Characterization of VdASP F2 Secretory Factor from *Verticillium dahliae* by a Fast and Easy Gene Knockout System

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The vascular wilt fungus *Verticillium dahliae* produces persistent resting structures known as microsclerotia, which enable long-term survival of this plant pathogen in soil. The completed genome sequence of *V. dahliae* has facilitated large-scale investigations of individual gene functions using gene-disruption strategies based on *Agrobacterium tumefaciens*-mediated transformation. However, the construction of gene-deletion vectors and screening of deletion mutants have remained challenging in *V. dahliae*. In this study, we developed a fast and easy gene knockout system for *V. dahliae* using ligation-independent cloning and fluorescent screening. We identified secretory factor VdASP F2 in a T-DNA insertion library of *V. dahliae* and deleted the VdASP F2 gene using the developed knockout system. Phenotypic analysis suggests that VdASP F2 is not necessary for *V. dahliae* growth on potato dextrose agar under various stress conditions. However, on semisynthetic medium or under limited nutrient conditions at lower temperatures, the VdASP F2 deletion mutant exhibited vigorous mycelium growth, less branching, and a significant delay in melanized microsclerotial formation. Further assessment revealed that VdASP F2 was required for the expression of *VDH1* and *VMK1*, two genes involved in microsclerotial formation. Cotton inoculated with the VdASP F2 deletion mutant wilted, demonstrating that VdASP F2 is not associated with pathogenicity under normal conditions. However, after inducing microsclerotial formation and incubation at low temperatures, cotton infected with the VdASP F2 deletion mutant did not exhibit wilt symptoms. In conclusion, our results show that VdASP F2 plays an important role in the response of *V. dahliae* to adverse environmental conditions and is involved in a transition to a dormant form for prolonged survival.

Verticillium dahliae is a widespread and destructive soilborne fungus that causes wilt disease in hundreds of dicotyledonous plant species, including herbaceous annuals, perennials, and woody plants (Fradin and Thomma 2006; Klosterman et al. 2009). This leads to severe economic losses, especially in cotton production (James 2002). *V. dahliae* is particularly difficult to control because the fungus produces melanized structures known as

microsclerotia (Rauyaree et al. 2005). These microsclerotia are dormant structures that allow the fungus to exist in the soil without a host for many years (Fradin and Thomma 2006). After stimulation by root exudates, microsclerotia germinate and subsequently enter into the root to initiate new infection cycles; thus, they are the primary source of *V. dahliae* infection (Fradin and Thomma 2006). The molecular mechanisms involved in *Verticillium* wilt have been gradually uncovered with the discovery of genes involved in virulence, growth, conidiation, and development. *VMK1* encodes a mitogen-activated protein kinase that is involved in signal transduction pathways that control cellular and developmental processes in response to environmental cues. This protein has been reported to be required for microsclerotial formation and pathogenicity in *V. dahliae* (Rauyaree et al. 2005). *VDH1*, a hydrophobin gene, is also involved in microsclerotial development but is not required for pathogenicity of *V. dahliae* (Klimes and Dobinson 2006; Klimes et al. 2008). Glutamic acid-rich protein 1 plays an important role in sensing limited nutrient conditions in *V. dahliae* and is involved in microsclerotial formation and virulence (Gao et al. 2010). Microsclerotial development also involves mitogen-activated protein kinase VdHog1 (Wang et al. 2016b), MADS-box transcription factor VdMcm1 (Xiong et al. 2016), and calcineurin-responsive zinc-finger transcription factor Crz1 (Xiong et al. 2015). Several additional proteins involved in virulence have been identified, including cAMP-dependent protein kinase A (VdPkc1) (Tzima et al. 2010), G-protein β subunit (Tzima et al. 2012), necrosis- and ethylene-inducing peptide-1-like proteins (Santhanam et al. 2013; Zhou et al. 2012), the transcriptional regulator VdSge1 (Santhanam and Thomma 2013), and Ave, an effector recognized by the tomato Ve1 immune receptor, which mediates tomato resistance against *V. dahliae* race 1 (de Jonge et al. 2012).

With the completion of the genome sequence of *V. dahliae*, large-scale gene functional analysis has become possible. *Agrobacterium tumefaciens*-mediated transformation (ATMT) is a convenient and effective method for transformation of *V. dahliae* conidia (Maruthachalam et al. 2011). However, the generation of gene-replacement constructs and identification of null mutants from a large number of transformants remain limiting factors. To construct gene-replacement vectors, enzyme digestion or fusion polymerase chain reaction (PCR) are conventionally used (Szewczyk et al. 2007); however, these are usually time consuming and, sometimes, appropriate double-restriction enzyme digestion sites cannot be found. Several convenient cloning techniques have been introduced to facilitate vector construction, such as the one-step construction of *Agrobacterium* recombination-ready plasmids method (Paz et al. 2011), the USER cloning technique (Wang et al. 2016a),

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and *Saccharomyces cerevisiae*-based recombination methods (Lu et al. 2014). Some methods have been employed to remove ectopic transformants. The herpes simplex virus thymidine kinase (HSVtk) is capable of converting 5-fluoro-2'-deoxyuridine to a compound that is toxic to fungi; therefore, some researchers weed out ectopic insertions using HSVtk as a negative selectable marker (Ma et al. 2009; Wang et al. 2016a). In addition, *KU70/KU80* (*mus-51/mus-52*) mutations have been used to construct fungal gene knockout background strains that exhibit a higher frequency of homologous recombination (Qi et al. 2015; Takahashi et al. 2006).

