Coordinated and Distinct Functions of Velvet Proteins in *Fusarium verticillioides*

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Velvet-domain-containing proteins are broadly distributed within the fungal kingdom. In the corn pathogen *Fusarium verticillioides*, previous studies showed that the velvet protein *F. verticillioides* VE1 (FvVE1) is critical for morphological development, colony hydrophobicity, toxin production, and pathogenicity. In this study, tandem affinity purification of FvVE1 revealed that FvVE1 can form a complex with the velvet proteins *F. verticillioides* VelB (FvVelB) and FvVelC. Phenotypic characterization of gene knockout mutants showed that, as in the case of FvVE1, FvVelB regulated conidial size, hyphal hydrophobicity, fumonisin production, and oxidant resistance, while FvVelC was dispensable for these biological processes. Comparative transcriptional analysis of eight genes involved in the ROS (reactive oxygen species) removal system revealed that both FvVE1 and FvVelB positively regulated the transcription of a catalase-encoding gene, *F. verticillioides* CAT2 (FvCAT2). Deletion of FvCAT2 resulted in reduced oxidant resistance, providing further explanation of the regulation of oxidant resistance by velvet proteins in the fungal kingdom.

The filamentous fungus *Fusarium verticillioides* (synonym *Fusarium moniliforme*, teleomorph *Gibberella moniliformis*, synonym *Gibberella fujikuroi* mating population A) is one of the most common corn pathogens. Infection by this fungus can cause stalk and ear rot on corn (1). This fungus can produce many toxic metabolites, such as fumonisins, fusarins, and bikaverin. Fumonisins, including FB1 (fumonisin B1, the most abundant fumonisin in corn), FB2, FB3, and FB4, are among the most important mycotoxins in grains and are able to inhibit ceramide synthase (2, 3). Fumonisins can cause neural tube and craniofacial defects in mice embryo culture (4). A gene cluster comprised of 17 genes is involved in fumonisin biosynthesis in *F. verticillioides* (5–7). Among these genes, disruption of FUM1, FUM6, and FUM8 abolishes fumonisin production (5, 7).

A velvet-domain-containing protein, *F. verticillioides* VE1 (FvVE1), regulates the biosynthesis of fumonisins in *F. verticillioides* (8). FvVE1 is the orthologue of *Aspergillus nidulans* VeA, the first characterized velvet-domain-containing protein (9). VeA contains one velvet domain, one nuclear localization signal sequence (NLS) at the N terminus, one nuclear export signal (NES) at the end of the velvet domain, and one PEST domain at the C terminus (10, 11). Velvet-domain-containing proteins are widely conserved in fungal species, particularly in ascomycetes (10, 12, 13). In addition to VeA, filamentous fungi also possess other velvet-domain-containing proteins, including VelB, VelC, and VosA. VelB in *A. nidulans* has one noncontinuous velvet domain, in which an insertion of 99 amino acids was found, but has no nuclear localization signal sequence. It shuttles between the cytoplasm and nucleus, dependent on VeA (14). VelC in *A. nidulans* contains a single velvet domain at its C terminus. VosA in *A. nidulans* contains a velvet domain and TAD (transcription activation domain) with a NLS between them (15–17). Recently, a DNA binding motif was found in the velvet domain of VosA (17). All of these velvet-domain-containing proteins form the velvet family (10, 18, 19). Previous studies demonstrated that VelB can interact with VeA and VosA (14, 16), and VelC can interact with VosA in *A. nidulans* (20). Unlike the case with *A. nidulans*, VosA is not present in *Fusarium oxysporum*, in which all three velvet proteins, VeA, VelB, and VelC, can interact with each other (21). These studies indicate that the composition of velvet protein complex varies among fungi. Further investigation of velvet protein complexes in other fungi will be helpful in fully understanding the composition, function, and evolution of velvet protein complexes in the fungal kingdom.

