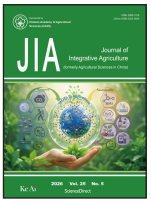
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RESEARCH ARTICLE

FvVam6 is associated with fungal development and fumonisin biosynthesis *via* vacuole morphology regulation in *Fusarium verticillioides*

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Highlights

- Deleting the *FvVam6* gene in *Fusarium verticillioides* significantly affects fungal development, virulenc, vacuolar morphology and FB_1 biosynthesis.
- The Vps39 and clathrin domains are critical for the biological functions of FvVam6.

Abstract

As the largest multifunctional and dynamic organelle in fungi, vacuoles are associated with different organelles through membrane contact sites and participate in various cellular processes. Vacuole and mitochondria patch (vCLAMP), the membrane contact site that tethers vacuoles and mitochondria, is indispensable for reciprocal interplay between these two organelles. The impairment of vacuoles and mitochondria significantly suppressed FB_1 production in *Fusarium verticillioides*. However, the understanding of how vCLAMP complex regulates fumonisin biosynthesis remained unknown. Herein, the biological functions of vCLAMP component Vam6 were investigated in *F. verticillioides*. Our results showed that *FvVam6* deletion mutant ΔFvVam6 exhibited palpable defects in fungal development, stresses responses and pathogenicity. In addition, abnormal vacuolar morphology and significantly reduced FB_1 production were observed in ΔFvVam6. Furthermore, we demonstrated that two vacuolar sorting protein 39 (Vps39) domains and clathrin domain were critical for the biological functions of FvVam6, while clathrin and Vps39-2 domains played dominant roles in the regulation of virulence and FB_1 production. Taken together, our results advanced our understanding of vCLAMP in fumonisin biosynthesis in plant pathogenic fungus *F. verticillioides*.

Keywords: *Fusarium verticillioides*, *FvVam6*, fumonisin B_1, vacuolar morphology

1. Introduction

Maize (*Zea mays*) is one of the most important cereal crops worldwide, with an estimated production of 1.16 billion tons in 2023. As the second largest maize producer in the world, the maize production in China was approximately 277 million tons in 2022 (China-NBS 2023). *Fusarium verticillioides* is an important maize pathogen that cause maize seedling blight, stalk rot and ear rot (Woloshuk and Shim 2013; Blacutt *et al.* 2018). These diseases not only lead to significant yield losses, the mycotoxins produced by the pathogen during plant infestation, including fumonisins, fusarins and fusaric acid, also pose serious threats to human and animal health (Missmer *et al.* 2006; Woloshuk and Shim 2013; Yan *et al.* 2023). Among all fumonisins, the B-series fumonisins pose the greatest adverse health risks in livestock and humans worldwide (Alexander *et al.* 2009), with fumonisin B_1 (FB_1) being the most frequent form in all naturally occurring fumonisins (Rheeder *et al.* 2002). Fumonisins have been reported to cause equine leukoencephalomalacia and liver cancer in livestock (Jaskiewicz *et al.* 1987; Marasas *et al.* 1988). In addition, a report by Gamanya and Sibanda (2001) linked fumonisin exposure to fever, dizziness, vomiting and

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other symptoms in humans.

Fumonisin biosynthesis requires 17 enzymatic activities encoded by 15 fumonisin biosynthetic genes, one global regulator gene *Fum21* and one ABC transporter gene *Fum19*, which are clustered in a 42-kb region on one chromosome (Desjardins *et al.* 1992; Proctor *et al.* 1999, 2003; Seo *et al.* 2001; Brown *et al.* 2005, 2007; Desjardins *et al.* 2007). Although the studies on the genetic basis of fumonisin production had been well investigated since 1992, the compartmentalization of fumonisin biosynthetic enzymes and how *F. verticillioides* regulates fumonisin biosynthesis remains limited.

As the largest multifunctional organelle in fungal and plant cells (Gow 1997; Shimada *et al.* 2018), vacuole functions in conventional cellular processes including maintenance of cellular homeostasis, degradation of cell components, storage of ions and metabolites (Klionsky *et al.* 1990; Weber 2002; Li and Kane 2009). However, certain specialized functions of vacuole are distinct across fungi kingdoms, which are associated with different environmental conditions and particular requirements of diverse fungal lifestyles (Veses *et al.* 2008; Veses and Gow 2008). Fungal pathogens alter their cellular morphology when they encounter their host or in response to specific environmental triggers, and vacuoles play important roles in the response of the niche-specific adaptation and morphological plasticity. In filamentous fungi such as *Ustilago maydis* and *Magnaporthe grisea*, the impairment of hyphal growth and virulence were accompanied with the alteration of vacuolar morphology and physiology (Steinberg *et al.* 1998; Weber *et al.* 2001). It is worth noting that a novel function of vacuole had been reported in *Aspergillus parasiticus* that the pathogen employed vacuole-vesicle system for the biosynthesis and the export of aflatoxins (Chanda *et al.* 2009). Significantly, our recent publication reported that three key fumonisin biosynthetic proteins co-localized with vacuoles, and vacuoles played crucial role for FB_1 production in the filamentous fungus *F. verticillioides* (Yan *et al.* 2023).

Accumulated evidence revealed that communications between subcellular organelles could maximize intracellular metabolic flux. A previous study reported that the purinosomes were embedded with the microtubule (MT) networks in HeLa cells, and the embedded locate mode regulates the spatial distribution and biological activity of purinosomes (An *et al.* 2010). Subsequent study revealed that both purinosomes and mitochondria could benefit from their reciprocal interactions, while their interactions were regulated by MT cytoskeleton (Chan *et al.* 2018). Significantly, the extensive subcellular network of membrane contact sites (MCSs) plays crucial roles in organelles communications. In yeast, the membrane site that contacts vacuoles and mitochondria was designated vacuolar and mitochondrial patch (vCLAMP) (Elbaz-Alon *et al.* 2014; Honscher *et al.* 2014). In vCLAMP complex, Vps39 acts as the linker that tethers the vacuole and mitochondrion by interacting with Rab GTPase Ypt7 and mitochondrial receptor Tom40, respectively (Elbaz-Alon *et al.* 2014; Honscher *et al.* 2014; Klecker and Westermann 2014). A study showed that Vps39 participated in vacuole fusion process and regulated vacuolar morphology in *Saccharomyces cerevisiae* (Montoro *et al.* 2018). Mao and colleagues (2021) reported that ScVps39 homologue Vam6 was important for the functions of mitochondrion and vacuole in human pathogen *C. albicans*. Even with past studies focus on the roles of Vps39 and its connection with mitochondrion, our understanding of Vps39 in phytopathogenic fungi is limited.

