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Septins regulate virulence in *Verticillium dahliae* and differentially contribute to microsclerotial formation and stress responses

Haifeng Wang[1†], Chen Tang[1†], Chenglin Deng[1], Wenwen Li[1], Steven J. Klosterman[2] and Yonglin Wang[1*]

Abstract

Septin proteins play a role in the formation of hyphal septa as well as in the division of nuclei, cytoskeletal organization, and cell morphogenesis in filamentous fungi. Herein, we investigated the functions of four septin-coding genes (*VdSep3, VdSep4, VdSep5,* and *VdSep6*) in the plant pathogenic fungus *Verticillium dahliae*. Microsclerotial formation was positively regulated by VdSep4 and VdSep6, whereas VdSep3 and VdSep5 had no effect on microsclerotial development but their deletion slightly reduced melanin production. Deletion of *VdSep4* or *VdSep5*, but not *VdSep3*, resulted in hypersensitivity to high-temperature stress. Deletion of *VdSep4* led to increased benomyl sensitivity whereas deletion of *VdSep3* showed increased benomyl resistance. The previously reported roles of VdSep3 and VdSep5 in virulence were confirmed. Disruption of each of the four septin-coding genes led to reduction in penetration peg formation and hyphal expansion into plant cells. Loss of *VdSep3* or *VdSep4* increased sensitivity to reactive oxygen species (ROS) and reactive nitrogen species (RNS) stress, whereas *VdSep6* played a role in RNS stress response but not in ROS stress response. In addition, all the septin gene deletion mutants showed an abnormality in chitin distribution but varied in their responses to several stresses examined. Taken together, our results indicate that members of the septin family in *V. dahliae* play different roles in regulating microsclerotial development, melanin synthesis, and stress responses, while they are all required for full virulence of the fungus.

Keywords: Septins, *Verticillium dahliae*, Microsclerotial formation, Melanin, Stress response, Fungal virulence

Background

The septins were first discovered in temperature-sensitive cell division cycle-deficient mutants of *Saccharomyces cerevisiae* and were named for their roles in cytokinesis and septum formation (Hartwell 1971). They represent an evolutionarily conserved family of GTP-binding proteins in diverse eukaryotic organisms, except for plants. In filamentous fungi, the septins play important roles in morphogenesis and virulence (Lindsey et al. 2010; Dagdas et al. 2012; Chen et al. 2016; Feng et al. 2017; Zhang et al. 2017; Zhou et al. 2017). Four septin genes (*FgCdc3*, *FgCdc10*, *FgCdc11*, and *FgCdc12*) in *Fusarium graminearum* are involved in fungal growth, septum formation, nuclear division, conidiation, and virulence (Chen et al. 2016). In *Magnaporthe oryzae*, assembly of septins at the penetration peg affects the initiation of infection and is required for pathogenicity (Dagdas et al. 2012). At the infection point, the septins are assembled into a heteromeric ring, and the organization of the septin ring requires a Nox2-NoxR complex (Gupta et al. 2015).

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In addition, the septin family performs convergent and distinctive functions. *Candida albicans* contains seven septins, CDC10 and CDC11, which function similarly in cytokinesis, chitin localization, and virulence but differently in bud formation (Warenda and Konopka 2002). *Cochliobolus heterostrophus* requires all four septins for reproductive propagule formation, but only CDC10 plays a role in virulence (Zhang et al. 2020). Five septin genes (*aspA*, *aspB*, *aspC*, *aspD*, and *aspE*) in *Aspergillus nidulans* are involved in conidiation, however, gene deletion mutants Δ*aspA*, Δ*aspB*, and Δ*aspC* but not Δ*aspD* and Δ*aspE* showed reduced septation and increased branching (Pan et al. 2007; Lindsey et al. 2010; Hernández-Rodríguez et al. 2012, 2014). Moreover, *aspE* is involved in hypothermal and osmotic stress responses (Hernández-Rodríguez et al. 2014).

Verticillium wilt is caused by *Verticillium dahliae*, a fungal plant pathogen that is extremely difficult to combat due to the long-term survival of its inoculum (microsclerotia) in soil and broad host range (over 200 plant species) (Bhat and Subbarao 1999; Bhat et al. 2003; Klosterman et al. 2009). *V. dahliae* infects through the roots and colonizes the plant vascular system to produce a large number of conidia, which are spread throughout the whole plant via the transpiration stream (Fitzell et al. 1980; Huisman 1982). Recently, it was demonstrated that the *V. dahliae* VdSep5 is required for the formation of a septin ring, which separates the hyphopodium from the invading hyphae and forms a specialized fungus-host interface regulated by NADPH oxidase B (*VdNoxB*); VdSep3 plays a role in vegetative growth and hyphopodium formation (Zhou et al. 2017). Furthermore, Zhou et al. (2017) discovered that deletion of *VdSep5* caused a decrease in delivery of secreted proteins to the penetrating hyphae near this interface.

Microsclerotia are the main dormant survival structures in *V. dahliae* and serve as a new source of the primary inoculum in the disease cycle (Klosterman et al. 2009). The melanized microsclerotia can survive for up to 14 years, exhibiting resistance to various stresses (Wilhelm 1955; Santhanam and Thomma 2013). Given these characteristics, microsclerotia of *V. dahliae* are extremely important to disease controls and have attracted the attention of many researchers worldwide (Neumann and Dobinson 2003; Duressa et al. 2013; Xiong et al. 2014; Wang et al. 2018). Melanin, whose biosynthesis is always tightly coupled with microsclerotial development in *V. dahliae*, can protect against adverse environments such as ultraviolet radiation and heat as well as increase cell rigidity and virulence (Bell and Wheeler 1986; Song 2018; Wang et al. 2018). Melanin is primarily deposited within the cell walls and between the cells at the final stage of microsclerotial maturation. The initial stages of microsclerotial development have also been studied via light and electron microscopy, revealing hyphal swellings and formation of septa (Griffiths 1970), suggesting that septum formation may play an important role during microsclerotial formation. Additionally, transcriptome analyses of *V. dahliae* in our previous research reveal that septin family members are differentially expressed at different stages of microsclerotial development (Xiong et al. 2014).

Although the functions of VdSep3 and VdSep5 in virulence have previously been studied in *V. dahliae*, the roles of septins in the morphogenesis of *V. dahliae*, such as the formation of microsclerotia, are unclear, and the mechanism by which *V. dahliae* responds to different stresses also needs to be investigated further. In this study, we identified and studied the basic functions of four septins in *V. dahliae*. The results show that these four septins in *V. dahliae* collectively play important but distinct roles in microsclerotial development, melanin biosynthesis, stress responses, and resistance against benomyl, while each contributes to virulence and chitin distribution in cell walls.

Results

Identification of septins in *V. dahliae*

VdSep3 (VDAG_00736) and *VdSep5* (VDAG_04382), the homologs of *M. oryzae sep3* and *sep5*, respectively, were identified in *V. dahliae* (Zhou et al. 2017). Here, we employed septins from *M. oryzae* to identify extra homologs in *V. dahliae*. Two septin-coding genes, *VdSep4* (VDAG_07169) and *VdSep6* (VDAG_01474), were identified in the genome of *V. dahliae* by BLASTP searches with the amino acid sequences of MGG_06726 and MGG_07466 as queries (Dagdas et al. 2012) (Additional file 1: Figure S1a). All the predicted septin proteins in *V. dahliae* contain a typical septin-type guanine nucleotide-binding domain (Additional file 1: Figure S1b). To investigate the functions of these septins in *V. dahliae*, single-gene deletion mutants designated as Δ*VdSep3*, Δ*VdSep4*, Δ*VdSep5*, and Δ*VdSep6* were generated with XS11 as the wild-type parental strain (Additional file 1: Figure S2a, b). Southern blot analyses were performed to confirm the mutation of each target gene.