Velvet proteins are involved in many important biological processes. First, some velvet proteins in pathogenic fungi have been found to be critical for virulence. Deletion of VeA orthologue-encoding genes reduced the virulence of *F. verticillioides* in corn and the virulence of *Fusarium graminearum* in wheat (8, 22). Additionally, the absence of VeA1 in *Histoplasma capsulatum* and deletion of vea and velB orthologues in *F. oxysporum* also decreased virulence in immunosuppressed mice (21, 23). Second, velvet proteins regulate sexual development and secondary metabolism. Deletion of vea orthologue completely impaired the formation of sexual fruiting bodies or resistant structures and the production of mycotoxins in *A. nidulans*, *Aspergillus flavus*, *Aspergillus parasiticus*, *F. verticillioides*, *Fusarium fujikuroi*, *Penicillium chrysogenum*, and *Cochliobolus heterostrophus* (24–31). Similar functions of VelB were also found in *F. oxysporum* (21). Third, VeA orthologues in *Fusarium* spp. are also critical for controlling conidial size and hyphal hydrophobicity (21, 22, 28), and the similar functions were also found in VelB in *F. oxysporum* (21).
Fourth, velvet proteins are required for oxidant resistance in A. nidulans. Deletion of velvet-protein-coding genes, including veA, velB, and vosA, made conidia hypersensitive to H₂O₂ in A. nidulans (16). The velB deletion mutants and vosA deletion mutants were more sensitive to H₂O₂ than veA mutants in A. nidulans (16). The regulatory mechanism of velvet proteins in oxidant resistance has been investigated for Cochliobolus heterostrophus, which demonstrated that the VeA orthologue ChVe1 positively regulates expression of the catalase-encoding gene C. heterostrophus CAT3 (ChCAT3) but not other catalase-encoding genes, including ChCAT1 and ChCAT2 (30).

In this study, we found, using a tandem-affinity-purification-based approach, that FvVe1, the VelB orthologue F. verticillioides VelB (FvVelB), and the VelC orthologue F. verticillioides VelC (FvVelC) form a complex in F. verticillioides. By phenotypic analysis of deletion mutants and transcriptional analysis, we determined the roles of the FvVelB and FvVelC proteins in the biosynthesis of fumonisin, conidial size control, and hyphal hydropathy. Furthermore, we revealed an association between velvet proteins and the oxidative stress response in F. verticillioides and showed a possible regulatory mechanism of velvet proteins for oxidative stress resistance.

**MATERIALS AND METHODS**

**Strains and media.** All *F. verticillioides* strains used in this study are listed in Table 1. YPG medium (0.3% yeast extract, 1% peptone, and 2% glucose), PDA medium (20% potato, 2% glucose, and 1.5% agar), and YPGA medium (0.3% yeast extract, 1% peptone, 2% glucose, and 1.5% agar) were used for characterization of vegetative growth and asexual development. The regeneration medium (yeast extract, 0.1%; casein hydrolysate, 0.1%; sucrose, 0.8 M; agar, 1.6%) was used for protoplast transformation. All cultures were grown at 28°C.

**Target gene knockout.** Target gene replacement was performed as detailed in Fig. S1 to S4 in the supplemental material, using the split-marker approach and homologous gene replacement method (32, 33). The gene replacement cassette containing *hph* (hygromycin phosphotransferase gene) was introduced into the wild-type strain by protoplast transformation. PCR analyses for *hph* and the target gene were used to confirm the knockout strains (primers are listed in Table S1).

**Construction of complemented strains.** The plasmid pKN, containing the neomycin phosphotransferase gene, which confers G418 resistance, was used to construct the vectors for complementation. A DNA fragment containing the corresponding gene and its native promoter and terminator regions was inserted into the multiple cloning sites of the plasmid. The constructed vectors were then introduced into the deletion mutant by protoplast transformation, and fungal transformants were selected on regeneration medium containing G418 (80 μg/ml).