Herein, we identified ScVps39 homologue Vam6 in *F. verticillioides* and generated a gene deletion mutant ΔFvVam6. We found that *FvVam6* is indispensable for fungal development, stresses responses and pathogenicity. In addition, ΔFvVam6 exhibited severe defects in vacuolar morphology and fumonisin biosynthesis. Further research demonstrated that two vacuolar sorting protein 39 (Vps39) domains and clathrin domain but not citron homology (CNH) domain were crucial for the biological functions of FvVam6 in fumonisin biosynthesis. Taken together, our findings provide important information on the role of vCLAMP complex in fumonisin biosynthesis regulation in *F. verticillioides*.

2. Materials and methods

2.1. Fungal strains, culture conditions and fungicides

Fusarium verticillioides wild-type strain M3125 (Yan *et al.* 2020, 2023; Yu *et al.* 2022) was used for the construction of the derived mutants in the current study. All strains were grown at 28°C on potato dextrose agar (PDA) (20 g glucose, 200 g potato, 10 g agar, and 1 L water) for mycelial growth. Conidia production on PDA plate was measured as described previously (Zhou *et al.* 2022). For conidia germination assay, conidia collected from PDA plates were cultivated on water agar (WA) (1 g agar and 1 L water), then incubated at 28°C for 8 h in dark. For fumonisin biosynthesis gene expression and FB_1 production assays, all strains were cultured in Myro medium (1 g $NH_4H_2PO_4$, 3 g KH_2PO_4, 2 g $MgSO_4{\cdot}7H_2O$, 5 g NaCl, 40 g sucrose, and 1 L water, pH 5.9) at 28°C for 2 and 7 days, respectively (Zhou *et al.* 2022). For measurement of fungal growth under different stress agents, mycelial plugs (4 mm in diameter) taken from the periphery of a 4-day-old colony of each strain were inoculated on PDA plates amended with 1 mol L^{-1} NaCl, 0.01% (w/v) SDS, 20 mmol L^{-1} H_2O_2, 6.5 μg mL^{-1} natamycin, 90 μg mL^{-1} thymol and 160 μg mL^{-1} eugenol, respectively. After 4 days of incubation at 28°C, fungal colony diameter on all plates was measured. Technical grade pyraclostrobin (98%, Jiangsu Huifeng Agrochemical Co., Ltd., China) and picoxystrobin (98%, Shandong Weifang Rainbow Chemical Co., Ltd., China) were each dissolved in methanol (10 mg mL^{-1}) and stored at 4°C as a stock solution. All experiments were performed in triplicate with 3 replicates.

2.2. Strain construction

The *FvVam6* gene deletion mutant ΔFvVam6 was generated *via* the homologous recombination protocol described previously (Yan *et al.* 2020; Zhou *et al.* 2022). All transformants were screened by PCR assays and further verified by qRT-PCR assays. For complementation, the fragment containing the entire *FvVam6* gene and geneticin-resistant gene was constructed, and then transformed into the protoplast of the ΔFvVam6 mutant. All primers used in the current study are listed in Appendix A.

2.3. Maize seedling infection assay

Seedling rot virulence assays were conducted using B73 cultivar as previously described (Yan *et al.* 2020). After growth on PDA plates for 5 days, conidia of M3125 and all mutants were collected and resuspended in sterilized water to a concentration of 1×10^7 mL^{-1}. Seedlings were inoculated with 2.5 μL conidia suspension. After 7 days of incubation at room temperature (20°C–25°C) in the dark, the infected seedlings were recorded and imaged. The experiments were performed in triplicate with 10 inoculated seedlings for each strain.

2.4. Microscopic examinations

Mitochondrial structure, vacuolar structure and endocytosis were observed with a Leica TCS SP5 confocal microscope (Wetzlar, Hessen, Germany). The wild-type M3125 and all mutants were cultured in Myro for 1 day before staining with different dyes. The mitochondrion was stained with Mito Tracker Red CMXRos (Yeasen, Shanghai, China), the vacuolar structure was visualized with CMAC (KeyGen, Nanjing, China), and the endocytosis was visualized with FM4-64 (Thermo Fisher, MA, USA). The laser excitation wavelength was set at 353, 579, and 579 nm for CMAC, FM4-64 and Mito Tracker Red CMXRos, respectively.

For transmission electron microscopy (TEM) examination, M3125 and each mutant strains were cultured in Myro medium for 2 days at 28°C at 175 r min^{-1}, then the mycelia of each strain were washed in deionized water and fixed in 2.5% glutaraldehyde at 4°C at least for 2 h. The sample preparation and analysis were performed by Wuhan Servicebio Co., Ltd., China.

2.5. Assays for fumonisin biosynthesis gene expression

To measure the expression levels of fumonisin biosynthesis genes, mycelia were harvested from 2-day-old Myro cultures and used for RNA extraction and subsequent cDNA synthesis. The qPCR assays were conducted with a ChamQTM SYBR qPCR Master Mix Kit (Vazyme, Nanjing, China). The GAPDH of *F. verticillioides* was used as the internal control. The relative expression of each gene was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak *et al.* 2001). The experiments were performed in triplicate with 3 replicates.

2.6. Analysis of FB_1 production

To determine the FB_1 production, M3125 and all derived mutants were cultured in liquid Myro medium. After incubation at 28°C, 175 r min^{-1} for 7 days, mycelia along with 2 mL culture broth of each strain were collected. Subsequently, FB_1 production was assayed with a competitive ELISA Detection Kit (Aijude, Nanjing, China) following the manufacturer's protocols. The experiments were performed in triplicate with 3 replicates.

2.7. Assays for *FvVam6* deletion on ATP biosynthesis and H_2O_2 production

Mycelia samples of M3125, ΔFvVam6 and ΔFvVam6-C grown in Myro medium for 2 days were used for the measurement of ATP biosynthesis and H_2O_2 production with ATP Content Assay Kit (Solarbio, Beijing, China) and H_2O_2 Content Assay Kit (Solarbio, Beijing, China), respectively. Briefly, samples of liquid nitrogen-ground mycelia (0.05 g) were added to 0.5 mL lysis buffer in the ATP Detection Kit (Solarbio, BC0305) or H_2O_2 Detection Kit (Solarbio, BC3595). Subsequently, quantification of ATP or H_2O_2 content was conducted following the manufacturer's protocols. The experiments were performed in triplicate with 3 replicates.

3. Results

3.1. Mitochondria are involved in FB_1 biosynthesis in *F. verticillioides*

In our recent publication, we showed that vacuoles are important cellular organelles for FB_1 biosynthesis (Yan *et al.* 2023). Herein, we determined how FB_1 biosynthesis responds to quinone outside inhibitors (QoIs) pyraclostrobin and picoxystrobin, the fungicides that target mitochondria, one of the organelles that closely communicate with vacuoles. After treatment with 0.25 and 0.60 μg mL^{-1} pyraclostrobin and picoxystrobin (approximately EC_{50} against mycelial growth), respectively, the FB_1 production and the expression of 4 fumonisin biosynthesis genes (*Fum1*, *Fum6*, *Fum8*, and *Fum21*) were significantly decreased when compared to the control non-treated group (Appendix B), indicating that mitochondrion was involved in FB_1 biosynthesis in *F. verticillioides*.