V. dahliae septins are involved in regulating the formation of microsclerotia

Based on our RNA-seq data of *V. dahliae*, four septin-coding genes were differentially expressed during microsclerotial development. To investigate the roles of septins in this biological process, we compared microsclerotial production between the wild-type strain XS11 and mutants (Δ*VdSep3*, Δ*VdSep4*, Δ*VdSep5*, and Δ*VdSep6*) on complete medium (CM). The results showed that all

the mutants except Δ*VdSep4* were melanized at the colony center after incubation for 5 days on CM. Δ*VdSep3* and Δ*VdSep5* produced larger melanized areas, indicating that they formed more microsclerotia (Fig. 1a). Microsclerotial development of all these strains was also microscopically observed. Compared with XS11, melanin production in all the mutants was repressed, and Δ*VdSep4* could not form microsclerotia after incubation for 4 days (Fig. 1b). Mature microsclerotia could only be observed in Δ*VdSep4* from the 12th-day onward, but were markedly less in number compared with those of other strains.

Growth on cellulose membrane was examined to further determine the roles of septins in microsclerotial production and melanin biosynthesis. The Δ*VdSep4* and Δ*VdSep6* mutants did not form microsclerotia and exhibited no melanin deposition until 12 days of incubation, whereas the complemented strains Δ*VdSep4*-C and Δ*VdSep6*-C showed no difference in microsclerotial production when compared with XS11 (Fig. 1c). Statistical analysis revealed that melanin deposition produced by Δ*VdSep4* or Δ*VdSep6* was significantly lower than that produced by XS11 at 35 days of incubation (Fig. 1d).

We performed reverse transcription-quantitative polymerase chain (RT-qPCR) analysis to evaluate the expression of five genes with reported roles in melanin biosynthesis (*VDAG_00183*, *VDAG_00190*, *VDAG_03393*, *VDAG_03665*, and *VDAG_03674*) in *V. dahliae* (Wang et al. 2018; Xiong et al. 2014). Among them, four genes were down-regulated by more than 99% in the Δ*VdSep6* strain, but deletion of *VdSep6* had no impact on *VDAG_03674* expression compared with that in XS11 (Fig. 1e). In contrast, significant up-regulation of these five melanin biosynthesis-related genes was observed in *VdSep3*, *VdSep4*, and *VdSep5* deletion mutants compared with XS11. The results indicated that VdSep4 and VdSep6 are indispensable for the regulation of microsclerotial development, and septins play different roles in melanin biosynthesis in *V. dahliae*.

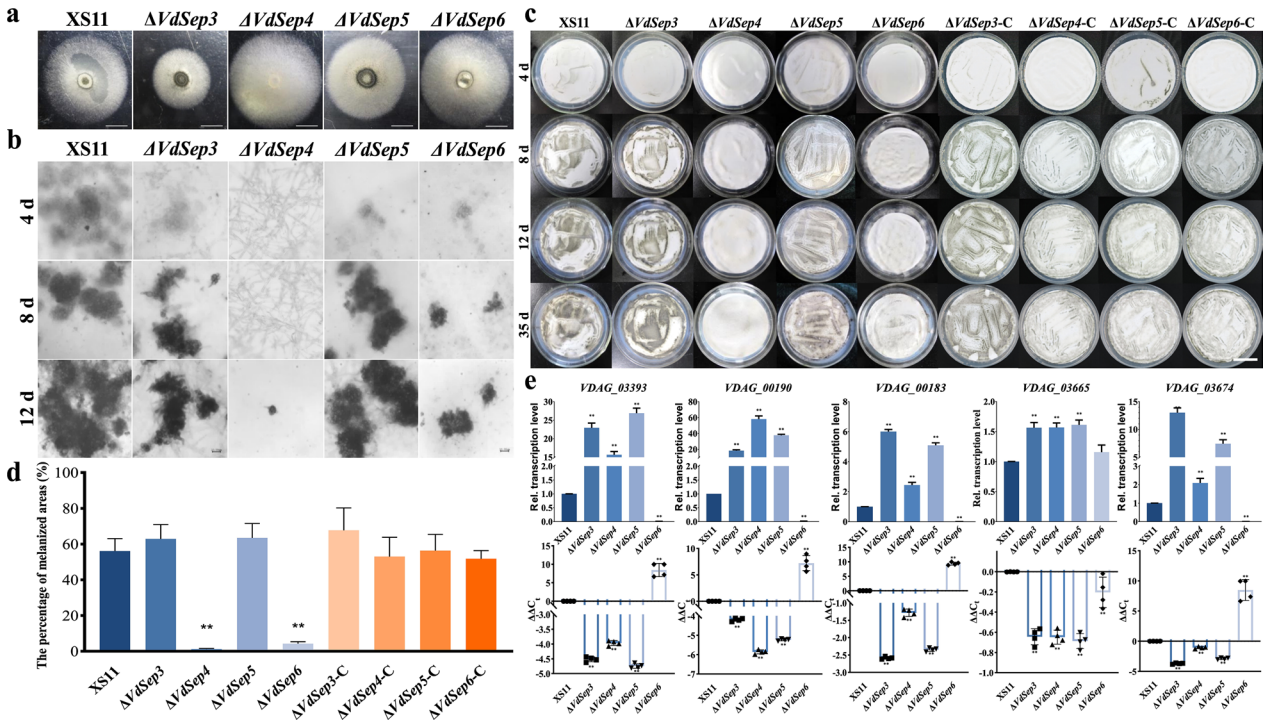

Fig. 1 Septins regulate the formation of melanized microsclerotia. **a** Colony phenotype of the wild-type strain XS11 and mutants (Δ*VdSep3*, Δ*VdSep4*, Δ*VdSep5*, and Δ*VdSep6*) on complete medium (CM). Conidial suspension (10^6 conidia/mL) of each strain was dropped onto a glass slide covered with CM and incubated at 25 °C for 5 days. Bar = 0.28 cm. **b** Microscopic observation of microsclerotial development at 4, 8, and 12 days of incubation. Bar = 20 μm. **c** Colony phenotype of the indicated strains on nitrocellulose membrane overlayed on basal medium (BM). Conidial suspension (10^6 conidia/mL) of each strain was dropped onto the membrane and incubated at 25 °C. Bar = 3 cm. **d** Melanized areas of the colonies were determined using ImageJ at 35 days of incubation. Error bars represent standard deviations based on three independent replicates. Asterisks indicate significant differences (**, $P < 0.01$). **e** Evaluation of relative expression levels of the genes involved in melanin synthesis in *V. dahliae* via RT-qPCR. The average gene expression values were normalized against the *V. dahliae β-tubulin* gene. Error bars represent standard deviation based on four independent technical replicates, and the determined ΔΔCt values were shown in the bottom panel (**, $P < 0.01$)

The septins of *V. dahliae* play different roles in response to high-temperature stress

Previous research has shown that melanin can protect against high-temperature stress (Bell and Wheeler 1986; Song 2018; Wang et al. 2018), and the *V. dahliae* septins were down-regulated under this type of stress (Fang et al. 2019). To further study the roles of the four septins in the response to high-temperature stress, the mycelial growth rates were measured. The growth of the Δ*VdSep4* strain nearly ceased at 30 °C, while that of the Δ*VdSep5* strain was also inhibited (Fig. 2a). Statistical analyses indicated that the Δ*VdSep3* strain was more resistant to thermal stress than XS11, and the Δ*VdSep6* strain exhibited similar inhibition rate to that of XS11 (Fig. 2b). This result suggested that, except for VdSep6, all the septins participate in the high-temperature stress response.

Septins regulate hyphal septum formation and cell wall integrity in *V. dahliae*

Septum formation occurs during microsclerotial development (Griffiths 1970). Considering the anticipated role of septins in septum formation in *V. dahliae*, CFW-stained hyphae were observed in septin gene deletion mutants. Compared with XS11 and complemented strains, the mutants Δ*VdSep4*, Δ*VdSep5*, and Δ*VdSep6* exhibited a longer inter-septal distance, whereas Δ*VdSep3* showed a shorter inter-septal cell distance (Additional file 1: Figure S3a). Chitin is a hallmark component of fungal cell wall and also required for septum formation (Langner and Göhre 2016). We therefore examined the expression of five chitin synthesis-related genes in the mutants via RT-qPCR. All five septin genes examined in the Δ*VdSep3* strain were up-regulated relative to those in XS11 (Additional file 1: Figure S3b). In Δ*VdSep6*, the expression level of *VDAG_08591* did not significantly differ from that in XS11, but the expressions of the remaining four genes were significantly down-regulated. The expression levels of *VDAG_03141* and *VDAG_08591* were independent of *VdSep5*, while those of the other three genes were affected by its deletion. In the Δ*VdSep4* and Δ*vdSep5* strains, *VDAG_08428* was significantly down-regulated but *VDAG_02341* was significantly up-regulated (Additional file 1: Figure S3b). These results indicated that the septin family genes regulate chitin synthesis-related genes in *V. dahliae* and this likely influences normal septum formation.

