and cYFP were ligated into the *Spe*I/*EcoR*I-digested pSur vector and *Pac*I/*Spe*I-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 *Pac*I/*Spe*I-digested pSur-nYFP and *Spe*I/*Eco*RI-digested pG418-cFYP 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 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 *Pac*I/*Eco*RI-linearized pG418 and *Pac*I/*Eco*RI-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 5f/5r and VdTRP 3f/3r from the genomic DNA of *V. dahliae* strain V991. The Hyg^{r} 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, ground 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 remaining 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 Acclaim 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 conidia 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::nYFP/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), H_2O_2 (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 26°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 (*ΔVdTRP-1*/*ΔVdTRP-2*) and complemented strains (COM) were grown on Czapek–Dox medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% KCl, 0.001% $FeSO_4{\cdot}7H_2O$ and 1.5% agar) with 1.5% glucose, 1.5% sucrose and 0.5% cellulose as the sole carbon source at 26°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, *ΔVdTRP*, COM) were inoculated on the nitrocellulose membrane, which was overlaid on PDA and MIM medium (glucose, 5.0 g l^{-1}; $NaNO_3$, 0.2 g l^{-1}; KCl, 0.52 g l^{-1}; $MgSO_4{\cdot}7H_2O$, 0.52 g l^{-1}; K_2HPO_4, 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, *ΔVdTRP*, 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 *ΔVdTRP*) used for transcriptome profile analyses were first inoculated on a cellophane membrane overlaid 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 Tbtools software (Chen *et al*., 2020).

RT-qPCR and gene expression analysis

Total RNA was extracted from the *ΔVdTRP* 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 ($2^{-\Delta\Delta Ct}$).

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 TMHMM 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).

Acknowledgements

This research was funded by the National Natural Science Foundation of China [31701734] and the Natural Science Foundation of Chongqing [cstc2021jcyj-msxmX0098, cstc2017jcyjAX0190].

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; HL.S. 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.

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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^R). 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.