3.2. *FvVam6* is involved in vegetative growth, asexual development and virulence

To determine the biological functions of *FvVam6*, the core subunit that connected vacuoles and mitochondria, we generated the deletion mutant ΔFvVam6 from the wild-type progenitor M3125. The vegetative growth of ΔFvVam6 was significantly decreased when compared to M3125 and ΔFvVam6-C strains on PDA plates (Fig. 1-A). When we inoculated conidial suspensions of M3125, ΔFvVam6 and ΔFvVam6-C on maize seedlings, M3125 and ΔFvVam6-C exhibited severe seedling rot symptoms after 7 days of incubation, while ΔFvVam6 only caused minute disease symptoms on maize seedlings (Fig. 1-B). Moreover, significant reductions in conidia germination rate as well as conidia production were observed in ΔFvVam6 when compared to the wild-type progenitor M3125 (Fig. 1-C and D). Collectively, our data suggested that *FvVam6* is indispensable for fungal development and virulence in *F. verticillioides*.

3.3. *FvVam6* is involved in responses to a variety of stresses

To characterize the role of *FvVam6* in responses to environmental stresses, we determined the sensitivity of ΔFvVam6 strain to osmotic stress, cell wall-damaging agent and oxidative stress. As indicated, ΔFvVam6 exhibited increased sensitivity to osmotic stress induced by 1 mol L^{-1} NaCl but decreased sensitivity to 0.01% (w/v) cell wall-damaging agents SDS. However, no sensitivity change was found in M3125, ΔFvVam6 and ΔFvVam6-C when exposed to oxidative stress triggered by 20 mmol L^{-1} H_2O_2 (Fig. 2). Taken together, our findings indicated critical roles of Vam6 in osmotic stress response and cell wall

integrity in *F. verticillioides*.

We further determined the sensitivity of wild-type M3125 and mutant ΔFvVam6 to natamycin, thymol and eugenol, the compounds considered to disrupt the integrity of fungal cell membranes by forming polyene-sterol complex (Zuzarte *et al.* 2011; Marchese *et al.* 2017; Meena *et al.* 2021; Gou *et al.* 2023). As results showed, ΔFvVam6 exhibited increased sensitivity to natamycin and thymol when compared to M3125 and ΔFvVam6-C. It is also noteworthy that ΔFvVam6 did not show growth on PDA plates containing 6.5 μg mL^{-1} natamycin and 90 μg mL^{-1} thymol (Table 1), suggesting that *FvVam6* is involved in the regulation of fungal cell membrane integrity.

3.4. *FvVam6* is required for vacuolar morphology but not endocytosis

The deletion of *Vps39* leads to abnormal vacuolar morphology in *S. cerevisiae* (Nakamura *et al.* 1997; Montoro *et al.* 2018). We therefore investigated the role of *FvVam6* in maintaining vacuolar structure. CMAC staining showed that the wild-type M3125 and complemented strain ΔFvVam6-C exhibited spherical and large vacuoles, whereas ΔFvVam6 showed diffused fluorescence signal throughout the entire fungal cell (Fig. 3-A). TEM analysis further showed irregular and smaller vacuoles observed in ΔFvVam6 (Fig. 3-B). Previous studies showed that endocytosis was connected to vacuole fusion in

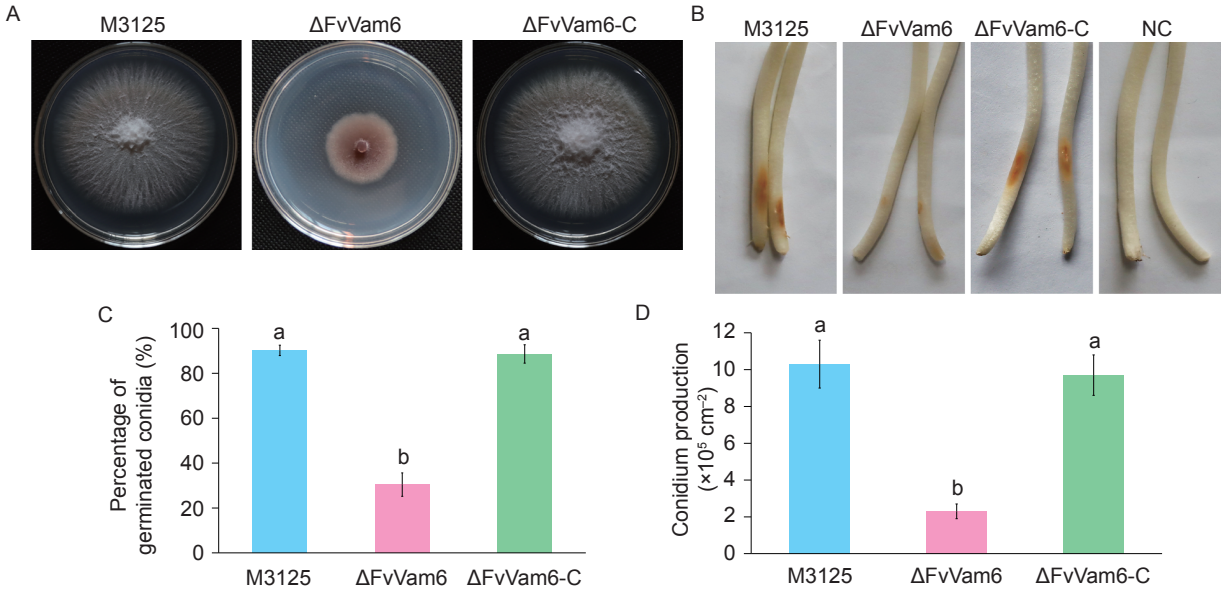


Fig. 1 *FvVam6* is important for vegetative growth, asexual development and virulence in *Fusarium verticillioides*. A, colony morphology of the wild-type M3125, ΔFvVam6 and complemented strain ΔFvVam6-C on PDA plates at 28°C for 5 days. B, FvVam6 is required for full virulence. Maize seedlings were grown in dark for 3 days, and then inoculated with conidia suspension of each mutant. The images were taken after 7 days of inoculation. Sterilized water was used as negative control. C, conidial germination of M3125, ΔFvVam6 and ΔFvVam6-C. Conidia of each strain were cultured on WA plates. After incubation at 28°C for 8 h in dark, conidial germination of 200 conidia was examined. D, conidial production of M3125, ΔFvVam6 and ΔFvVam6-C. Conidia were collected from the strains cultured on PDA plates at 28°C for 5 days. Bars denote standard errors from 3 repeated experiments. Values on the bars followed by the same letter are not significantly different (P<0.05) according to Fisher's LSD test.