On PDA and CM media, both the Δ*VdSep3* and Δ*VdSep6* exhibited a slower hyphal growth than XS11 (Figs. 2a, 3a, b), indicating that they are involved in hyphal growth in *V. dahliae*. Since chitin is the main structural component of fungal cell walls, and septins play roles in the regulation of chitin synthesis, we investigated the roles of these two septins in cell wall integrity by testing the sensitivity of gene deletion mutants to

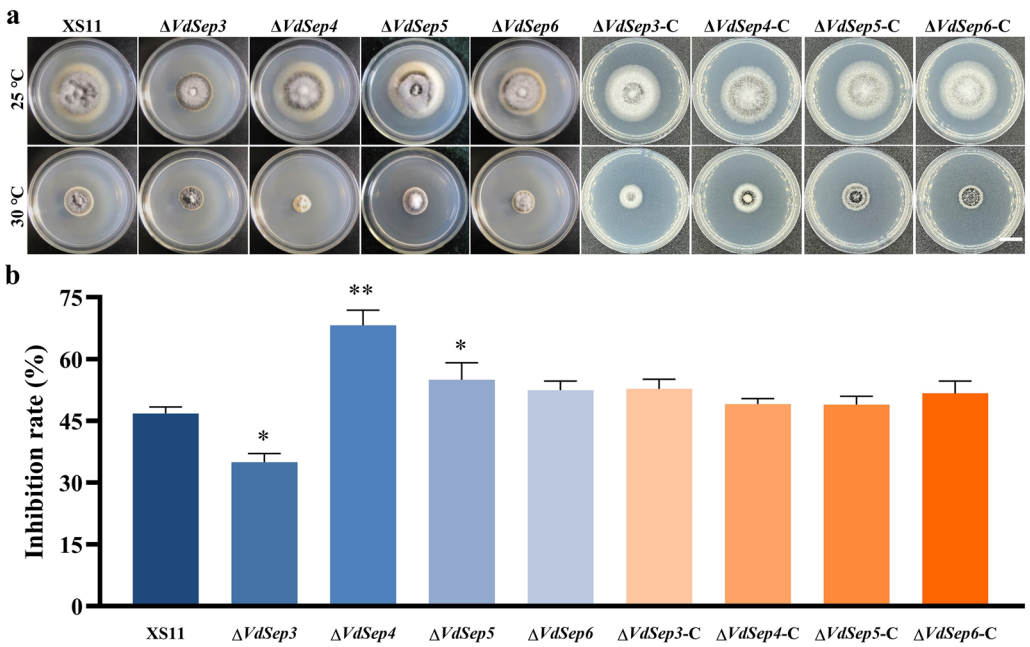

Fig. 2 Disruption of septins changes the sensitivity to high-temperature stress. **a** Colonies of XS11 and the mutants (Δ*VdSep3*, Δ*VdSep4*, Δ*VdSep5*, and Δ*VdSep6*) were grown on potato dextrose agar (PDA) for 10 days at 25 °C and 30 °C. Bar = 1.32 cm. **b** Column diagram showing growth inhibition rate at 30 °C. The inhibition rate (%) = [(Growth diameter (25 ℃) − Growth diameter (30 ℃)) / Growth diameter (25 ℃)] × 100. Error bars represent standard deviation based on three independent replicates (**, $P < 0.01$; *, $P < 0.05$)

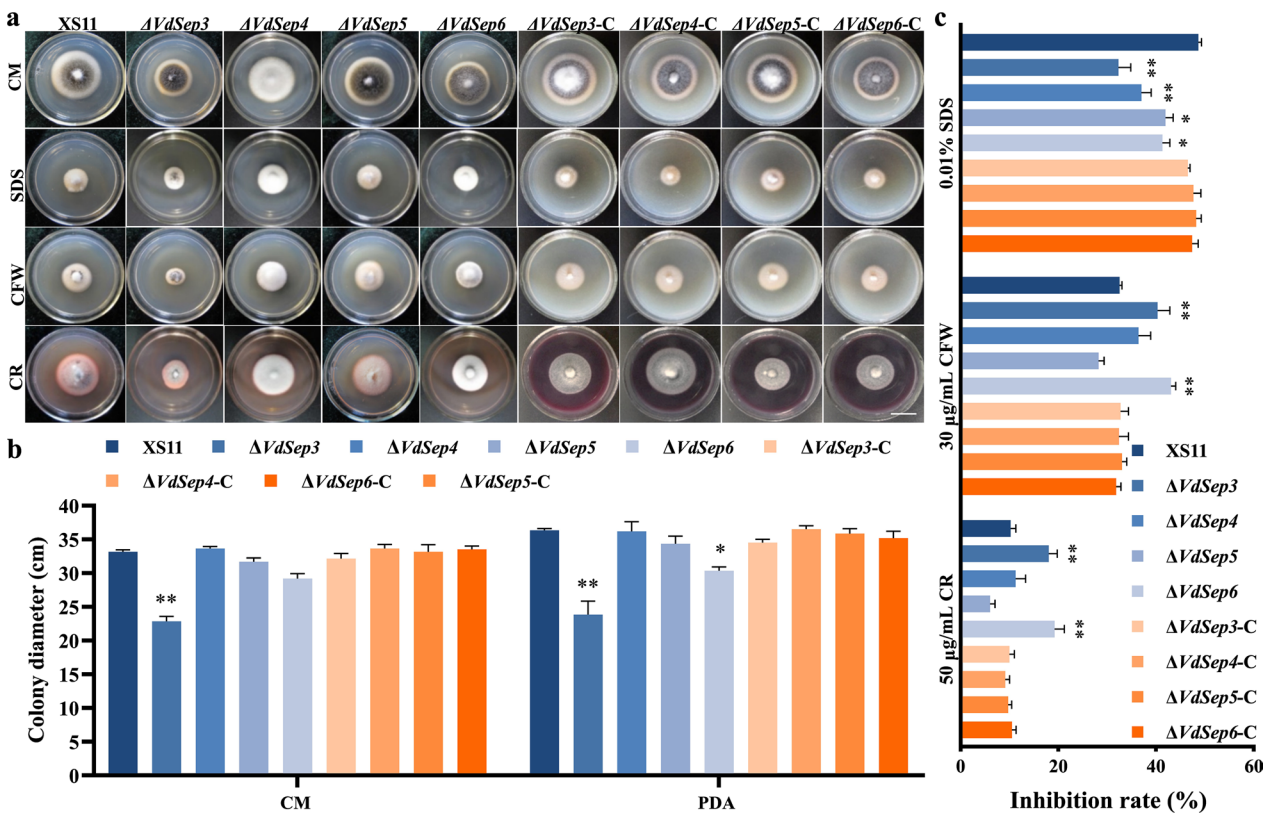

Fig. 3 Septins are involved in mycelial growth and cell wall integrity. **a** Colony of XS11 and mutants (Δ*VdSep3*, Δ*VdSep4*, Δ*VdSep5*, and Δ*VdSep6*) were grown on complete medium (CM) or CM containing 30 μg/mL calcofluor white (CFW), 25 μg/mL Congo red (CR), or 0.01% SDS for 10 days at 25 °C. Bar = 0.617 cm. **b** Column diagram showing colony diameters of the indicated strains grown on CM or potato dextrose agar (PDA) for 10 days at 25 °C. Error bars represent standard deviation based on three independent replicates (**, $P < 0.01$; *, $P < 0.05$). **c** Growth inhibition of the indicated strains by CFW (30 μg/mL), CR (25 μg/mL), or 0.01% SDS. The strains were cultured on CM with stress agents for 10 days at 25 °C. Error bars represent standard deviation based on three independent replicates (**, $P < 0.01$; *, $P < 0.05$)

the cell wall synthesis inhibitors calcofluor white (CFW) and Congo red (CR), and a well-known cell membrane disruptor sodium dodecyl sulfate (SDS) (Kopecká and Gabriel 1992; Ram and Klis 2006; Mazáň et al. 2008). Compared with XS11 and the complemented strains, the Δ*VdSep3* and Δ*VdSep6* strains exhibited higher growth inhibition rates when treated with CR and CFW. However, SDS, at the concentration used, did not inhibit the growth of the mutants as strongly as that of XS11 (Fig. 3a, c).