**Construction of the strain expressing FvVe1-Myc-His.** In order to identify proteins associated with FvVe1 in *F. verticillioides*, in vivo, a plasmid in which a 5x-c-Myc–6XHis coding sequence was added as a tag to the 3’ end of the *F. verticillioides* VE1 (FvVe1) gene (see Fig. S5A in the supplemental material) was constructed and then transformed into the *Fvvel1* deletion mutant. The transformants were verified by Western blotting with anti-c-Myc antibody (see Fig. S5B). A complemented transformant that showed wild-type phenotypes for conidial size and hyphal hydropathy (see Fig. S5C) was chosen for purification of FvVe1-Myc-His.

**Purification of FvVe1-Myc-His proteins.** The Fvvel1 [FvVe1-Myc-His] complemented strain and the wild-type strain (negative control) were cultured for approximately 17 h in constant darkness in YPG liquid medium. The purification procedure followed the method previously reported (34). Fractions containing purified FvVe1-Myc-His proteins were immunoprecipitated by adding 40 μl of anti-c-Myc antibody-attached beads (c-Myc [9E10] agarose conjugate sc-40 AC; Santa Cruz Biotechnology). The precipitates of FvVe1-Myc-His samples were analyzed by SDS-PAGE (4% to 15%), which was subsequently silver stained following the manufacturer’s instructions (ProteoSilver Plus; Sigma). Specific bands were excised and subjected to tryptic digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**HPLC-MS analysis of fumonisins.** Extracts from cultures of the wild type, *Fvvel1*, *FvvelB*, and *FvvelC* deletion mutants, and *FvvelB[FvvelB]* complemented strains grown on cracked corn medium in constant darkness for 11 days were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). Fumonisins were first extracted according to the acetonitrile method described by Rottinghaus et al. (35). HPLC-MS analysis of fumonisins was performed as described previously by Silva et al. (36) with some modifications.

Liquid chromatography separation was performed on an Agilent 1200 Series system (Agilent, USA) using an Agilent Zorbax Extend-C18 1.8-μm, 2.1- by 50-mm column. With a total flow rate of 0.5 ml/min, mobile phase A consisted of water with 0.3% formic acid, and mobile phase B consisted of methanol with 0.5% formic acid. The gradient began with 65% mobile phase B for 4 min, changed to 95% B over 4 min, and maintained a constant level at 95% B for 7 min. The injection volume was set to 10 μl.

Mass spectra were acquired using an Agilent 6520 accurate-mass quadrupole time-of-flight (Q-TOF) MS system equipped with an electrospray ionization (ESI) source. All MS experiments were detected in the positive ionization mode. For Q-TOF/MS conditions, fragmentor and capillary voltages were kept at 280 and 3500 V, respectively. Nitrogen was supplied as the nebulizing and drying gas. The temperature of the drying gas was set at 300°C. The flow rate of the drying gas and the pressure of the nebulizer were 13.0 liters/min and 30 lb/in², respectively. Full-scan spectra were acquired over a scan range of m/z 300 to 800. Fumonisins were identified by comparison to an FB1 standard, as well as previously described molecular weight and fragmentation spectrum (35, 36).

**H₂O₂ and menadione sensitivity test.** Conidia developed on YPGA plates)/mean colony diameter on control plates.

**RNA extraction and qRT-PCR analysis.** Mycelia were harvested and immediately frozen and ground into fine powder in liquid nitrogen. RNA extraction, cDNA synthesis, and quantitative reverse transcription-PCR (qRT-PCR) analysis was performed as described previously (37, 38). Each cDNA sample was analyzed in triplicate, and the average thresh-