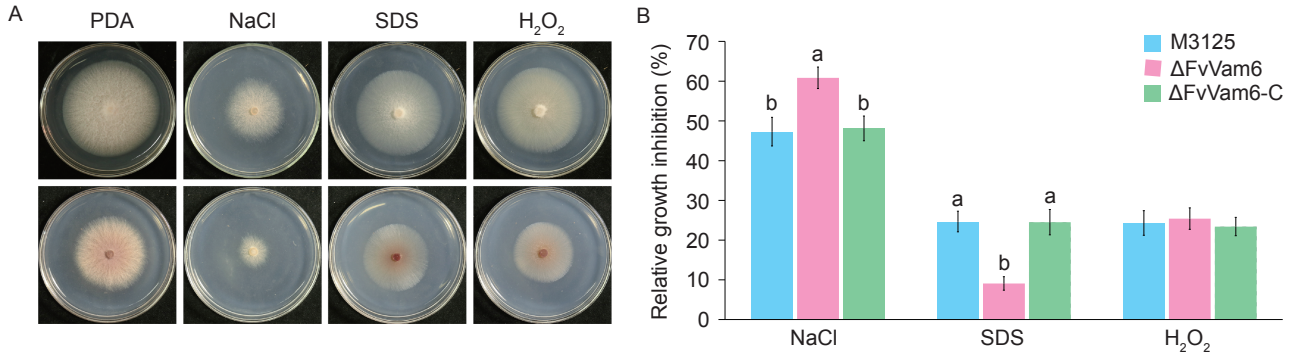


Fig. 2 Defects of the ΔFvVam6 in response to different stresses. A, the wild-type M3125, ΔFvVam6 and complemented strain ΔFvVam6-C were cultured on PDA medium supplemented with osmotic stressor (1 mol L^{-1} NaCl), cell wall-damaging agent (0.01% (w/v) SDS) and oxidative stressor (20 mmol L^{-1} H_2O_2). The photos were taken after 4 days of incubation. B, the growth inhibition rates of the indicated strains under different stress conditions (normalized to colonies diameter on PDA plates). Bars denote standard errors from 3 repeated experiments. Values on the bars followed by the same letter are not significantly different (P<0.05) according to Fisher's LSD test.

Table 1 The inhibition of three fungicides to M3125, ΔFvVam6 and ΔFvVam6-C[1)]

Inhibtion rate (%)	Natamycin (6.5 μg mL^{-1})	Thymol (90 μg mL^{-1})	Eugenol (160 μg mL^{-1})
M3125	87.84±1.81 b	95.27±1.05 b	67.57±1.34
ΔFvVam6	100 a	100 a	69.70±1.34
ΔFvVam6-C	86.39±1.73 b	94.77±0.78 b	66.66±1.61

[1)] Means and standard deviations (SD) were calculated from three independent experiments. Values in table followed by the same letter are not significantly different (P<0.05). The growth inhibition rate of different compounds were cultured on PDA plates for 4 days at 28°C.

eukaryotic cells (Wedlich-Soldner *et al.* 2000; Dou *et al.* 2011; Zhang *et al.* 2015). In the current study, we tested whether endocytosis was affected by *FvVam6* deletion using the FM4-64 staining assays. As indicated, ΔFvVam6, similar to M3125 and ΔFvVam6-C, all rapidly absorbed FM4-64 within 1 min after the dye treatment, indicating that the endocytosis is not affected by the deletion of *FvVam6* (Fig. 3-C). Taken together, these data suggested that *FvVam6* is essential for governing vacuolar structure but not endocytosis in *F. verticillioides*.

3.5. Deletion of *FvVam6* does not disrupts mitochondrial structure and functions

As an important subunit of vCLAMPs, Vam6 was proven indispensable for proper mitochondrial structure and functions in *Candida albicans* (Mao *et al.* 2021). In addition, the abnormal structures could impair the functions of mitochondria in *F. graminearum* (Duan *et al.* 2020). In the current study, the impact of FvVam6 deletion on mitochondria was visualized *via* MitoTracker Red CMXRos staining. As shown in Fig. 4-A, the mitochondria remained tubular structure in ΔFvVam6 when compared to M3125 and ΔFvVam6-C, indicated that FvVam6 is not associated with mitochondrial structure. Furthermore, we measured the effects of *FvVam6* deletion on ATP production and H_2O_2 accumulation since these substances are closely associated with mitochondrial activity (Vacca *et al.* 2004; Wang *et al.* 2017; Bulthuis *et al.* 2019). However, no significant difference was found in ATP production and H_2O_2 content among M3125, ΔFvVam6 and ΔFvVam6-C (Fig. 4-B and C). Taken together, our data indicated that *FvVam6* is not involved in the regulation of mitochondrial structure and functions in *F. verticillioides*.

3.6. *FvVam6* is required for fumonisin biosynthesis

When cultured in Myro medium for 7 days, ΔFvVam6 exhibited a substantial repression (approximately 48 folds) in FB_1 production compared to the wild-type M3125 and complemented mutant ΔFvVam6-C (Fig. 5-A). In addition, the expression levels of *Fum1*, *Fum6*, *Fum8* and *Fum21* were remarkably down-regulated in ΔFvVam6 when compared to M3125 and ΔFvVam6-C (Fig. 5-B). These findings suggested that *FvVam6* is indispensable for fumonisin biosynthesis in *F. verticillioides*.

3.7. The Vps39 and clathrin domains of FvVam6 are important for fungal development and full virulence

To explore the biological functions of CNH, Vps39-1, clathrin and Vps39-2 domains in the FvVam6 protein, we generated

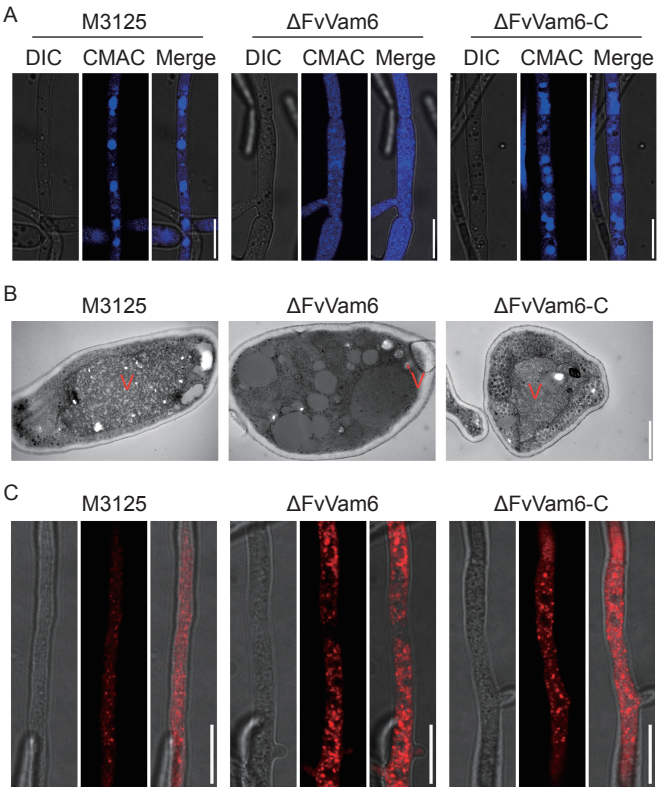


Fig. 3 Deletion of *FvVam6* affects on vacuolar morphology but not endocytosis in *Fusarium verticillioides*. A, vacuolar structure of the wild-type M3125, ΔFvVam6 and complemented mutant ΔFvVam6-C revealed by CMAC staining. Bar=10 μm. B, ultrastructural morphology of vacuole in M3125, ΔFvVam6 and ΔFvVam6-C were examined with a transmission electron microscopy. "V" represents fungal vacuole in *F. verticillioides*. Bar=1 μm. C, fresh hyphae of M3125, ΔFvVam6 and ΔFvVam6-C were stained with FM4-64. Photographs were taken at the indicated time points. Bar=10 μm.