Septins are required for full virulence of *V. dahliae*

Considering the contribution of VdSep5 to virulence of *V. dahliae*, we sought to determine whether other septins are required for fungal virulence. Tobacco plants inoculated with XS11 or complemented strains exhibited obvious disease symptoms, while those inoculated with the mutants exhibited decreased symptoms (Fig. 4a and Additional file 1: Figure S4a). Based on the disease indexes and survival rates of tobacco plants inoculated with the different strains, the Δ*VdSep3* and the Δ*VdSep5* strains exhibited attenuated virulence, consistent with previous results (Zhou et al. 2017). The virulence of the Δ*VdSep4* and Δ*VdSep6* strains were obviously decreased when compared to XS11 and their complemented strains (Fig. 4b, c and Additional file 1: Figure S4b). At 35 days post-inoculation (dpi), the presence of the pathogen *in planta* was examined by re-isolation. The results showed that all the inoculated fungal strains could be obtained from the root-stem junction (Fig. 4d). In addition, the biomass of *V. dahliae* in the tobacco stems was evaluated by quantitative real-time PCR (qPCR) at 5 dpi (Fig. 4e, f). The data revealed that these septins are required for the full virulence of *V. dahliae*.

To explore the clues causing virulence defect in the septin mutants, we carried out infection assays using onion epidermis. Except for Δ*VdSep3*, all of the tested strains could invade onion epidermal cells at 4 dpi (Additional file 1: Figure S5). We further investigated the penetration ability of each strain on cellophane. Δ*VdSep3* failed

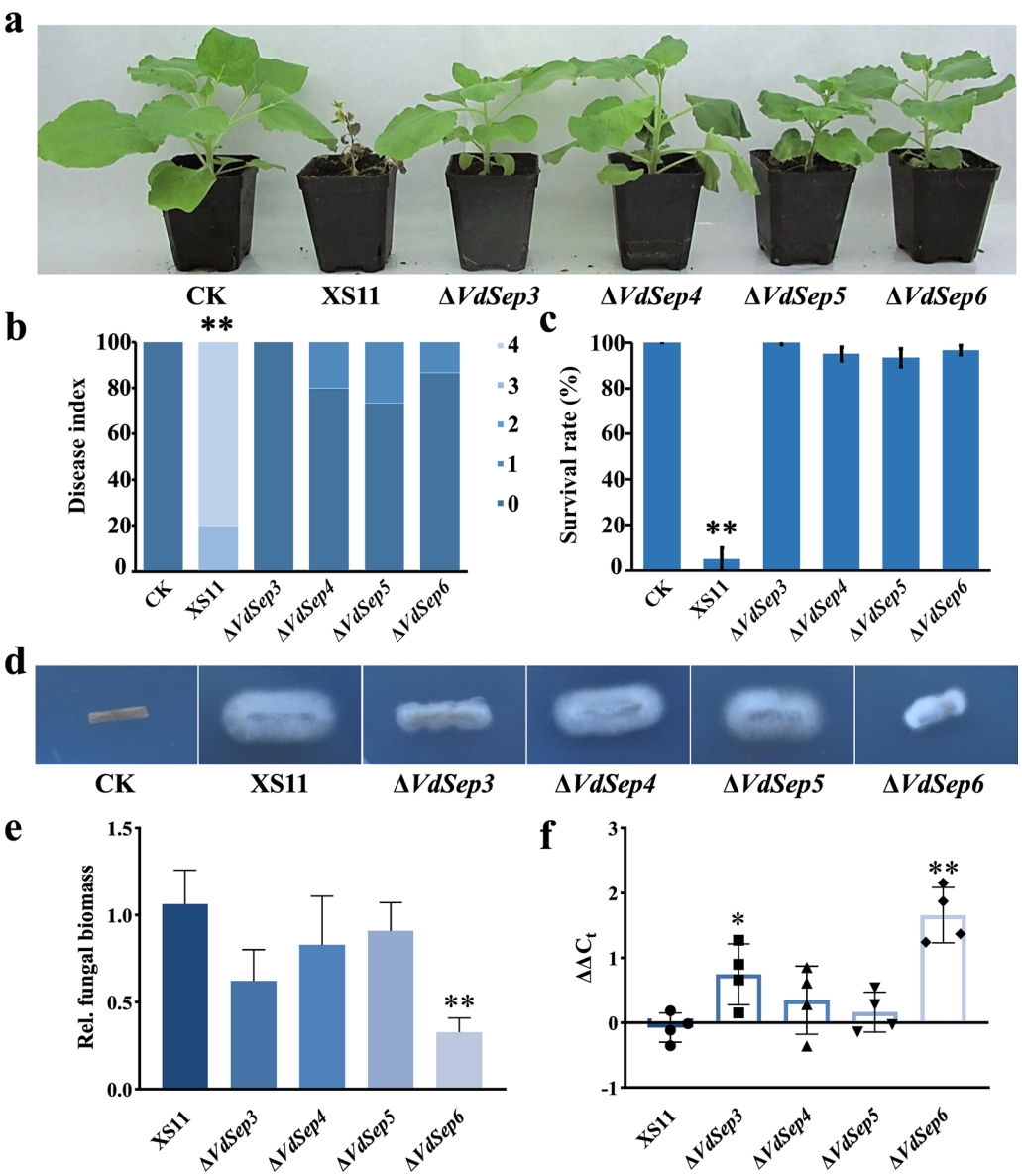

Fig. 4 The septin genes are required for the full virulence. **a** Symptom phenotype on *Nicotiana benthamiana*. One-month-old tobacco seedlings were immersed in conidial suspension (10^6 conidia/mL) of each strain for 10 min. Sterile distilled water was used as control (CK). After inoculation, all seedlings were replanted in soil and incubated in a greenhouse for 35 days at 25 °C. **b** Column diagram showing disease index at 35 days post-inoculation (dpi). The disease was scored on a scale of 0–4. 0, no wilting; 1, yellowing or wilting of < 2 leaves; 2, yellowing or wilting of one-fourth leaves; 3, yellowing or wilting of two-thirds leaves; and 4, more than 85% of the leaves wilted or the whole plant died. Bars represent the percentages of plants under each scale. The experiments were repeated three times with similar results (**, $P < 0.01$). **c** Column diagram showing survival rates of tobacco plants. The data were determined at 35 dpi. Error bars represent standard deviation based on three independent experiment replicates (**, $P < 0.01$). **d** Detection of the pathogen at the stem-root junction of inoculated plants at 35 dpi. **e** Fungal biomass detected by qRCR at 5 dpi. Error bars represent standard deviation based on four independent technical replicates (**, $P < 0.01$). **f** The ΔΔCt values of qPCR. Error bars represent standard deviation based on four independent technical replicates (**, $P < 0.01$; *, $P < 0.05$)

to penetrate cellophane after incubation for 3 days, while only a few hyphae penetrated through the membrane into the culture medium after incubation for 6 days. The other three mutants exhibited similar penetration ability to XS11 (Fig. 5a). Further microscopic examination revealed that all strains formed hyphopodia with no significant difference (Fig. 5b), but the numbers of penetration pegs formed by the XS11 or complemented strains were significantly higher than those formed by the mutants (Fig. 5c).