**TABLE 1 Strains used in this study**

<table>
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<th>Species</th>
<th>Strain name or description</th>
<th>Genotype</th>
<th>Source</th>
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<td><strong>WT</strong> Wild type</td>
<td>This study</td>
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<td><em>F. verticillioides</em></td>
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<td>Δ<em>fve1::fve1-Smyc-his</em></td>
<td>This study</td>
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<tr>
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<td><strong>FvvelB</strong> mutant</td>
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<td>This study</td>
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<td>This study</td>
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**HPLC-MS analysis of fumonisins.** Extracts from cultures of the wild type, *Fvvel1*, *FvvelB*, and *FvvelC* deletion mutants, and *FvvelB[FvvelB]* complemented strains grown on cracked corn medium in constant darkness for 11 days were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). Fumonisins were first extracted according to the acetonitrile method described by Rottinghaus et al. (35). HPLC-MS analysis of fumonisins was performed as described previously by Silva et al. (36) with some modifications.

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**H₂O₂ and menadione sensitivity test.** Conidia developed on YPGA medium were harvested into distilled water, and the final concentration of conidial suspensions was then adjusted to 10⁷ conidia/ml. Three microliters of conidial suspension was inoculated on YPGA medium with or without 25 μg/ml menadione or 3.27 mM H₂O₂. Colony diameters were recorded after 72 h of incubation at 28°C in the dark. The relative inhibition rate of each strain was calculated as follows: 100 × (mean colony diameter on control plates – mean colony diameter on oxidant-added plates)/mean colony diameter on control plates.

**RNA extraction and qRT-PCR analysis.** Mycelia were harvested and immediately frozen and ground into fine powder in liquid nitrogen. RNA extraction, cDNA synthesis, and quantitative reverse transcription-PCR (qRT-PCR) analysis was performed as described previously (37, 38). Each cDNA sample was analyzed in triplicate, and the average threshold.
old cycle was calculated. Relative expression levels were calculated using the $2^{- \Delta \Delta CT}$ method (39). The results were normalized to the expression level of $\beta$-tubulin. The primer pairs used for the qRT-PCR assay are shown in Table S2 in the supplemental material.

RESULTS

Identification of FvVE1-associated proteins. In order to identify proteins associated with FvVE1 in F. verticillioides in vivo, proteins from the strain expressing 5×c-Myc–6×His-tagged FvVE1 were extracted, enriched by using a nickel column, precipitated by an anti-c-Myc antibody, and isolated by electrophoresis. Three protein bands seen in the FvVE1-Myc-His strain but not in the wild-type strain were cut for protein identification by mass spectrometry. Two independent experiments were carried out, and proteins that were commonly identified in both experiments were chosen for further analysis in this study. These identified proteins included all three velvet proteins encoded by the F. verticillioides genome. Based on previously described phylogenetic analysis (21), these velvet proteins are the VeA orthologue FvVE1 (FVEG_09521), the VeB orthologue FvVelB (FVEG_01498), and the VeC orthologue FvVelC (FVEG_05214) (Fig. 1B). In addition, three other proteins that had not previously been implicated in interactions with velvet family members were also identified. Based on sequence homology, these proteins are likely homologues of CPSF5 (cleavage and polyadenylation specificity factor 5), UreG (urease accessory protein), and yeast Hrp1p. We gave FVEG_02467 the name F. verticillioides Hrp1 (FvHrp1) (Fig. 1B).

Velvet proteins regulate morphogenesis. In order to analyze the function of each individual velvet protein, single gene knock-out mutants for FvVELB and FvVELC were generated by homologous gene replacement with hygromycin as the selective marker. Due to the absence of aerial hyphae, the hydrophobicity of the cell surface was investigated. Water droplets (30 µl each) with or without acid fuchsin were placed on the surfaces of 7-day-old colonies of wild-type, FvVE1, FvVelB, FvVelC, and complemented strains grown on PDA medium. On colonies of wild-type, FvvelB, and FvvelC strains, the water droplets remained on the surface of the mycelium without extending or being absorbed for quite a long time (at least 1 h). In contrast, the strains carrying mutations in FvVE1 and FvVelB displayed a phenotype quite different from those of wild-type and FvVelC strains. The water droplets on the FvvelB strain were immediately spread and absorbed (Fig. 2A). FvVel1 and FvVelB play almost equal roles in balanced production of two different types of conidia, while FvVelC is dispensable in this process.