To explore the mechanism of microsclerotial development, we screened the *V. dahliae* T-DNA insertion transformant library and identified a mutant with less melanized microsclerotial formation. Using hiTAIL-PCR (Liu and Chen 2007), the T-DNA was identified to be inserted in a gene encoding allergen Asp F2-like proteins (VDAG_04551). These proteins are highly conserved sequences among fungi with signal peptides and are considered cell-wall components (He et al. 2015; Sentandreu et al. 1998). He et al. (2015) suggest that the disruption of the *cerevisin* gene affects microsclerotial formation and the virulence of *V. dahliae*, and *VdASP F2* expression is significantly reduced in the *cerevisin* mutant (He et al. 2015). Asp F2 is a major allergen in *Aspergillus fumigatus* (Banerjee et al. 1996, 1998). Pra1, a pH-regulated Asp F2-like protein in *Candida albicans*, is a surface-associated and secreted Asp F2-like protein. Pra1 may play a role in cell morphogenesis by gene disruption (Sentandreu et al. 1998), inhibiting the activation of innate immune cells and neutrophil responses (Losse et al. 2011; Luo et al. 2010; Soloviev et al. 2007). However, the function of Asp F2 in plant-pathogenic fungi is poorly understood. In this study, we established a fast and easy gene knockout system for *V. dahliae*, which we used to create a *V. dahliae* V991 deletion mutant lacking *VdASP F2* in order to determine its role in *V. dahliae*.

RESULTS

Gene-replacement constructs.

Exonuclease III (ExoIII) catalyzes the stepwise removal of mononucleotides from the 3'-hydroxyl termini of double-stranded DNA to generate long, compatible, cohesive ends between DNA inserts and cloning vectors. The annealed DNA complex is stable, and the ligation reaction can be omitted. The annealed DNA complex is transformed into *Escherichia coli*, and the noncomplementary regions (gaps and overhangs) are repaired by the bacteria. This technique has come to be known as ligation-independent cloning (LIC) (Liu et al. 2012).

PCR primers were designed to amplify two homologous arms of the *VdASP F2* gene with 15 bp of the sequence overlapping the ends of the digested pGFP vector, which was constructed based on the pPK2 and constitutively expresses enhanced green fluorescent protein gene (eGFP) (Fig. 1A) (P1 and P4) or the hygromycin B phosphotransferase resistance gene cassette (*Hyg^r*) (P2 and P3). The digested pGFP vector, *Hyg^r* cassette, and two homologous *VdASP F2* arms were mixed with ExoIII (Fig. 1A) to generate long, compatible, cohesive ends between the pGFP vector, *Hyg^r* cassette, and *VdASP F2* arms. The longer cohesive ends were able to anneal to each other and were more stable and, therefore, the ligation reaction could be omitted. Finally, the bacterial repair system resolved the overhangs or gaps in a predictable fashion to generate excisable inserts (Fig. 1A) (Li and Evans 1997). The gene-replacement construct was named pGFP-*VdASP F2*, and approximately 15 to 30 colonies grew on the medium each time, all of which were correctly constructed vectors.

The gene disruption cassette was introduced into the host genome, where it was integrated by either homologous recombination or by ectopic recombination. Even if the target gene

has been deleted in some transformants, extra ectopic integration of T-DNA is possible. Therefore, null mutants, ectopic insertions, and null mutants with ectopic insertions are all possible after *Agrobacterium tumefaciens* transformation (Fig. 1B).

Gene deletion and complementation of *VdASP F2* in *V. dahliae*.

To verify gene-replacement constructs and analyze the function of *VdASP F2* in *V. dahliae*, we constructed a gene knockout vector of *VdASP F2* using the developed system. The knockout vector was transformed into *V. dahliae* via *A. tumefaciens* AGL1. As a negative selection marker, eGFP virtually eliminates all ectopic insertions in the knockout system. Green fluorescent screening was carried out to identify *VdASP F2* null mutants (Fig. 2A). To confirm that *VdASP F2* had been knocked out, primers were used to amplify the genome sequence at the insertion site (Fig. 1A), which returned a longer fragment if *VdASP F2* had been knocked out (Fig. 2B).

We obtained candidate null mutants based on the results of green fluorescent screening and PCR amplification (Fig. 2A and B). For further confirmation, null mutants, ectopic insertions, and null mutants with ectopic insertions were analyzed by Southern blotting. The expected 2.6-kb band was observed on the Southern blot after *Xba*I digestion in null mutants, whereas multiple bands were observed in ectopic insertion mutants and null mutants with ectopic insertions (Fig. 2C). Reverse-transcription (RT)-PCR was performed to confirm the absence of *VdASP F2* mRNA in *VdASP F2* deletion strains (Fig. 2D). The results clearly indicate that the developed knockout system was successfully used to create a *V. dahliae* gene deletion mutant.

VdASP F2 is a secretory protein that is highly conserved among fungi.