the domain deletion constructs, namely FvVam6$^{\Delta CNH}$, FvVam6$^{\Delta Vps39-1}$, FvVam6$^{\Delta clathrin}$ and FvVam6$^{\Delta Vps39-2}$, then transformed into the wild-type M3125 (Fig. 6-A). The results revealed that FvVam6$^{\Delta Vps39-1}$, FvVam6$^{\Delta clathrin}$ and FvVam6$^{\Delta Vps39-2}$ mutants had noticeable defects in mycelial growth, conidia production and conidia germination in comparison with M3125 and FvVam6$^{\Delta CNH}$. These defects were more severe in FvVam6$^{\Delta clathrin}$ and FvVam6$^{\Delta Vps39-2}$ (Fig. 6-B–D). In addition, the mutants FvVam6$^{\Delta Vps39-1}$, FvVam6$^{\Delta clathrin}$ and FvVam6$^{\Delta Vps39-2}$ and ΔFvVam6 caused little seedling rot symptoms after 7 days of inoculation when compared with M3125 (Fig. 6-E). Taken together, these results suggested that two Vps39 and clathrin domains of FvVam6, but not the CNH domain, are important for fungal development and virulence.

3.8. The Vps39 domains of FvVam6 are critical for vacuole morphology

We examined the vacuolar structure by staining the hyphae of M3125 and mutant strains with CMAC. As indicted, while the wild-type M3125 maintained circular vacuoles, ΔFvVam6, FvVam6$^{\Delta Vps39-1}$, FvVam6$^{\Delta clathrin}$ and FvVam6$^{\Delta Vps39-2}$ mutants exhibited irregular and drastically diffused vacuole staining.

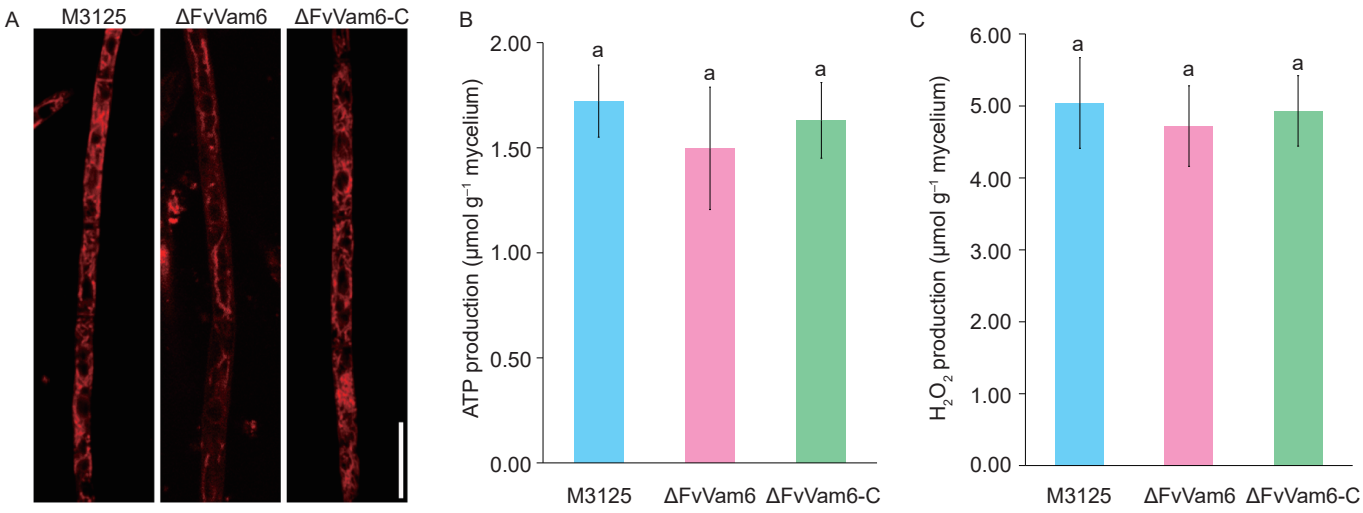


Fig. 4 *FvVam6* is dispensable for mitochondrial structure and functions in *Fusarium verticillioides*. A, mitochondrial structure of the wild-type M3125, ΔFvVam6 and complemented mutant ΔFvVam6-C revealed by Mito Tracker Red CMXRos staining. Bar=10 μm. B, ATP production in M3125, ΔFvVam6 and ΔFvVam6-C. C, H_2O_2 content in M3125, ΔFvVam6 and ΔFvVam6-C. All strains were cultured in Myro medium at 28°C for 2 days. Bars denote standard errors from 3 repeated experiments. Values on the bars followed by the same letter are not significantly different (P<0.05) according to Fisher's LSD test.

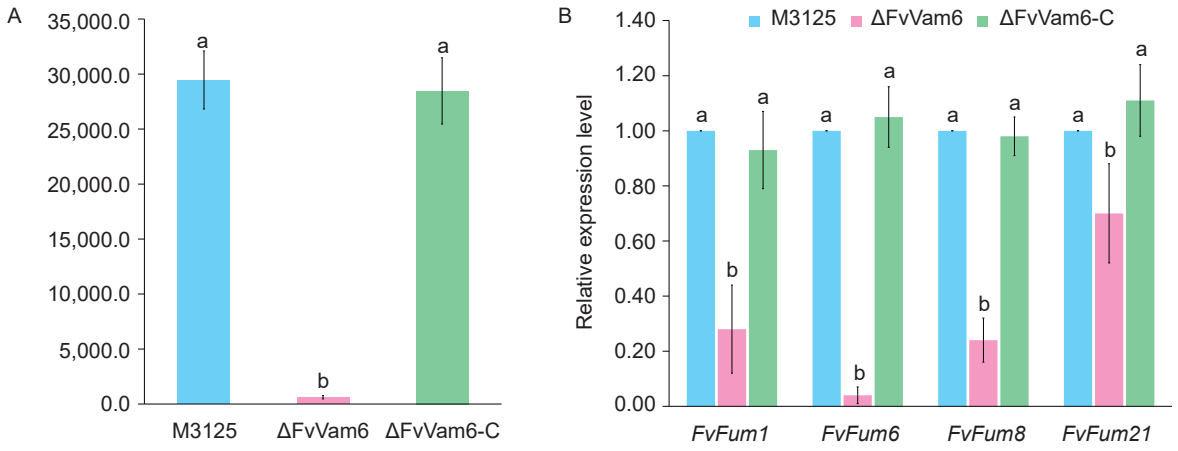


Fig. 5 *FvVam6* is indispensable for FB_1 biosynthesis in *Fusarium verticillioides*. A, FB_1 production of the wild-type M3125, ΔFvVam6 and complemented strain ΔFvVam6-C in Myro medium at 28°C for 7 days. B, relative expression level of fumonisin biosynthetic genes were significantly suppressed in ΔFvVam6 when cultured in Myro medium at 28°C for 2 days. Bars denote standard errors from 3 repeated experiments. Values on the bars followed by the same letter are not significantly different (P<0.05) according to Fisher's LSD test.