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together with findings of our previous study (28), demonstrated that, as in the case of FvVE1, FvVelB is also critical for normal morphological development and cell surface hydrophobicity in *F. verticillioides*. Thus, similar to FvVE1, FvVelB is critical in maintaining normal hydrophobicity of cells in *F. verticillioides*.

**Velvet proteins regulate fumonisin biosynthesis.** FvVE1 plays a key role in regulating biosynthesis of fumonisins (8). To determine whether other members of the velvet complex also regulate fumonisin production, extracts from cultures of the wild-type, *Fvve1*, *FvvelB*, *FvvelC*, and *FvvelB* [FvVELB] strains, grown on cracked corn medium in constant darkness for 11 days, were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). As shown in Fig. 3A, all fumonisins, including FB1, FB2, FB3, and FB4, were not detected in the *Fvve1* mutant. In the *FvvelB* mutant, production of FB1, FB2, FB3, and FB4 was reduced by 70.8%, 84.2%, 87.3%, and 93.6%, respectively, compared with that of the wild type. The *FvvelB* [FvVELB] strain reverted to wild-type levels of fumonisin production. In the *FvvelC* mutant, fumonisins were at levels similar to those for the wild type.

We then examined transcriptional levels of *FUM1* and *FUM8* in the *FvvelB*, *FvvelB* [FvVELB], *Fvvel1*, and wild-type strains. As shown in Fig. 3B, gene expression of *FUM1* and *FUM8* was dramatically reduced in the *FvvelB* and *Fvvel1* strains, but the reduction was more severe in the *Fvvel1* strain. Therefore, different components of the velvet complex differentially contribute to fumonisin biosynthesis. As previously shown (8), our data further verified that FvVE1 plays an essential role in the regulation of fumonisin biosynthesis, while FvVelB, although not essential for this process, also positively regulates fumonisin production. FvVelC, however, is dispensable for fumonisin biosynthesis.

**Velvet proteins regulate oxidative stress tolerance.** In *A. nidulans*, VelB and VosA play more important roles than VeA in tolerance against various stresses, including UV and H₂O₂, although all three velvet proteins contribute to tolerance to these stresses (16). Since ROS (reactive oxygen species) play a key role in plant-pathogen interactions, we examined the sensitivities of each velvet protein null mutant to H₂O₂ and menadione (a ROS-inducing chemical, 2-methyl-1,4-naphthoquinone, also known as vitamin K3). As shown in Fig. 4, the *Fvve1* mutant was hypersensitive to H₂O₂ and menadione. On solid medium with 25 μg/ml menadione, the *Fvve1* mutant could not form colonies, while the wild type and the *FvvelB* strain could. No significant difference in growth inhibition between the *FvvelB* mutant and the wild type were observed. On solid medium with 3.27 mM H₂O₂, growth inhibition rates of the *Fvve1* mutant and the *FvvelB* mutant were significantly higher than that of the wild type (Fig. 4A). However, the *FvvelB* mutant was less sensitive to H₂O₂ than the *Fvve1* mutant; the relative growth inhibition rates of the *Fvve1* mutant and the *FvvelB* mutant were 46.3% and 18.7%, respectively (Fig. 4B). All these observations indicate that FvVE1 plays a more important role in oxidative stress resistance than FvVelB and the relative roles of FvVE1 and FvVelB of *F. verticillioides* are different from those of *A. nidulans* in the regulation of oxidative stress tolerance.
The FvvelC mutant displayed wild-type sensitivities to H$_2$O$_2$ and menadione (Fig. 4).