The *VdASP F2* gene was present as a single copy in this *V. dahliae* strain. It has an open reading frame of 894 nucleotides with no introns and encodes a protein of 297 amino acids. BLAST searches against the National Center for Biotechnology Information GenBank database were performed to identify orthologs of *VdASP F2* in order to examine the conservation of allergen Asp F2-like proteins in other organisms. Amino acid sequence analysis revealed that the resulting *VdASP F2* orthologs were highly homologous, and the amino acid sequence was highly conserved among fungi. Alignments against orthologs from *S. cerevisiae*, *Aspergillus fumigatus*, and a few typical plant pathogens (*Fusarium oxysporum* and *Sclerotinia sclerotiorum*) are shown in Figure 3A.

Bioinformatics analysis revealed that Asp F2-like proteins contain a predicted N-terminal signal peptide. The signal peptide domain of *VdASP F2* is located in the region between amino acid positions 1 and 18, suggesting that *VdASP F2* may be a secreted protein (Fig. 3A). To confirm this hypothesis, eGFP and eGFP-tagged *VdASP F2* (*VdASP F2*:eGFP) constitutive expression strains were constructed. Intense green fluorescence was detected in eGFP strains under fluorescence microscopy but only faint green fluorescence was observed in *VdASP F2*:eGFP strains (Fig. 3B). Strains were further inoculated in liquid Czapek-Dox medium and cultured with shaking. Western blotting revealed that *VdASP F2*:eGFP could be detected in the supernatant of the culture medium, indicating that *VdASP F2* is indeed a secreted protein (Fig. 3C).

To analyze whether *VdASP F2* is localized in plant tissue, we also expressed and purified fusion *VdASP F2*:eGFP without signal peptide (Δ SP *VdASP F2*:eGFP-6His) (Supplementary Fig. S1A and B) because the signal peptide was cleaved off after the *VdASP F2* was secreted by the *V. dahliae* strain. The locations of Δ SP *VdASP F2*:eGFP-6His and eGFP-6His are not significantly different in plant cells, as indicated by GFP

fluorescence. In the *in vitro* root uptake assay, the purified proteins of both Δ SP VdASP F2:eGFP-6His and eGFP-6His were inoculated with cotton roots for 12 h, and GFP fluorescence was observed on the root cells. However, the GFP fluorescence did not remain after washing twice (Supplementary Fig. S1C). Therefore, the results of the Δ SP VdASP F2:eGFP-6His uptake assay suggested that the secreted protein VdASP F2 cannot enter the plant cells.

Fungal growth and susceptibility to abiotic stress.

We constructed a geneticin (G418)-resistant vector to confirm that the phenotypic differences observed in the VdASP F2 deletion mutants were associated with the gene replacement event (Supplementary Fig. S2). A wild-type VdASP F2 gene under the control of its native promoter and terminator was introduced into the gene deletion mutant, and one G418-resistant transformant was selected for RT-PCR analysis of VdASP F2

Fig. 1. Overview of fast and easy gene-knockout system in *Verticillium dahliae*. **A**, Two homologous arms of the target gene are amplified using polymerase chain reaction with primers containing 15 bp of sequence at the 5' end and exonuclease III (ExoIII) digest to generate overhangs. P1 and P4 contain overhangs homologous to the pGFP vector, and P2 and P3 have overhangs homologous to the hygromycin B phosphotransferase resistance gene cassette (*Hyg^r*), which allow for annealing. The annealing mixture is transformed into *Escherichia coli*, and gaps and overhangs are repaired by the bacteria to generate the circular knockout vector. The gene-deletion construct is then transformed into *V. dahliae* via *Agrobacterium tumefaciens*-mediated transformation (ATMT). Transformants are screened for GFP fluorescence under a microscope. WT = wild type and eGFP = *eGFP*. **B**, Homologous recombination creates three types of transformants: null mutants, ectopic insertion transformants, and null mutants with ectopic insertions. Null mutants do not have GFP fluorescence because the GFP gene was eliminated in the null mutants but ectopic transformants and null mutants with ectopic insertions exhibit green fluorescence. Primers P5 and P6 are used for identification of recombined DNA fragments, and a probe is used for Southern blotting.

expression. This complemented mutant was then used in further phenotypic analyses for comparison with *VdASP F2* knockout mutants. We assessed the vegetative growth and microsclerotial formation of the *VdASP F2* deletion mutant, ectopic insertion transformant, complemented mutant, and wild-type strains under a variety of abiotic stresses. We found that vegetative growth and microsclerotial formation were not significantly different among the strains in the presence of NaCl, KCl, H₂O₂, high pH, or sorbitol (Fig. 4A).

To investigate the role of *VdASP F2* in *V. dahliae* growth during carbon uptake or utilization of specific carbon sources, the *VdASP F2* deletion mutant, ectopic insertion transformant, complemented mutant, and wild-type strains were grown on Czapek-Dox medium with different carbon sources (glucose, sucrose, or cellulose). Analysis of colony diameters indicated no significant differences in radial growth rates among the different strains (Supplementary Fig. S3). All results indicated that *VdASP F2* is not involved in carbon utilization or growth processes in *V. dahliae*. The *VdASP F2* deletion strain, however, did exhibit less pigment and microsclerotial formation on

carbon- or nitrogen-deficient medium and on semisynthetic (Czapek-Dox) medium (Fig. 4B).