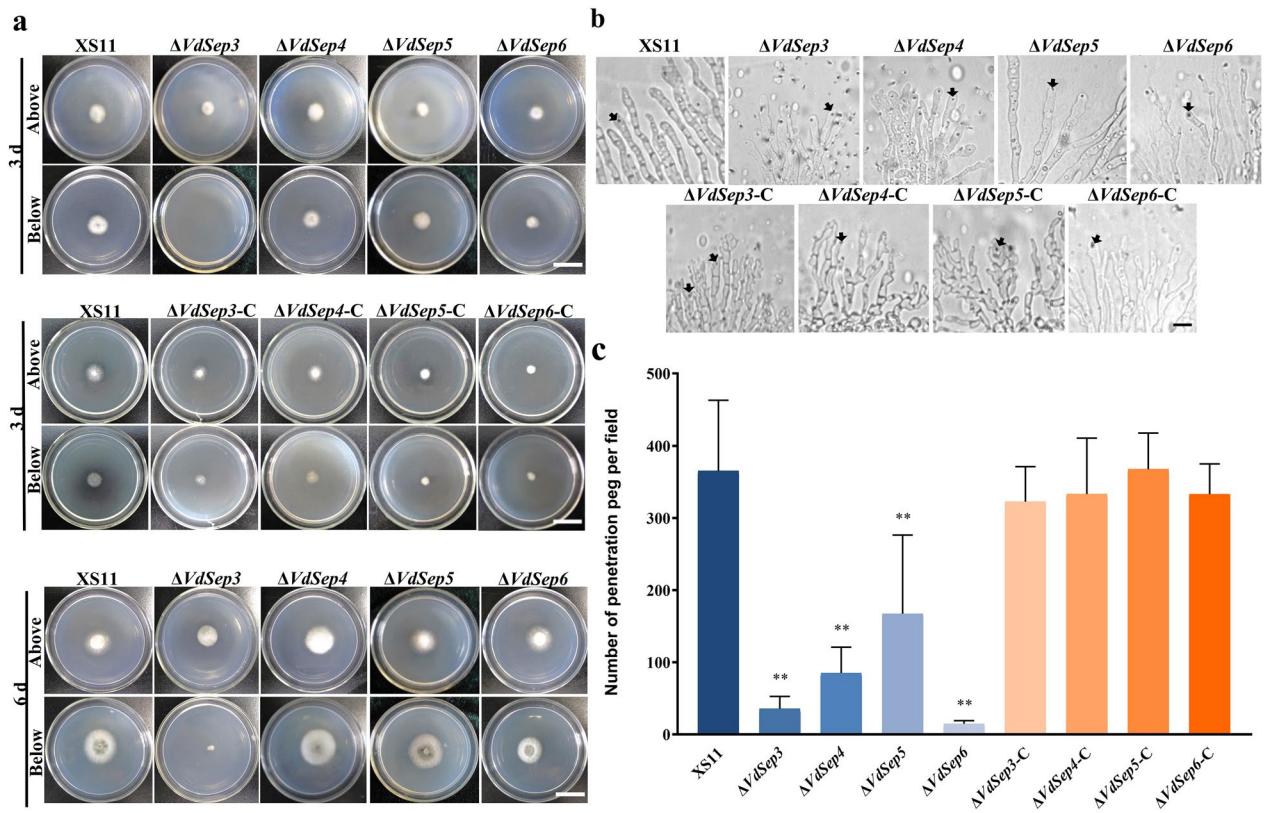

Fig. 5 Penetration peg formation on cellophane. **a** Colonies of XS11 and mutants (Δ*VdSep3*, Δ*VdSep4*, Δ*VdSep5*, and Δ*VdSep6*) grown on minimal medium (MM) overlaid with (upper panel) or without a cellophane membrane (lower panel). The strains were grown on MM plates overlaid with a cellophane membrane at 25 °C for 3 days or 6 days, and incubated for another 3 days after the membrane was removed. Bar = 2 cm. **b** Hyphopodia and penetration pegs formed on a cellophane membrane at 6 dpi. Bar = 10 μm. **c** Column diagram showing the number of penetration pegs formed by each strain on cellophane. The error bars represent standard deviations based on three independent replicates (**, $P < 0.01$)

Septin gene deletion mutants exhibit varied responsiveness to ROS/RNS

Hydrogen peroxide (H_2O_2) and nitric oxide (NO), representative of two families of related compounds designated as reactive oxygen and nitrogen species (ROS and RNS, respectively), are important signaling molecules and antimicrobial substances in the prevention of pathogen invasion and spread during plant-pathogen interactions (Hong et al. 2008). We analyzed the effects of deletion of septin genes on responses of *V. dahliae* to H_2O_2 and nitrosative stresses. The expression of the septin genes except for *VdSep3* was up-regulated in XS11 after being treated with H_2O_2, suggesting that septins in *V. dahliae* may play roles in response to H_2O_2 stress (Fig. 6a). Sensitivity assays to H_2O_2 revealed that the Δ*VdSep3* and Δ*VdSep4* strains exhibited increased sensitivity to different concentrations of H_2O_2, but the Δ*VdSep5* strain was more resistant to high concentrations of H_2O_2 compared with XS11 and the complemented strains (Fig. 6b, c).

The roles of each of the septins in nitrosative stress responses were also examined. On medium containing 10 mM sodium nitroprusside dehydrate (SNP), a NO donor, the growth of all strains was inhibited to varying degrees: the Δ*VdSep4* and Δ*VdSep6* strains could hardly grow (Fig. 6d); the inhibition rate of the Δ*VdSep3* strain was higher than that of XS11 but lower than those observed for the Δ*VdSep4* and Δ*VdSep6* strains. The Δ*VdSep5* strain displayed slightly decreased sensitivity to NO compared with XS11 and the complemented strains (Fig. 6e). Combining these results, we concluded that VdSep3 and VdSep4 play a positive role in the resistance to both H_2O_2 and NO stress, whereas VdSep5 plays a negative role in response to H_2O_2 stress, and VdSep6 only responds to NO stress.

V. dahliae septins have different resistance to benomyl

Benomyl is a broad-spectrum fungicide that interferes with fungal cell division by disrupting the microtubules (Hauptmann et al. 1985). In recent years, relevant studies have shown that septins are important for the organization and function of microtubules (Spiliotis and Kesisova 2021). To examine the role of