**FvVE1 and FvVELB positively regulate FvCAT2 expression.** To test how FvVE1 and FvVELB regulate antioxidant activity, transcriptional levels of eight genes involved in ROS detoxification during H$_2$O$_2$ treatment were comparatively analyzed in the Fvve1 deletion mutant, the FvvelB deletion mutant, and the wild-type strain by qRT-PCR. These genes included GST (FVEG_07456; a glutathione S-transferase), GLT2 (FVEG_08420; encoding a glutathione transferase), GLRX3 (FVEG_07558; encoding a glutaredoxin), CCP (FVEG_01375; encoding a copper chaperone), putative GST (pu-GST, FVEG_00418), catalase-peroxidase 1 (FVEG_10866), CAT1 (FVEG_05591), and catalase-peroxidase 2 (FVEG_12888).

When mycelium was treated with 1.96 mM H$_2$O$_2$, all 8 genes showed transcriptional increases after 2 h of H$_2$O$_2$ treatment for the wild type, while after 4 h of treatment, their transcriptional levels were reduced relative to those after 2 h of treatment (Fig. 5A). Among these genes, FVEG_12888, which is predicted to encode catalase peroxidase 2, was the only gene showing a significant reduction in transcription upon Fvve1 or FvvelB deletion. Its transcriptional levels were significantly lower in both the Fvve1 deletion mutant and the FvvelB deletion mutant than those in the wild type at 2 h but not at 4 h after treatment. One gene, GLRX3 (FVEG_07558; encoding a glutaredoxin), displayed significantly higher transcriptional levels in the FvvelB deletion mutant, but not in the Fvve1 deletion mutant, than in the wild type at 2 h after H$_2$O$_2$ treatment (Fig. 5A).

In conidia before H$_2$O$_2$ treatment, none of above-described genes whose transcription was tested showed significant differences among the wild-type strain, the Fvve1 deletion mutant, and the FvvelB deletion mutant (Fig. 5B). Transcriptional levels of these genes were dramatically increased after wild-type conidia were incubated in liquid medium with 3.02 mM H$_2$O$_2$ for 0.5 to 2 h. However, at 2 h after H$_2$O$_2$ treatment, transcriptional levels of FVEG_12888 and CAT1 were significantly lower in both the Fvve1 deletion mutant and the FvvelB deletion mutant than in the wild type. Similar to results observed in mycelium, Fvvel deletion has a greater effect on the transcription of FVEG_12888 than FvvelB deletion. Transcription levels of the putative GST gene at both 0.5 h and 2 h and of CCP at 2 h and of CAT1 at 2 h were significantly lower in the Fvve1 deletion mutant, but not in the FvvelB deletion mutant, than the wild type. GLRX3 and GLT2, however, displayed higher transcriptional levels in both the Fvve1 deletion mutant and the FvvelB deletion mutant than in the wild type at 2 h after H$_2$O$_2$ treatment. Increased expression of GLRX3 and GLT2 is likely to complement expression defects in FVEG_12888 and CAT1.
The above-described results with mycelium and conidia indicate that deletion of \textit{Fve1} and \textit{FvvelB} impaired the normal transcription of several ROS-removal-related genes, and transcriptional profiles of affected genes shared some similarity in the \textit{Fve1} deletion mutant and the \textit{FvvelB} deletion mutant, suggesting that the two velvet proteins coordinately activate the same set of genes for ROS detoxification. Overall, \textit{Fve1} deletion had greater influence on the expression of these ROS-removal-related genes, providing an explanation of why the \textit{Fve1} deletion mutant was more sensitive to oxidants than the \textit{FvvelB} deletion mutant.

\textbf{FvCat2 contributes to ROS antioxidant activity and morphological development.} All the above-described data indicate that \textit{FVEG\_12888} is the most important gene regulated by velvet proteins among tested genes involved in antioxidant activity. Phylogenetic analysis (see Fig. S6 in the supplemental material) showed that \textit{FVEG\_12888} is the orthologue of \textit{Neurospora crassa} \textit{CAT-2} (40) and \textit{A. fumigatus} CAT2 (41, 42), and thus it was named FvCat2 in this study.