Disruption of *VdASP F2* causes significantly delayed development of melanized microsclerotia in *V. dahliae*.

To explore the role of *VdASP F2* in microsclerotial development, we grew the *VdASP F2* deletion mutant, ectopic insertion transformant, complemented mutant, and wild-type strains on microsclerotia-inducing medium (MIM) to induce near-synchronous development of microsclerotia (Neumann and Dobinson 2003). After 15 days of growth, melanized microsclerotial production was observed in the ectopic insertion transformant, complemented mutant, and wild-type strains but not in the *VdASP F2* deletion mutant. Melanized microsclerotial formation was observed in the *VdASP F2* deletion mutant after 60 days (Fig. 5A). These results suggest that the *VdASP F2* deletion delayed microsclerotial formation and development rather than eliminating it. The results are also consistent with growth on Czapek-Dox medium, where the *VdASP F2* deletion mutant exhibited less production of microsclerotia in response

Fig. 2. Green fluorescent protein (GFP) screening and verification of *VdASP F2* deletion mutants in *Verticillium dahliae*. **A**, GFP is not present in wild-type (WT) or null mutants but is present in ectopic insertion transformants and null mutants with ectopic insertions. **B**, A pair of primers complementary to the region outside of the two homologous arms of the *VdASP F2* gene-deletion cassettes was used to verify the gene-deletion event. Open circles, closed circles, and open squares are used to represent ectopic insertion mutants (EC), null mutants (KO), and null mutants with ectopic insertions (KO+EC), respectively. **C**, Southern blotting indicates a single-copy insertion of the hygromycin B phosphotransferase resistance gene cassette (*Hyg^r*) in null mutants. Additional bands in the other lanes suggest ectopic insertions in ectopic insertion transformants and null mutants with ectopic insertions. **D**, reverse-transcription polymerase chain reaction reveals the elimination of the *VdASP F2* gene in gene-deletion mutants. β -Tubulin was used as a reference gene.

to nutrient limitation. Moreover, *VDH1* and, particularly, *VMK1*, two genes that are involved in microsclerotial formation (Klimes and Dobinson 2006; Klimes et al. 2008; Raayaree et al. 2005), exhibited lower expression in the *VdASP F2* deletion mutant than in the wild type on MIM (Fig. 5B). These results demonstrate that *VdASP F2* is related to microsclerotial formation.

Finally, we examined the mycelial growth and melanized microsclerotial formation of the *VdASP F2* deletion mutant under extremely limited nutrient conditions by analyzing microsclerotial morphology in water agar medium (López-Escudero et al. 2012). Mycelial growth was extremely slow in the ectopic transformant, complemented mutant, and wild-type strains but the *VdASP F2* deletion mutant exhibited vigorous aerial mycelium growth (Fig. 5C). In addition, the mycelia of the *VdASP F2* deletion mutant formed fewer branching hyphae than those of other strains (Fig. 5C). In particular, some substrate mycelium aggregates containing a dark, melanized outer layer formed in the ectopic transformant, complemented mutant, and wild-type strains but not in the *VdASP F2* deletion mutant

(Fig. 5C and D). These hyphal aggregates eventually developed into microsclerotia.

We also examined the mycelial growth and melanized microsclerotial production of the *VdASP F2* deletion mutant under low temperature. None of the *V. dahliae* strains exhibited microsclerotial formation at 26°C; however, except for the *VdASP F2* deletion mutant, they formed microsclerotia more quickly when inoculated at 8°C. The *VdASP F2* deletion strain always exhibited vigorous mycelial growth on potato dextrose agar (PDA) with nitrocellulose membranes (Fig. 5E and F). These results suggest that *VdASP F2* is involved in the response of *V. dahliae* to environmental conditions such as nutritional deficiency or low temperature but it is not necessary for microsclerotial formation under nutrient-rich conditions.

VdASP F2 is not required for pathogenicity but is necessary for survival of *V. dahliae* under adverse circumstances.

We subsequently assessed the role of *VdASP F2* in the pathogenicity of *V. dahliae* by inoculating 3-week-old upland

Fig. 3. *VdASP F2* is a secreted protein in *Verticillium dahliae*. **A**, Multiple alignments indicate that *ASP F2* is highly conserved among fungi. Protein sequences from top to bottom are derived from *V. dahliae*, *Fusarium oxysporum*, *Saccharomyces cerevisiae*, *Sclerotinia sclerotiorum*, and *Aspergillus fumigatus*. **B**, Under differential interference contrast (DIC) and fluorescence (UV) microscopy, intense green fluorescence was detected in constitutively expressed eGFP strains, while only faint green fluorescence was observed in *VdASP F2*:eGFP strains. WT = wild type. **C**, Western blot analysis with total proteins isolated from liquid Czapek-Dox medium. Bands (58 kDa) were detected in *VdASP F2*:eGFP strains but not in WT or eGFP strains. Coomassie brilliant blue staining reveals equal loading of proteins used for Western blot analysis.

cotton seeds (*Gossypium hirsutum* ‘Xinluzao-13’) with the *VdASP F2* deletion mutant, ectopic insertion transformant, complemented mutant, and wild-type strains using root-dip inoculation. All four strains showed wilting symptoms 20 days after *V. dahliae* inoculation (Fig. 6A). Thus, *VdASP F2* does not appear to be required for pathogenicity against *G. hirsutum*.