Intriguingly, although irregular and smaller vacuoles were distributed in fungal hyphae, large and round-like vacuoles were also observed in FvVam6$^{\Delta CNH}$ mutant (Fig. 7-A). In addition, TEM analysis showed similar results with CMAC staining assays (Fig. 7-B). Taken together, our data suggested that both of CNH, clathrin and two Vps39 domains participate in the fusion and division of vacuoles, while the clathrin and two Vps39 domains play major role in regulating vacuole morphology.

3.9. Clathrin and Vps39-2 of FvVam6 are the major domains that regulate fumonisin biosynthesis

To assess the regulatory role of CNH, Vps39-1, clathrin and Vps39-2 domains in fumonisin biosynthesis, we measured the FB_1 production in wild-type M3125 and mutant strains. After incubating in Myro medium for 7 days, we determined that FB_1 levels were repressed approximately 2.0-, 80- and 47-fold in FvVam6$^{\Delta Vps39-1}$, FvVam6$^{\Delta clathrin}$ and FvVam6$^{\Delta Vps39-2}$ in comparison with the wild-type M3125, respectively (Fig. 8-A). Subsequently, we determined the transcriptional levels of 4 fumonisin biosynthesis genes through qRT-PCR to verify the role of each domain in fumonisin biosynthesis. The results showed that *Fum1*, *Fum6*, *Fum8* and *Fum21* were drastically down-regulated in mutants FvVam6$^{\Delta Vps39-1}$, FvVam6$^{\Delta clathrin}$ and FvVam6$^{\Delta Vps39-2}$ but not in FvVam6$^{\Delta CNH}$ when compared to M3125 (Fig. 8-B). Taken together, these findings suggested that 2 Vps39 and clathrin domains are important for FvVam6 functions, while clathrin and Vps39-2 are the dominant domains for FvVam6 in fumonisin biosynthesis regulation.

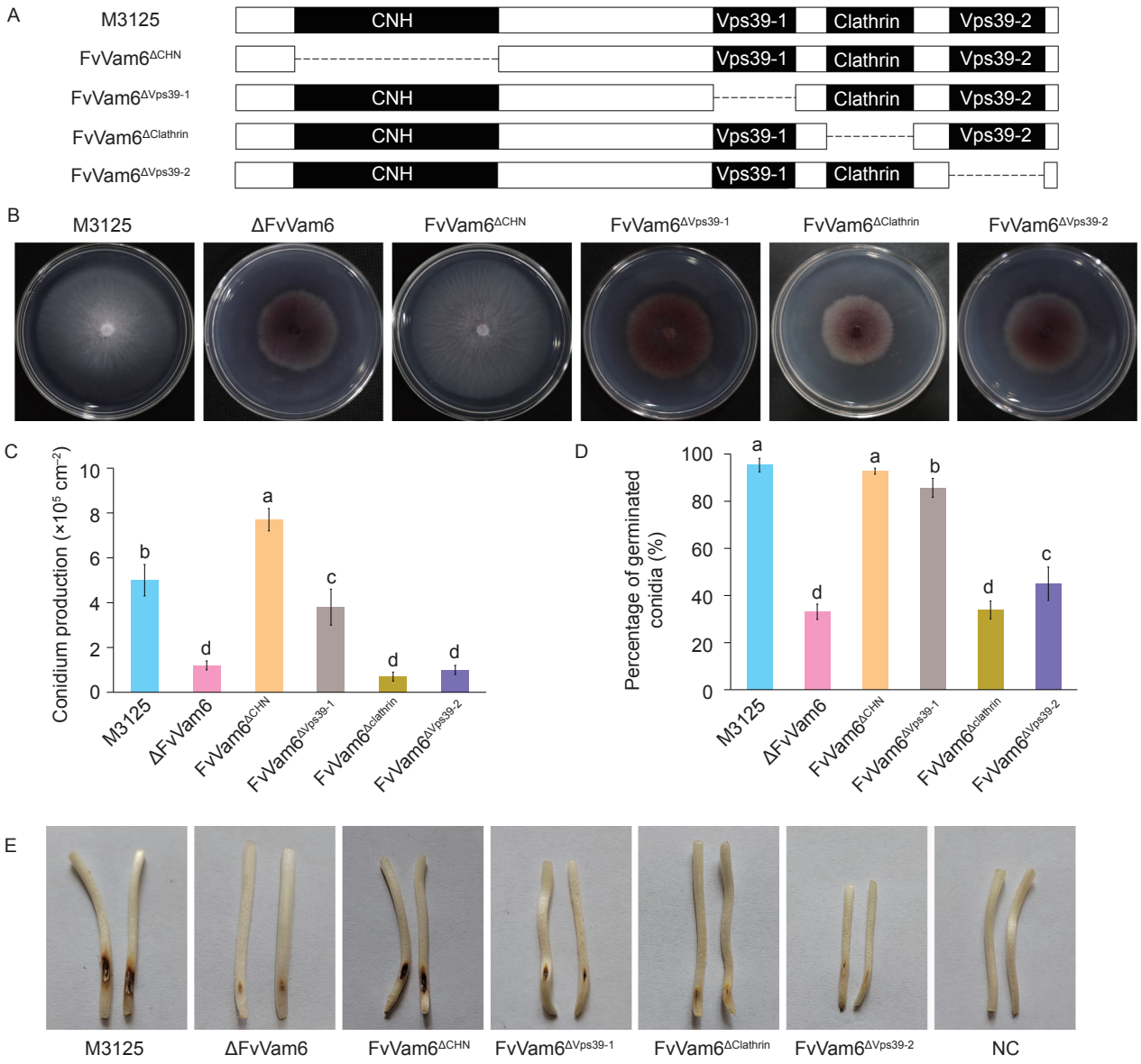


Fig. 6 Functional analysis of the CNH, clathrin and Vps39 domains of FvVam6 in fungal development and virulence. A, diagram and strategy showing domain deletion of FvVam6. B, colony morphology of the wild-type M3125, ΔFvVam6 and derived mutants on PDA plates. Photos were taken after incubation at 28°C for 5 days. C, conidial production of M3125 and the indicated mutants. Conidia were collected from the strains cultured on PDA plates at 28°C for 5 days. D, conidial germination of M3125 and the indicated mutants. Conidia of each strain were cultured on WA plates. After incubation at 28°C for 8 h in dark, conidial germination of 200 conidia was examined. E, infection assay with maize seedlings. Maize seedlings were grown in dark for 3 days, and then inoculated with conidia suspension of each mutant. The images were taken after 7 days of inoculation. Sterilized water was used as negative control. Bars denote standard errors from three repeated experiments. Sterilized water was used as negative control (NC). Values on the bars followed by the same letter are not significantly different (P<0.05) according to Fisher's LSD test.