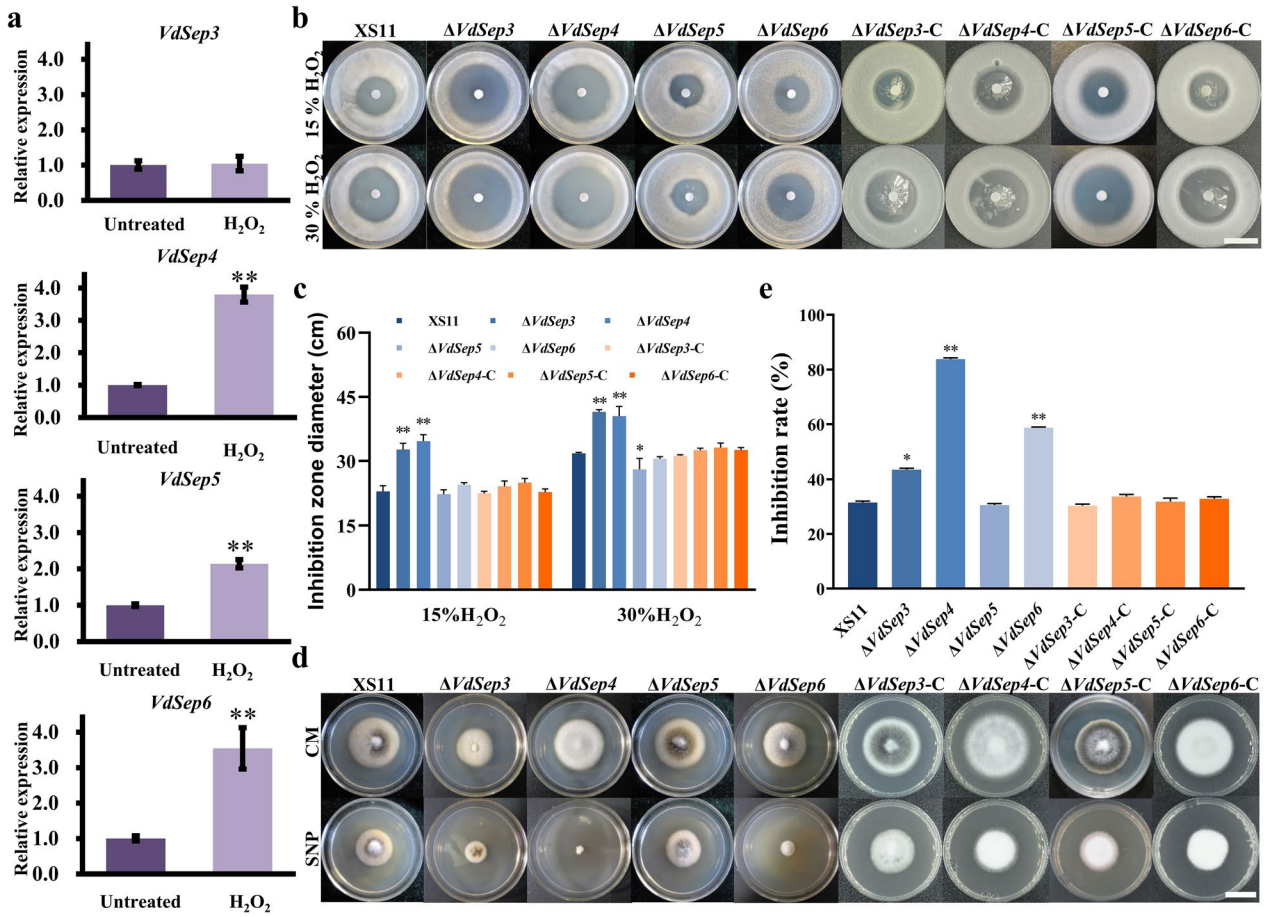

Fig. 6 The septin gene deletion mutants display distinct sensitivities to oxidative and nitrosative stress. **a** Reverse transcription-quantitative PCR analyses of the expression levels of the four septin genes *VdSep3*, *VdSep4*, *VdSep5,* and *VdSep6* in XS11 in response to H_2O_2 stress. Gene expression values were normalized against an endogenous control (*β-tubulin* gene). Error bars represent standard deviation based on three independent replicates (**, $P < 0.01$). **b** Colony growth assay under stress by 15% H_2O_2 (upper panel) or 30% H_2O_2 (lower panel). Conidial suspension of each indicated strain was mixed with melted potato dextrose agar (PDA) medium to a final concentration of 10^7 conidia/mL and poured into a petri dish to prepare plates. A sterile filter paper disk (5 mm in diameter) was transferred to the center of the plate and 5 μL H_2O_2 (15% or 30%) was then added to each paper disc. The diameters of the inhibition zone were measured after incubation at 25 °C for 3 days. Bar = 2 cm. **c** Statistic analysis of growth inhibition in **b.** Error bars represent standard deviation based on three independent replicates (**, $P < 0.01$; *, $P < 0.05$). **d** Colony growth assay in the presence of sodium nitroprusside dehydrate (SNP, 10 mM). All the strains were grown on complete medium (CM) or CM containing 10 mM SNP for 10 days. Bar = 2 cm. **e** Statistical analysis of growth inhibition in **d**. Error bars represent standard deviation based on three independent replicates (**, $P < 0.01$; *, $P < 0.05$)

septins in the regulation of microtubules in *V. dahliae*, we incubated all the strains on PDA medium containing 0.6 μmol/L benomyl (Fig. 7a). The growth of the Δ*VdSep4* strain was significantly inhibited by benomyl compared with that of XS11 and the complemented strains, but the Δ*VdSep3* strain was not affected. The Δ*VdSep5* and Δ*VdSep6* strains showed similar inhibition to that of XS11 and the complemented strains (Fig. 7b). These results showed that the septins in *V. dahliae* may have different functions in response to benomyl stress.

Discussion

Septins have important roles in fungal morphogenesis and virulence (Warenda et al. 2003; Alvarez-Tabarés and Pérez-Martín 2010; Dagdas et al. 2012; Chen et al. 2016; Zhang et al. 2017). Among the septins in *V. dahliae,* a previous study of VdSep5 mainly concentrated on its roles in virulence and delivery of secretory proteins to the penetration interface (Zhou et al. 2017). In this study, we demonstrated that all the four septins in *V. dahliae* participate in virulence, impact hyphal septum formation, and regulate fungal response to SDS stress.

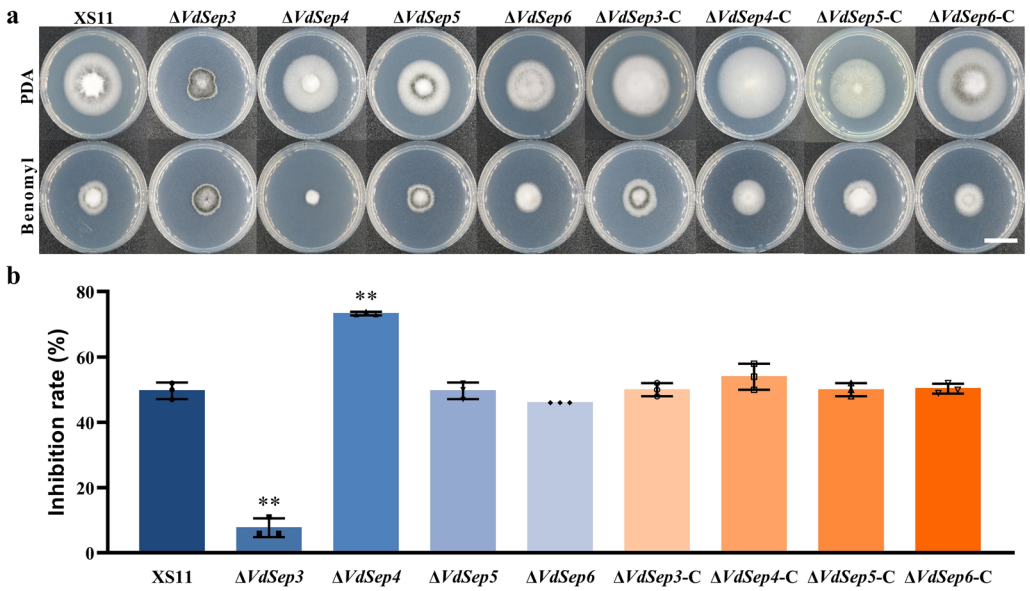

Fig. 7 Septins play different roles in response to benomyl. **a** Colony growth assay under stress by 0.6 μmol/L benomyl at 25 °C for 7 days. Bar = 2 cm. **b** Column diagram showing growth inhibition in **a**. Error bars represent standard deviation based on three independent replicate experiments (**, $P < 0.01$)

Specifically, VdSep3 is involved in hyphal growth as well as stress responses caused by ROS, RNS, CR, CFW, and high temperature; VdSep4 plays roles in microsclerotial formation, stress responses to ROS, RNS, and high temperature, and stability of tubulin; VdSep6 has a role in responses to NO, CR, and CFW, and in melanin biosynthesis; VdSep5 is involved in high-temperature stress response and negatively regulates the response to ROS.

The septins in *V. dahliae* are involved in microsclerotial formation and thermal stress resistance

Microsclerotia are the primary source of infection and important dormant structures that can survive in the soil for years (Wilhelm 1955). In *V. dahliae*, the formation of mature microsclerotia is normally accompanied by melanin synthesis (Duressa et al. 2013; Xiong et al. 2014). In our current study, deletion of different septins resulted in different effects on the formation of microsclerotia and melanin biosynthesis in *V. dahliae*. As a result, all septins are important in the formation of melanized microsclerotia. VdSep3 and VdSep5 inhibit melanin biosynthesis, whereas VdSep6 promotes it. VdSep4 regulates microsclerotial development while suppressing melanin biosynthesis gene expression. Further analyses of these unique differences may yield more insight into these processes.

Melanin has been linked to microsclerotial resistance because it can protect against adverse environmental factors such as hyperthermia, UV irradiation, enzymatic lysis, nutrient deprivation, and fungicidal application (Wang et al. 2018). We previously investigated how melanin biosynthesis protects microsclerotia against temperature extremes in *V. dahliae* (Fang et al. 2019), and herein we revealed that each of the septin gene deletion mutants of *V. dahliae* could form melanized microsclerotia eventually. Although Δ*VdSep5* exhibited no difference in melanin formation with the wild-type strain, this mutant was still sensitive to high temperature. This suggests that besides melanin biosynthesis, there are other mechanisms underlying high-temperature resistance.