V. dahliae is able to survive for 10 to 15 years in soil owing to the formation of microsclerotia (Fradin and Thomma 2006). To form microsclerotia and mimic *V. dahliae* overwintering in soil, we mixed the four *V. dahliae* strains with soil and incubated them at 8°C for 1 week to form microsclerotia. We then incubated them for 2 months at –20°C to simulate winter conditions, and 6-week-old *G. hirsutum* seedlings were transferred into soil with these strains. Cotton plants infected with the ectopic transformant, complemented mutant, and wild-type strains exhibited chlorosis, wilting, and necrosis symptoms. However, those infected with the *VdASP F2* deletion mutant did not (Fig. 6B).

In order to analyze the correlation between pathogen growth within plant tissues and symptom severity, quantitative PCR analysis was used to analyze the level of *V. dahliae* DNA in the aboveground stem of cotton. We seldom detected *V. dahliae* DNA in *G. hirsutum* infected by the low-temperature pretreated *VdASP F2* deletion mutant (Fig. 6C). We speculate that the low infection rate of *G. hirsutum* is the result of low survival of the *V. dahliae VdASP F2* mutant in the soil, due to its impaired ability to form microsclerotia.

DISCUSSION

Since the sequencing of the genome of *V. dahliae*, there has been a surge in investigations into the gene functions of *V. dahliae* using high-throughput gene deletion techniques.

ATMT is an effective method for the transformation of *V. dahliae*. However, the construction of gene-deletion vectors and screening of gene-deletion mutants limit the efficiency of ATMT. LIC offers an alternative to restriction enzyme or ligase cloning. Although the use of T4 DNA polymerase and ExoIII provides an efficient technique for cloning (Li and Elledge 2007; Liu et al. 2012), the resulting constructs are not stable, particularly when dealing with multifragment ligations. In addition, accurate deletion of endogenous genes remains challenging in *V. dahliae* because of nonhomologous end joining (Qi et al. 2015). In this study, we presented an approach for gene deletion in *V. dahliae* that is suitable for high-throughput manipulation, in which several time-consuming steps can be performed in a high-throughput manner. To increase the cloning efficiency of the gene-replacement construct, pGFP was digested by two 5′ overhang cutters (*Xba*I and *Bam*HI) rather than a blunt-end cutter (Liu et al. 2012), and a larger amount of the *Hyg*^r cassette was prepared through PCR rather than from the enzyme digestion vector (Liu et al. 2012). To eliminate the most ectopic insertion transformants, eGFP-fused nuclear localization signal (eGFP NLS), rather than eGFP, was used as a negative marker, making fluorescence easier to observe.

There is little information available regarding the function of *VdASP F2* in plant-pathogenic fungi. To investigate the function of *VdASP F2*, we used a fast and easy gene-deletion system to delete *VdASP F2* in *V. dahliae*. The *VdASP F2* deletion mutant exhibited vigorous aerial mycelium growth and reduced the ability to produce microsclerotia at low temperatures and under limited nutrient conditions. However, the mutant could still form microsclerotia on the PDA medium. Microsclerotia are the primary long-term survival structures when *V. dahliae*

Fig. 4. Susceptibility of *VdASP F2* deletion mutants to abiotic stress. **A**, Growth of wild-type (WT) and mutant strains on potato dextrose agar (PDA) containing 0.5 M NaCl, 0.7 M KCl, 1.5 mM H₂O₂, or 1 M sorbitol or grown at pH 11. **B**, Growth comparisons among mutant and WT strains on Czapek-Dox medium without nitrogen or carbon source were conducted. $\Delta VdASP F2$ = *VdASP F2* deletion mutant, Compl = $\Delta VdASP F2$ mutant complemented with *VdASP F2* WT allele, and Ect = ectopic transformant.

encounters inhospitable environmental conditions. The results of the present study indicate that VdASP F2 cannot enter the plant cell and is considered a putative cell-wall-associated component (He et al. 2015), similar to the Asp F2-like protein Pra1, which was detected in the cell walls of *C. albicans* (Sentandreu et al. 1998).

We found that *V. dahliae* could form microsclerotia more quickly at 8 than at 26°C. *V. dahliae* will survive if it can form microsclerotia at 8°C before it is transferred to -20°C. It is difficult for the VdASP F2 deletion mutant to survive at -20°C because of the lack of microsclerotia. Liquid water turns into ice crystals below freezing temperatures and kills the cells. Thus, VdASP F2 is important for the quick formation of microsclerotia, helping *V. dahliae* survive exposure to poor environmental conditions. We mixed *V. dahliae* and soil and then subjected them to low temperatures, where it was difficult to maintain normal growth due to both limited soil nutrition and low temperature. In this case, the formation of microsclerotia is vital for *V. dahliae* survival. The VdASP F2 deletion mutant was impaired by its lack of microsclerotial protection. However, further research is required for a deeper understanding of the

mechanism by which VdASP F2 regulates microsclerotial formation in the cell.