To gain further insight into the function of FvCat2, a null mutant of FvCat2 was generated (see Fig. S4 in the supplemental material). \textit{H}_{2}\textit{O}_{2} and menadione sensitivities between the wild type and the Fvcat2 deletion mutant were compared. On normal medium, the growth rate of the deletion mutant was similar to that of the wild type (Fig. 6A and B). However, when the strains were grown on the medium supplemented with \textit{H}_{2}\textit{O}_{2} or menadione, growth of the Fvcat2 mutant displayed a higher level of inhibition than that of the wild type (Fig. 6A and B). The \textit{N. crassa} gene knockout mutant for the FvCat2 orthologue CAT-2 also displayed increased sensitivity to \textit{H}_{2}\textit{O}_{2} or menadione compared with that of the wild-type \textit{N. crassa} strain (Fig. 6A and B), suggesting the general contribution of CAT-2 to oxidant resistance in filamentous ascomycetes.

The Fvcat2 mutant also exhibited several morphological defects, including reduced conidial production and increased conidial size relative to those of the wild type (Fig. 6D and E). The hydrophobicity of the hyphal surface was also slightly reduced compared with that of the wild type (Fig. 6C). Fumonisins were present at levels similar to those of the wild type (Fig. 6F).

All mutant phenotypes were restored in the complemented strains, confirming that the above phenotypes in the Fvcat2 mutant were caused by loss of the Fv\textit{CAT2} gene. These data suggest that Fv\textit{VE1}-dependent expression of Fv\textit{Cat2} plays a role in maintaining normal oxidant resistance, conidial development, and cell hydrophobicity but has no influence on fumonisin production.

\textbf{DISCUSSION} The velvet proteins are widely distributed in filamentous fungi. In \textit{A. nidulans}, VeA can interact with VelB (14), VelB can interact with VosA (16), and VosA can interact with VelC (20). Yeast two-hybrid experiments demonstrated that all velvet proteins, including VeA, VelB, and VelC, can interact with each other in \textit{Fusarium oxysporum} (21). In consistence with the results in \textit{F. oxysporum} (21), here we demonstrated that Fv\textit{VE1} (the VeA orthologue) can form a complex with other velvet proteins, Fv\textit{VelB}, and Fv\textit{VelC}, in \textit{F. verticillioides}. These phenomena, together with the fact that the \textit{Fusarium} genus lacks VosA (21), indicate that the composition of velvet proteins and the interaction among velvet proteins in the \textit{Fusarium} genus are different from those in the \textit{Aspergillus} genus. Furthermore, this study identified several new proteins that possibly interact with velvet proteins, including CPSFS (cleavage and polyadenylation specificity factor 5), UreG (urease accessory protein), and FvHrp1. The yeast FvHrp1 homologue Hrp1p is a sequence-specific RNA-binding protein required for mRNA 3’-end formation. Hrp1p shuttles between the nucleus and the cytoplasm.
(43, 44). However, this is the first time c-Myc has been used as a tag to isolate velvet proteins; whether the c-Myc tag has interaction with CPSF5, UreG, and FvHrp1 is unknown. Their interactions with velvet proteins still remain to be confirmed by other methods. Both CPSF5 and FvHrp1 are involved in RNA processing (43, 45–49); if they interact with velvet proteins, the velvet protein complex might also be involved in posttranscriptional regulation.