V. dahliae septins are important for chitin distribution and play different roles in the regulation of microtubules

The fungal cell wall, a dynamic structure with both rigidity and plasticity, plays prominent roles in fungal differentiation and morphogenesis (Bowman and Free 2006). Glucan and chitin are the most important structural polysaccharides in fungal cell walls. (Lopez-Romero and Ruiz-Herrera 1986). These polysaccharides are normally bound together in a balanced manner to reconstitute the cell wall in preparation for expansion or stress adaptation (Bowman and Free 2006). However, when chitin biosynthesis or decomposition is disrupted, the imbalance leads to an uneven chitin distribution, which subsequently inhibits hyphal growth (Tang et al. 2020b). In addition, a lack of stable unidirectional cell polarity in septin gene deletion mutants also causes abnormalities in hyphal morphology and chitin distribution (Gladfelter

2006). Herein, we observed abnormal chitin dispersion in cell walls of septin gene deletion mutants of *V. dahliae*, similar to that observed in the *AspB* mutant of *A. nidulans* and in the *BcSep4* mutant of *Botrytis cinerea* (Westfall and Momany 2002; Feng et al. 2017). The abnormal chitin distribution in these mutants could be due to that disrupted septin proteins cannot properly support the scaffold to maintain the normal cell wall diffusion barrier.

The cell wall integrity inhibitors CR and SDS work in different ways. CR binds specifically to β-1,3-glucan, preventing normal cell wall assembly and inhibiting cell growth (Kopecká and Gabriel 1992; Ram and Klis 2006). SDS disrupts hydrophobic interactions between noncovalently bound soluble cell wall glycoproteins and β-1,3-glucan chains as well as causes plasma membrane permeabilization and cell lysis, all of which affect cell wall integrity. (Mazáň et al. 2008). Interestingly, deletion of *VdSep5* caused slight resistance to CR and CFW stresses, while deletion of *VdSep3* or *VdSep6* led to sensitivity to CR and CFW stresses. There are other instances for members in a gene family function as mutual antagonists to coordinately modulate a certain pathway (Foos et al. 1992; Wu et al. 2000). In this study, SDS stimulated growth in all septin mutants, possibly demonstrating that SDS destruction of cell wall integrity affects all septins. As a result, all four septins may function concertedly to maintain normal morphogenesis and melanin biosynthesis in *V. dahliae*.

Linkages were previously revealed between septin and tubulin functions in *Aspergillus fumigatus* (Juvvadi et al. 2013). Considering that microtubules play an important role in the growth and development of filamentous fungi, we hypothesized that the response to RNS might be related to tubulin activity. We conducted experiments with the tubulin-targeting fungicide benomyl, indicating that septins are differentially involved in tubulin function. However, the mechanism by which VdSep3 increases benomyl resistance remains unknown. It could be related to its increase in melanin formation.

Septins are required for full virulence of *V. dahliae*

During infection and colonization of host plants, pathogenic fungi form hyphopodia and infectious hyphae or other differentiated infection structures, and these processes are closely linked to cell polarization. (Momany and Talbot 2017). Septins play an important role in regulating cell polarity through their action on a protein scaffold and the maintenance of a diffusion barrier. Consequently, most pathogenic fungi exhibit reduced virulence after the deletion of septin genes (Momany and Talbot 2017). For example, FgCdc3, FgCdc11, and FgCdc12 in *F. graminearum* are important for virulence to wheat, but FgCdc10 contributes minimally to the virulence (Chen et al. 2016). Deletion of septin-coding genes in *M. oryzae* impedes infection structure formation, leading to loss of pathogenicity (Dagdas et al. 2012; Momany and Talbot 2017). In *V. dahliae*, VdSep3 and VdSep5 play an important role in hyphopodium development, and the corresponding gene deletion mutants were compromised in virulence to cotton (Zhou et al. 2017). VdSep4 and VdSep6 were also important to the virulence of *V. dahliae* as observed in this current study. VdSep4 regulates the timing of penetration, and the remaining septins mediate hyphal growth in plant cells. However, each of the septin gene deletion mutants, including Δ*VdSep3*, could be re-isolated from the infected plants, which is different from a previous study suggesting no plant penetration by a Δ*VdSep3* mutant strain (Zhou et al. 2017). Potentially, the differences in these findings could be explained by different genetic backgrounds of the two strains.

The ROS/RNS burst is an early defense response of plant hosts to prevent pathogen invasion. To combat oxidative and nitrosative stresses, pathogens utilize H_2O_2 and NO detoxification mechanisms (Hong et al. 2008). In *B. cinerea*, deletion of *Sep4* caused significantly faster mycelial growth under H_2O_2 stress (Feng et al. 2017). Yet few studies have examined the relationship between septins and NO stress responses in plant pathogenic fungi. Our previous study revealed that all of the septin-encoding genes in *V. dahliae* were slightly down-regulated in XS11 in response to SNP treatment (Tang et al. 2020a). Herein, the Δ*VdSep3* and Δ*VdSep4* mutants were hypersensitive to both H_2O_2 and NO stress, VdSep5 played a negative role in H_2O_2 resistance, and the Δ*VdSep6* mutant was only sensitive to NO stress. This may indicate that the virulence of these mutants was reduced partly because of their defective response to ROS/RNS stress. The roles of septins in the fungal response to H_2O_2 stress appear to be highly variable. However, the Δ*VdSep5* mutant showed a lower rate of growth inhibition in response to H_2O_2 and NO treatments than XS11, possibly due to an excess of melanized microsclerotia.

Conclusions

This study sheds light on the roles of all *V. dahliae* septin members in microsclerotial formation as well as their involvement in stress resistance and contribution to fungal virulence. The results complement and extend those of a previous study (Zhou et al. 2017) and clearly indicate that the septins in *V. dahliae* play different roles in regulating microsclerotial development, melanin synthesis, and stress responses, while each is important for fungal virulence. The insights gained into the functions of the septins in *V. dahliae* are helpful for understanding the its pathogenesis.

Methods

Fungal strains and culture conditions

The wild-type strain XS11 of *V. dahliae* was isolated from a smoke-tree in Fragrant Hills, Beijing, China (Wang et al. 2013), which was used as a parent strain to generate septin gene deletion mutants. Gene deletion mutants and their complemented strains were obtained using polyethylene glycol (PEG)-mediated genetic transformation (Wang et al. 2013). All strains were purified by single-spore isolation. All fungal strains were maintained as conidial suspension (10^6 spores/mL) in 30% glycerol at -80 °C.

For analyses of the effects of cell wall inhibitors or nitrosative stress on mycelial growth, strains were grown on CM (1 L solid CM: 50 mL 20 × nitrate salts, 1 mL 1000 × trace, 10 g D-glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, 1 mL vitamin solution, 15 g agar) plates and CM plates supplemented with 25 μg/mL Congo red (CR), 0.01% sodium dodecyl sulfate (SDS), 30 μg/mL calcofluor white (CFW) or 10 mM sodium nitroprusside dehydrate (SNP) at 25 °C for 10 days. For high-temperature stress, strains were grown on PDA at 25 °C or 30 °C for 10 days. To test benomyl stress, strains were grown on PDA or PDA containing 0.6 μM/mL benomyl at 25 °C for 7 days.

For testing responses to H_2O_2 stress, a piece of sterile filter paper disc (Φ = 5 mm) was placed in the center of the plate containing a 1 mL conidial suspension (10^7 conidia/mL) of each *V. dahliae* strain and then dropped with 5 μL of hydrogen peroxide solution (15% or 30%) (Sangon Biotech). The diameter of the inhibition zone was measured after incubation at 25 °C for 3 days.

Observations of microsclerotial formation were carried out on both CM and basal media (BM: 10 g/L glucose, 0.2 g/L sodium nitrate, 0.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7H_2O$, 1.52 g/L KH2PO4, 3 μM thiamine HCl, 0.1 μM biotin, and 15 g/L agar). A 0.5-μL conidial suspension (10^6 conidia/mL) of *V. dahliae* was dropped onto a glass slide overlaid with 300 μL of molten CM. For analyses on BM, conidial suspension (10^6 conidia/mL) of each strain was evenly spread on sterile cellulose membrane (Whatman, the UK, Φ = 80 mm, pore size = 0.22 μm) overlaid on a BM plate, and cultured at 25 °C for 35 days. Observations were performed under light microscopy (DM2500 Leica) after incubation for 4, 8, 12, and 35 days. The melanized areas of fungal colonies were measured using ImageJ (Papadopulos et al. 2007) under the default settings (the threshold of all images was 190). All the experiments were repeated three times. For comparison, conidia of each tested strain were also inoculated into 80 mL of liquid CM and incubated at 25 °C with shaking at 150 rpm on an orbital shaker for 5 days.