MATERIALS AND METHODS

Strains and plants.

V. dahliae strain V991, *E. coli* strain DH5 α , and *Agrobacterium tumefaciens* strain AGL1 were used in this study, as was *G. hirsutum* L. Plants were grown in the greenhouse at 25°C under a daily cycle of 16 h of light and 8 h of darkness.

Construction of binary and marker vectors.

To construct a binary vector for pGFP, the 1-kb eGFP-NLS was amplified by PCR with PrimeSTAR HS DNA Polymerase (TaKaRa Dalian Biotechnology) and was ligated into the pSilent-1 vector between *Xho*I and *Kpn*I. The eGFP-NLS will highly express under the control of the *trpC* promoter (PtrpC). Using the pSilent-eGFP-NLS plasmid as a template, PtrpC-eGFP-NLS was amplified and was ligated into the pPK2 vector between *Xba*I and *Eco*RI. The resulting plasmid was named pGFP. All primers in this study are listed in Supplementary Table S1.

Fig. 5. Microsclerotial formation of VdASP F2 deletion mutant and pathogenicity analysis. **A**, Strains were cultured on solid microsclerotia-inducing medium (MIM) to induce microsclerotial formation. Mycelia and microsclerotia were scraped off the membrane for examination and counting under the microscope. The VdASP F2 deletion mutant exhibited denser mycelia with delayed and reduced microsclerotial development. Photos of the same Petri dishes were taken 15 days (left) and 60 days (right) postinoculation. **B**, Microsclerotial formation-related gene expression on MIM. Expression of mitogen-activated protein kinase (VMK1) and a hydrophobin gene (VDH1) were downregulated in the VdASP F2 deletion mutant. Δ VdASP F2 = VdASP F2 deletion mutant, Compl = Δ VdASP F2 mutant complemented with VdASP F2 WT allele, and Ect = ectopic transformant. Experiments were performed in triplicate. **C**, Colony, mycelial, and microsclerotial morphology. After 15 days of growth on water agar, the VdASP F2 deletion mutant exhibited vigorous aerial mycelia with reduced branching and did not form microsclerotia. Arrows indicate small microsclerotia. **D**, The histogram represents the number of microsclerotia from **C**. **E**, The microsclerotial formation was analyzed at both 26 and 8°C. Pictures were taken 10 days after inoculation onto potato dextrose agar. **F**, The histogram represents the number of microsclerotia for strains inoculated at 8°C. Experiments (**D** and **F**) were performed with nine replications, and the error bars represent standard deviations. **G**, Arrangement of all strains on the petri dish and microscope images.

To produce the *VdASP F2* gene-replacement vector, *Hyg^r* was amplified by PCR from pSilent-1. pGFP was digested with 5' overhang cutters (*Xba*I and *Bam*HI). The *Hyg^r* cassette, digested pGFP, and two homologous arms were purified by DNA Gel Extraction Kit (TaKaRa Dalian Biotechnology) and eluted using double-distilled H₂O rather than elution buffer, because EDTA can inhibit ExoIII cleavage. The *Hyg^r* cassette and digested pGFP can be prepared and stored for future use. Two homologous arms, each comprising 1.5 kb from one side of the *VdASP F2* open reading frame, were amplified using the *V. dahliae* genome database (www.broadinstitute.org). PrimeSTAR HS DNA Polymerase (TaKaRa Dalian Biotechnology) was used, attaining maximum fidelity and avoiding addition of 3' A overhangs, which inhibit ExoIII cleavage. The underlined

sequences in Supplementary Table S1 are reverse-complemented counterparts of the pGFP (P1 and P4) or *Hyg^r* cassette (P2 and P3). The pGFP vector (25 to 50 ng), *Hyg^r* cassette (50 to 100 ng), two homologous *VdASP F2* arms (25 to 50 ng each), and 1 µl of 10× ExoIII buffer were mixed in one reaction tube. The tube was prechilled on ice for 2 min, and then 1 µl of ExoIII (20 units) was added to the tube and gently mixed. After 90 min of incubation at 0°C, 1 µl of 0.5 M EDTA was added to stop the reaction. The enzyme was inactivated at 65°C for 5 min. After cooling on ice, the mixture was transformed into *E. coli* component cells and cultured at 37°C overnight. Positive transformants were identified by colony PCR.

For the complementation assay, a 3.4-kb PCR product containing a 1.5-kb upstream sequence, the full-length *VdASP F2*

Fig. 6. Reduced virulence of the *VdASP F2* mutant against *Gossypium hirsutum*. **A**, One-month-old *G. hirsutum* was planted in soil and kept at room temperature with conidia of wild-type (WT), $\Delta VdASP F2$ mutant, ectopic insertion mutant (Ect), and complementation (Compl) strains. **B**, *G. hirsutum* was planted in soil and kept at low temperature. Photographs were taken 20 days postinoculation. **C**, *Verticillium dahliae* DNA levels were estimated by quantitative polymerase chain reaction using total *G. hirsutum* DNA isolated from the aboveground stems of three plants per sample. The experiment was repeated three times, and the vertical bars indicate the standard errors. These results were automatically calculated by the CFX Manager Software (Bio-Rad).

coding region, and a 1-kb downstream sequence was amplified from *V. dahliae* V991 genomic DNA using primers compl F/R and cloned into the *EcoRV/XbaI* sites of the pG418 vector to generate pG418-VdASP F2, thereby conferring G418 resistance used for reintroduction of the wild-type *VdASP F2* gene into the gene-deletion mutant. All gene-deletion vectors and gene-complementation vectors were transformed into *A. tumefaciens* strain AGL1 for subsequent *V. dahliae* transformation. The pG418 vector was constructed in our lab based on the pPK2 backbone.