4. Discussion

Vacuole is a dynamic cellular organelle that establish connection with various organelles in a eukaryotic cell. In higher plants, actin microfilaments were found to interact with vacuoles, thereby regulating vacuolar morphogenesis and dynamics (Palevitz and O'Kane 1981; Kutsuna *et al.* 2003; Mathur *et al.* 2003; Higaki *et al.* 2006). Microtubule cytoskeletons were required for vacuolar morphology and functions in moss plants (Ligrone and Duckett 1998; Oda *et al.* 2009). In addition, the interactions between mitochondria and vacuoles had been reported in different organisms (John Peter *et al.* 2017; Sousa *et al.* 2011; Yu *et al.* 2014). It is worth noting that the communications among subcellular organelles and the coordination at the cellular level largely dependent on MCSs, which were critical for intracellular signaling and membrane biogenesis (Eisenberg-Bord *et al.* 2016; Malia and Ungermann 2016). As the core member of vCLAMP complex, Vps39 connects vacuole and mitochondrion by binding with vacuole membrane protein Ypt7 and mitochondrial receptor Tom40, thereby affecting the biological activities of these two organelles (Elbaz-Alon *et al.* 2014; Honscher *et al.* 2014; Klecker and Westermann 2014).

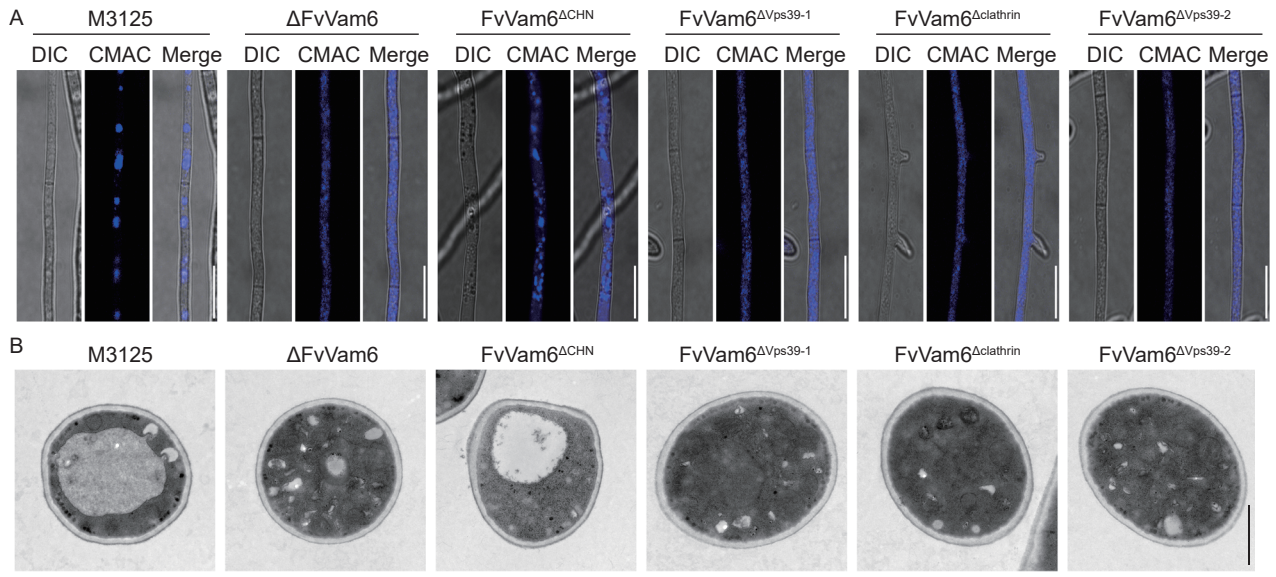


Fig. 7 Deletion of the Vps39 and clathrin domains of FvVam6 leads to vacuole fragmentation. A, vacuolar structure of the wild-type M3125, ΔFvVam6 and derived mutants revealed by CMAC staining. Bar=10 μm. B, ultrastructural morphology of vacuole in M3125, ΔFvVam6 and derived mutants were examined with a transmission electron microscopy. Bar=1 μm.

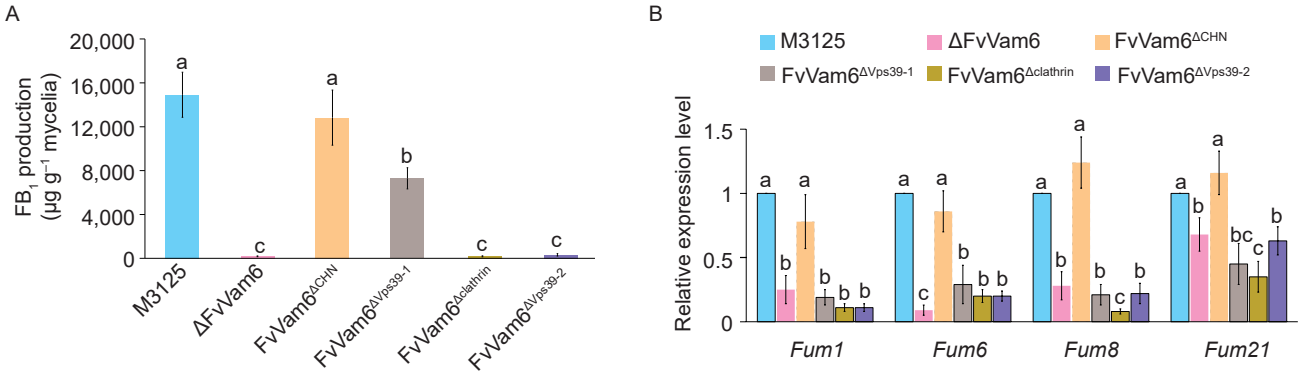


Fig. 8 Functional dissection of the CNH, clathrin and Vps39 domains of FvVam6 in fumonisin biosynthesis. A, FB_1 production of M3125, ΔFvVam6 and derived mutants in Myro medium at 28°C for 7 days. B, relative expression level of fumonisin biosynthetic genes in each strain after cultured in Myro medium at 28°C for 2 days. Bars denote standard errors from 3 repeated experiments. Values on the bars followed by the same letter are not significantly different (P<0.05) according to Fisher's LSD test.