Bioinformatic analysis

Complete sequences of the *V. dahliae* septins were downloaded from JGI (https://genome.jgi.doe.gov/Verda1/Verda1home.html) following BLASTP search with known septin orthologs from *M. oryzae* as queries (Dagdas et al. 2012). The sequences of septin orthologs in other fungi were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/) and JGI databases. Predictions of functional domain in septins of *V. dahliae* were performed using InterPro (http://www.ebi.ac.uk/interpro/). Amino acid sequence alignments were performed using ClustalX 2.0 (Larkin et al. 2007) and the phylogenetic tree was constructed using MEGA 6.0 (Tamura et al. 2013).

Strain construction and verification

The entire open reading frame (ORF) of each septin genes (*VdSep3*, *VdSep4*, *VdSep5*, and *VdSep6*) was replaced with a hygromycin resistance gene cassette constructed by the split-marker method (Goswami 2012). All the primers used for vector construction and verification are listed in Additional file 2: Table S1. After transformation, the transformants were screened with 50 μg/mL of hygromycin. To complement the mutants, the DNA fragment carrying the native promoter of septins was amplified with the primers in Additional file 2: Table S1. The resulting PCR products were co-transformed with the fragment of G418 by PEG-mediated genetic transformation. The complemented strains were screened with 50 μg/mL of G418.

The genomic DNA of the transformants was extracted by the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich 1994). The target gene deletion mutants were verified by PCR with internal and external primers (Additional file 2: Table S1). Southern blotting was performed to confirm the deletion of the target gene with the DIG High Prime DNA Labeling and Detection Starter Kit I in accordance with the manufacturers' protocol (Roche, Germany). The probe fragment used for Southern blot analysis was amplified from the *HPH* gene with primers Hyg-tzs and Hyg-tzx (Additional file 2: Table S1) and labeled with the DIG. *Eco*RI was used to digest the genomic DNA extracted from the tested strains.

Gene expression analyses

Fungal strains were cultured on PDA at 25 °C for 10 days, and their mycelia were collected for RNA extraction. The total RNA was extracted with TRIzol reagent (Invitrogen, USA) and purified using a PureLink RNA Minikit (Ambion, USA). Reverse transcription of RNA using the QuantScript RT kits (Tiangen, China) was performed to obtain cDNA. RT-qPCR was performed with the

SuperReal PreMix Plus (SYBR Green) using SYBR green dye and an ABI 7500 real-time PCR system (Applied Biosystems, USA). The *V. dahliae β-tubulin* gene was used as the internal reference for all the qPCR analyses (Xiong et al. 2014). Analyses of each gene were conducted in three biological and four technical replicates. The results of RT-qPCR were analyzed by the threshold cycle method using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). To evaluate the expression levels of the genes involved in oxidative responses, all strains were treated with or without 2.5 mM H_2O_2 for 1 h before RNA isolation. Primer sequences are listed in Additional file 2: Table S1.

Fungal inoculation assays

One-month-old tobacco plants (*Nicotiana benthamiana*) and conidial suspensions (10^6 spores/mL) of the *V. dahliae* strains were used for root-dip inoculation experiments. The roots of tobacco seedlings were immersed in conidial suspension of each strain for 10 min, with sterile water as control. All the inoculated plants were incubated in a greenhouse at 25 °C with a photoperiod of 14 h light/10 h dark for symptom development, and observations were performed every 5 days. The disease was scored at 35 dpi on a scale of 0–4. 0, no wilting; 1, yellowing or wilting of < 2 leaves; 2, yellowing or wilting of one-fourth leaves; 3, yellowing or wilting of two-thirds leaves; and 4, more than 85% of the leaves wilted or the whole plant died, and the disease index was determined. Survival rates were scored according to the number of seedlings that had died. Twenty seedlings were inoculated with each strain.

At 35 dpi, the sections of stem-root junction of the tobacco plants were used for re-isolation of the fungus. The steps are consistent with our previous research (Tang et al. 2020b).

To examine the biomass of pathogens in the plant vascular, stem sections of tobacco plants at 5 dpi were used for DNA extractions, performed using the Plant Genomic DNA Kit (Tiangen, China) according to the manufacturer's instructions. qPCR was carried out as previously described (Wang et al. 2013). The tobacco *GAPDH* gene was used as the internal reference for all the qPCR analyses. Analysis of *V. dahliae* ITS gene was performed in three biological and four technical replicates. The results of qPCR were analyzed by the threshold cycle method using the formula $2^{-\Delta\Delta CT}$.

Penetration assays on plant and cellophane membrane

Conidia of each strain were inoculated into 80 mL of liquid CM at 25 °C with shaking at 150 rpm on an orbital shaker for 5 days prior to use. Pieces of fresh onion epidermal tissue were soaked in alcohol for 7 days and then in sterile water for 1 h before use. The surface of the treated onion epidermis was overlaid on a sterile glass slide and placed in a sterile petri dish. Fresh conidial suspension (10^5 conidia/mL) of each strain was inoculated on the surface of the onion epidermis and drops of sterile water were then added to the petri dish to keep the environment moist. The culture was maintained at 25 °C and observed every 2 days.

For the penetration assays on cellophane, conidial suspensions (10^5 conidia/mL) of each strain of *V. dahliae* were inoculated in the center of a cellophane membranes overlaid on MM plate and cultured at 25 °C. The cellophane was removed from the plate surface after incubation for 3 and 6 days, and the plate was maintained for another 3 days to observe if there was colony on MM and thus determine if penetration had occurred before removing the membrane. The colonies on the cellophane membrane were removed with sterile water, and the invading hyphae, hyphopodium, and hyphae remaining on the membrane were observed under microscope (Leica DM 2500).

Statistical analysis

Data are presented as mean value ± standard error of the mean. Statistical analyses were performed by ANOVA and Student's *t*-test (SPSS 16.0). A P value < 0.05 (*) and P value < 0.01 (**) was considered statistically significant in this study.

Abbreviations

aa: Amino acid; acetyl-CoA: Acetyl-coenzyme A; BM: Basic media; cDNA: Complementary DNA; CFW: Calcofluor white; CM: Complete medium; CR: Congo red; CTAB: Cetyltrimethylammonium bromide; DIG: Digoxigenin; dpi: Days post-inoculation; G418: Geneticin; GTP: Guanosine triphosphate; H_2O_2: Hydrogen peroxide; HPH: Hygromycin; JGI: Joint Genome Institute; MM: Minimal medium; NADPH: Nicotinamide adenine dinucleotide phosphate; NCBI: National Center for Biotechnology Information; NO: Nitrogen monoxide; PDA: Potato dextrose agar; PEG: Polyethylene glycol; RNS: Reactive nitrogen species; ROS: Reactive oxygen species; RT-qPCR: Reverse transcription-quantitative PCR; qPCR: Quantitative real-time PCR; SDS: Sodium dodecyl sulfate; SNP: Sodium nitroprusside dehydrate; UV: Ultraviolet.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00145-x.

Additional file 1: Figure S1. Phylogenetic tree constructed on the basis of the amino acid sequences of septins from *Verticillium dahliae* and their homologs in other fungi, and schematic diagram of *V. dahliae* septins**. Figure S2.** Verification of septin gene deletion mutants and complemented strains in *Verticillium dahliae*. **Figure S3.** Septins are involved in hyphal septum formation in *Verticillium dahliae*. **Figure S4.** Virulence of the complemented septin deletion mutants of *Verticillium dahliae*. **Figure S5.** Penetration assay of *Verticillium dahliae* on onion epidermal cells.

Additional file 2: Table S1. Information of primers in this study

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Author contributions
YW and CT conceived the experiments; HW, CT, CD, and WL performed the experiments; CT, HW, and YW analyzed the data; HW, CT, SJK, and YW wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

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Competing interests
The authors declare that they have no competing interests.

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