To characterize the VdASP F2 protein, VdASP F2 was tagged with eGFP using fusion PCR. VdASP F2:eGFP and eGFP were first cut with *Sall* and *KpnI* and inserted into pSilent-1 (digested with *XhoI* and *KpnI*); next, expression cassettes (PtpC-VdASP F2:eGFP-TtrpC and PtpC-eGFP-TtrpC) were amplified and cloned into the *EcoRV/XbaI* sites of the pG418 vector to generate the pG418-VdASP F2:eGFP and pG418-eGFP vectors. These were then used for further characterization assays (see below).

In order to express proteins, VdASP F2-eGFP without signal peptide and eGFP was amplified by PCR with PrimeSTAR HS DNA Polymerase (TaKaRa Dalian Biotechnology) from VdASP F2:eGFP. Δ SPVdASP F2-eGFP-6His and eGFP-6His were digested by the restriction enzyme *KpnI*, followed by ligation with the binary vector PS1300, kindly provided by Dr. Jing Maofeng (Nanjing Agricultural University), which was digested with *SmaI* and *KpnI*.

Fungal transformations.

ATMT of *V. dahliae* was performed as described previously (Maruthachalam et al. 2011). pGFP-VdASP F2 transformants were selected on PDA supplemented with cefotaxime at 500 μ g/ml and hygromycin B at 50 μ g/ml, whereas pG418-VdASP F2 transformants were selected on PDA supplemented with cefotaxime at 500 μ g/ml and G418 at 60 μ g/ml. To verify homologous recombination, the transformants were inoculated into six-well plates and then observed under fluorescence microscopy to exclude ectopic transformations. *V. dahliae* genomic DNA was extracted by the cetyltrimethylammonium bromide method, as described previously (Saghai-Marouf et al. 1984). The genomic DNA was used as a template for PCR amplification with primers P5 and P6 to confirm the targeted gene knockout. The deletion of *VdASP F2* and its transcripts were further confirmed by Southern hybridization and RT-PCR. Complementation of *VdASP F2* deletion mutants was also confirmed by Southern and RT-PCR.

Bioinformatics analysis.

VdASP F2 homologs from other fungi were identified by BLASTP. Sequences were aligned using MegAlign software (DNA STAR, Inc.). Signal peptide analysis was carried out using the signal peptide prediction program SignalP 4.1 Server (Petersen et al. 2011).

Protein precipitation and Western blotting.

To determine whether VdASP F2 was secreted into the liquid cultures, the VdASP F2:eGFP, eGFP, and wild-type strains were cultured in liquid Czapek-Dox medium at 25°C for 15 days at 180 rpm. The culture broth was centrifuged at 12,000 $\times$ g for 15 min and filtered through three layers of Whatman filter paper to remove hyphal fragments. The volume of supernatant was reduced using a dialysis bag (MWCo 10 kDa; Sangon Biotech) and PEG 20,000, and then 30% (wt/vol) sucrose was added into the concentrated supernatant. The protein was extracted using an equal volume of 1 M Tris-saturated phenol (pH 8.0). The mixture was homogenized and incubated on ice for 5 min before the upper phenol phase was collected and the proteins were

precipitated with five volumes of 0.1 M ammonium acetate in methanol overnight at -20°C. After centrifugation at 12,000 $\times$ g for 15 min, the protein pellets were washed once with methanol and 80% (vol/vol) acetone. The protein pellets were resuspended in protein loading buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (12.5%) before they were transferred onto a polyvinylidene difluoride membrane using a Western blotting apparatus (Bio-Rad). A monoclonal GFP antibody (Sino Biological Inc.) and a goat antimouse immunoglobulin G conjugated with HRP (OriGene) were used as primary and secondary antibodies, respectively. Blot signals were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

Fungal growth and susceptibility to stress conditions.

The *VdASP F2* deletion mutant, ectopic transformant, complemented mutant, and wild-type strains were grown on modified Czapek-Dox solid medium (2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄·7H₂O, and 1.5% agar). The Czapek-Dox solid medium was supplemented with 1.5% glucose, 1.5% sucrose, or 0.5% cellulose as the sole carbon source. Mycelial growth was measured by colony diameter, and the hyphae and microsclerotia were visually estimated and compared.

Because microsclerotia play an important role in protecting *V. dahliae* from various types of stress, we evaluated the effects of H₂O₂, pH, salt, sorbitol, and temperature stress on the vegetative growth of *VdASP F2* deletion mutants inoculated on modified PDA.

Microsclerotial production on microsclerotia-inducing or limited nutrient agar medium and RT-PCR.