A recent publication showed that the deletion of *ScVps39* homologue *Vam6* led to profound defects in hyphal development and virulence in *C. albicans* (Mao *et al.* 2021). In phytopathogenic fungus *F. graminearum*, *FgVam6* was found indispensable for fungal development and virulence (Li *et al.* 2022). In the current study, we identified ScVps39 homologue FvVam6 and generated a gene deletion mutant in *F. verticillioides*. The mutant ΔFvVam6 exhibited severe defects in vegetative growth, conidia production and germination. In addition, the pathogenicity of ΔFvVam6 was severely attenuated on maize seedlings. These results demonstrated that *Vam6* is indispensable for fungal development and virulence in *F. verticillioides*.

Previous studies reported that the deletion of *CaVam6* or *ScVps39* led to reduction of vacuole size in *C. albicans* and *S. cerevisiae*, respectively (John Peter *et al.* 2017; Montoro *et al.* 2018; Mao *et al.* 2021). In addition, Yang and colleagues (2016) demonstrated that the loss of *CmVps39* resulted in smaller vacuoles in *Coniothyrium minitans*. Similar outcomes were also observed in *F. graminearum* (Li *et al.* 2022). Consistently, our research revealed that the mutant ΔFvVam6 exhibited abnormal vacuolar morphology and formed smaller vacuoles when compared with the wild-type M3125 *via* microscopic examination and TEM analysis. Since fusion and division of vacuoles were vital for maintaining normal cell viability (Richards *et al.* 2012; Shimada *et al.* 2018), we postulated that the defects in fungal development and virulence are due to the irregular vacuolar morphology.

In addition to the basic functions mentioned above, vacuoles perform several unique and conditional functions under specific nutrient conditions or at different developmental stages. These specific functions including the biosynthesis of alkaloids and flavonoids in plants (Tanaka *et al.* 2008; Ziegler

and Facchini 2008), the production of various secondary metabolites in filamentous fungi (Lendenfeld *et al.* 1993; Hoppert *et al.* 2001; Hong and Linz 2008; Chanda *et al.* 2009). Intriguingly, our recent studies showed that three key fumonisin biosynthesis enzymes co-localized with vacuoles under fumonisin-inducing conditions, and the integrity of vacuolar structure was essential for FB_1 production (Yan *et al.* 2023). In the current study, we investigated the impacts of *FvVam6* deletion on fumonisin biosynthesis genes expression and FB_1 production. The results revealed that the deletion of *FvVam6* significantly repressed transcription of *Fum1*, *Fum6*, *Fum8* and *Fum21*, thus led to the decreased FB_1 production. These findings suggested that *FvVam6* is important to fumonisin biosynthesis in *F. verticillioides*.

As the core subunit of vCLAMP complex, Vps39 regulates mitochondrial morphology and functions in several microorganisms. In *C. albicans*, the deletion of *CaVam6* resulted in fragmented mitochondria, thereby leading to mitochondrial dysfunctions including mtDNA stability, ATP production and mitochondrial membrane potential (Mao *et al.* 2021). Similar results were obtained in *Staphylococcus aureus* (Qu *et al.* 2019). However, no significant differences were detected between ΔFvVam6 and M3125 on mitochondrial morphology, ATP biosynthesis and H_2O_2 production. Therefore, we hypothesize that FvVam6 holds distinct roles in regulating mitochondrial structure and functions in *F. verticillioides* compared with its homologues in other microorganisms. A recent publiction found that there were two functionally distinct vacuole-mitochondria contact sites in *S. cerevisiae*, one was composed of Vps39, Ypt7 and Tom40, while another consists the linker Vps13, mitochondrial receptor Mcp1 and an unknown vacuole membrane protein (Montoro *et al.* 2018). Therefore, we speculated that Vps13 might invloved in the regulation of mitochondrial structure and functions in *F. verticillioides*.

Protein sequence analysis indicated that FvVam6 contains a CNH domain, which has been found in many eukaryotic proteins. A previous study reported that the CNH domain of Rom2 protein works as a stabilizer for the small GTPase Rho1, thereby affecting glucan biosynthesis and regulating the cell wall integrity signaling pathway in *A. fumigatus*, one of the most dangerous human pathogenic fungi (Bartual *et al.* 2021). Moreover, CNH domain of Vps39 protein was found critical for lysosome clustering and fusion in human cells (Caplan *et al.* 2001). In the current study, both abnormal as well as circular vacuoles were observed in *F. verticillioides* hyphae, indicated that the deletion of CNH domain in FvVam6 disrupts vacuoles fusion and division of vacuoles partially. Subsequent study showed that FvVam6$^{\Delta CNH}$ mutant exhibited a slightly reduction in FB_1 production when compared to the wild-type M3125. Therefore, we hypothesized that a proportion of large and circular vacuoles in fungal cells are sufficient to support fumonisins biosynthesis in *F. verticillioides*.

Clathrin participates in a diverse range of cellular functions and is well known for its contribution to clathrin-mediated endocytosis (Shafaq-Zadah *et al.* 2020; Wood and Smith 2021; Smith and Smith 2022). In current study, a clathrin protein domain was found in FvVam6. The results showed that the mutant FvVam6$^{\Delta clathrin}$ exhibited obvious defects in fungal development processes, virulence and fumonisin biosynthesis. However, both of FvVam6 protein and its clathrin domain were not involved in endocytosis, suggesting that functional difference between clathrin domain of FvVam6 and those classic clathrin proteins. An earlier publication by Hu *et al.* (2021) showed that two Vps39 domain-containing proteins Vam6 and Vps3 were critical for vacuolar morphology and virulence in human fungal pathogen *Cryptococcus neoformans*. In addition, Fan and Liu (2021) reported that two Vps39 domains exist in a Vam6-like protein Vlp1 in *C. neoformans*, and the deletion mutant ΔCnvlp1 exhibited proliferation and viability defects in host tissues, which further contributes to attenuated pathogenicity. In this study, we showed that Vps39-1 and Vps39-2 domains of FvVam6 are critical for fungal development, vacuole morphology and FB_1 biosynthesis. Furthermore, the defects were more severe in FvVam6$^{\Delta Vps39-2}$ when compared to FvVam6$^{\Delta Vps39-1}$. Therefore, we concluded that 2 Vps39 domains are important for FvVam6 functions, while Vps39-2 is the dominant domain that affects FvVam6 function in FB_1 biosynthesis.

5. Conclusion

In this study, we identified ScVps39 homologue FvVam6 in *F. verticillioides*. We determined that *FvVam6* is involved in fungal development, stresses responses, virulence, vacuolar morphology and fumonisin biosynthesis. Importantly, the clathrin and Vps39-2 domains were the major domain that affected FvVam6 functions in virulence and fumonisin biosynthesis. Taken together, our findings help advance the current understanding of the regulatory mechanism in fumonisin biosynthesis, and provided theoretical molecular target for the management of fumonisin contamination in agricultural commodities.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2024.05.023

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