This study also analyzed the relative contributions of FvVelB and FvVelC to morphological development, fumonisin biosynthesis, and oxidant resistance and compared their roles with those of the previous characterized FvVE1 (8, 26). Our results demonstrated that FvVE1 and FvVelB play similar roles in controlling balanced production of microconidia and macroconidia and in maintaining cell hydrophobicity. For fumonisin production, FvVE1 is essential. FvVelB is also important, but its absence did not completely abolish fumonisin production. Thus, its importance is less than that of FvVE1. In oxidant resistance, FvVE1 plays a more important role among velvet proteins than in other biological processes. All these facts indicate that the relative roles of VeA and VelB vary among different biological processes. It is possible that the velvet proteins might be able to form different protein complexes to fulfill different functions. In regulation of secondary metabolism, conidial development, and hyphal hydrophobicity, a complex composed of at least FvVE1 and FvVelB might be required. Regarding the oxidative stress response, FvVE1 alone or FvVE1 with proteins other than FvVelB or FvVelC might be sufficient. In addition, this study reveals the difference between Aspergillus and Fusarium in the relative contributions of VeA and VelB to oxidant resistance, since the contribution of VeA to oxidative stress resistance is lower than that of VelB in A. nidulans (16). VosA is required for trehalose accumulation in conidia and positively contributes to resistance to H2O2 and ultraviolet irradiation in A. nidulans (16). Since the transportation of VelB into nuclei depends on the formation of heterodimers with other velvet proteins with the nuclear localization signal (14, 16), the absence of VosA might make VeA more important in Fusarium than in Aspergillus. Although VelC negatively regulates conidial production in A. nidulans (20), similar to our observation for F. verticillioides, deletion of VelC caused less phenotypic alteration than that of VeA and VelB in A. nidulans and Aspergillus flavus (16, 18), indicating that VelC is less important than other velvet proteins.

Our study together with previous observations for C. heterosporus (30) provides a possible mechanism to explain how velvet proteins regulate oxidative stress resistance. The mechanisms of adaptation to oxidative stress are complicated in fungi. Several proteins with regulatory roles in oxidant resistance have been identified, but linkages among these regulatory proteins are not understood. Yap1p, a mammalian AP-1-like bZIP-type transcription factor in Saccharomyces cerevisiae, is the best-known regulator of oxidative stress resistance. Deletion of YAP1 or its orthologues increases sensitivity to oxidants in S. cerevisiae and other fungal species (50–53). Three new transcription factors, SRE, MIT-2, and MIT-4, were recently found to be important for resistance to menadione in N. crassa (54), but the mechanism by which they confer resistance is still unknown. In addition to these transcription factors, a mitogen-activated protein kinase (MAPK) pathway,
which is composed of Sty1 (MAPK), Wis1 (MAPKK), and Wis4 (MAPKKK) in Schizosaccharomyces pombe, is also implicated in the regulation of oxidative stress responses (55, 56). The Sty1 homologue SakA in Aspergillus fumigatus and the Sty1 homologue OS-2 and Wis1 homologue RRG-2 in velvet proteins. OS-2 and Wis1 homologue RRG-2 in velvet proteins.

In this study, we found that FvVE1 and FvVelB were the major cause of changes in conidial size and hydrophobicity in the Fvcat2 mutant or the Fvcat2 Knockout mutant (Nc-cat-2) of incubation at 28°C in darkness. (A) Oxidant sensitivity test. Conidia were inoculated onto YPGA medium with or without 3.27 mM H2O2 or 25 μg/ml menadione. Images were captured after 72 h (for F. verticillioides), 10 h (for the N. crassa wild type), or 16 h (for the N. crassa mutant) of incubation at 28°C in darkness. (B) Relative inhibition of strain colony growth by oxidants. Values for three replicates were used for statistical analysis. Standard deviations are marked with bars. (C) Hydrophobicity test. Conidia were inoculated onto PDA plates and cultured for 6 days. Thirty microliters of 1% acid fuchsin or water was loaded onto the surfaces of colony. (D) Comparison of conidial size. Micrographs of conidia produced by indicated strains grown on PDA plates are shown. Images were taken by a Zeiss Imager camera. Bar, 50 μm. (E) Quantification of conidia. Values are the mean numbers of conidia per cm². (F) HPLC-MS chromatogram of fumonisin extracts of indicated strains (WT, wild-type strain). RT, retention time (min).

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