To determine whether VdASP F2 is required for microsclerotial production on MIM (Hu et al. 2013), the ectopic transformant, *VdASP F2* knockout, complemented mutant, and wild-type strains were inoculated on MIM covered with a nitrocellulose membrane. After 15 or 60 days, hyphal and microsclerotial samples were taken for microscopic observation. To analyze whether the knockout of *VdASP F2* influenced the expression of other genes related to microsclerotial formation, total RNA was extracted from hyphae and spores were collected from the nitrocellulose membrane covering the MIM. RNA integrity was confirmed by agarose gel electrophoresis. RT-PCR was performed with PrimeScrip RT Reagent Kit (TaKaRa Dalian Biotechnology). Expression of mitogen-activated protein kinase (*VMK1*) and a hydrophobin gene (*VDH1*) were analyzed by RT-PCR. PCR cycling consisted of an initial step of denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. All primers used in this study are listed in Supplementary Table S1).

The ectopic transformant, *VdASP F2* deletion mutant, complemented mutant, and wild-type strains were inoculated on water agar to simulate limited nutrient conditions. Agar plugs were taken with a puncher for microscopic observation or counting. In order to view formation of microsclerotia under different temperatures, nitrocellulose membranes (80 mm in diameter) were placed on PDA medium. Microsclerotia were scraped from membranes and counted using a hemocytometer. This experiment was repeated three times.

Expression and purification of His-tagged proteins.

For in planta expression, *A. tumefaciens* strain GV3101 with respective constructs was grown in Luria-Bertani media supplemented with appropriate antibiotics. For agroinfiltration assays, *A. tumefaciens* was resuspended in infiltration buffer (10 mM MgCl₂ and 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.6; and 100 μ M acetosyringone). The

resuspended *A. tumefaciens* cells were diluted and mixed with P19 silencing suppressor in a 1:1 ratio at an optical density at 600 nm of 0.5 for each construct. Agroinfiltration experiments were performed on leaves of 6-week-old *Nicotiana benthamiana* plants.

Protein extraction was carried out according to the Lombardi et al. (2009) method, with minor modifications. *N. benthamiana* leaves were ground in liquid nitrogen in a precooled mortar and pestle. The samples were thawed in 3 ml of cold extraction buffer (50 mM sodium phosphate [pH 8.0], 500 mM NaCl, 0.1% Nonidet P-40, 0.1% β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) per gram of plant material. The samples were centrifuged at $12,000 \times g$ for 30 min at 4°C to pellet tissue debris. Imidazole was added to the supernatant for a final concentration of 10 mM. Then, 0.5 ml of Ni-NTA agarose (Qiagen), preliminarily washed with extraction buffer, was added to the supernatant and incubated at 4°C on a rocker for 1 h. Agarose was washed in 15 ml of wash buffer (extraction buffer with 20 mM imidazole). Bound His-tagged GFP was eluted with 250 mM imidazole.

In vitro cotton root uptake protein uptake.

The experiment was carried out according to the Gu et al. (2011) method, with modifications. Cotton roots were washed with water thoroughly to remove any debris. Small root tips (approximately 1 cm) were cut and placed into the protein solution (25 mM MES [pH 5.8] and protein at 1 mg/ml) and incubated for 12 h at 28°C. Half of the roots were washed twice in water for 2 h on an orbital shaker at 120 rpm. The roots were observed with an OLYMPUS DP70 microscope at excitations of 450 or 490 nm.

Infection assays.

Six-week-old *G. hirsutum* seedlings were used in infection assays to evaluate the effect of *VdASP F2* knockout on virulence using a root-dip inoculation method, as described previously (Santhanam and Thomma 2013). To prepare the inoculation, fungal cultures grown for 7 days in liquid potato dextrose broth medium were passed through several layers of cheesecloth to remove mycelia. Next, conidia (10 ml ; 1×10^7) were mixed with the soil and kept at room temperature for 2 months. To mimic overwintering, *V. dahliae* spores were mixed with soil and kept at 8°C for 2 weeks to induce microsclerotial formation. They were then transferred to -20°C for 2 months. Six-week-old *G. hirsutum* seedlings were planted in the soil with *V. dahliae* spores to assess pathogenicity. *G. hirsutum* was kept in an environmentally controlled growth room at $25 \pm 1^\circ\text{C}$ and 60 to 70% relative humidity under a daily cycle of 12 h of light and 12 h of darkness. Pictures were taken 20 days after *V. dahliae* inoculation.

V. dahliae biomass quantification in cotton.

Aboveground stems of cotton were harvested and flash frozen in liquid nitrogen. The samples were ground to powder, approximately 1 g of which was used for DNA isolation. Quantitative RT-PCR was conducted by the CFX Connect Real-Time PCR Detection System (Bio-Rad) with SYBR Green Master Mix (TaKaRa Dalian Biotechnology). For *V. dahliae* biomass measurement, the primer pair Vd-F and Vd-R was designed for the internal transcribed spacer (ITS)1 and ITS2 regions of the 5.8S ribosomal RNA gene of *V. dahliae*. The osmotic gene from *G. hirsutum*, amplified using the primer pair Gh-F and Gh-R, was used as an internal standard to normalize for differences in DNA template amounts. The average fungal biomass was determined using at least three *V. dahliae*-inoculated cotton samples. The experiments were repeated three times. PCR cycling started with an initial step of denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s.

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