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FvVE1 Regulates Biosynthesis of Fumonisins and Fusarins in *Fusarium verticillioides*

KYUNG MYUNG[†], **SHAOJIE LI**[†], **ROBERT A.E. BUTCHKO**[‡], **MARK BUSMAN**[‡], **ROBERT H PROCTOR**[‡], **HAMED K. ABBAS**[§], and **ANA M. CALVO**[†,*]

†*Department of Biological Sciences, Northern Illinois University, 1425 W. Lincoln Hwy., Dekalb, Illinois 60115. USA*

‡*Mycotoxin Research Unit, US Department of Agriculture-ARS, 1815 N. University Street, Peoria, Illinois 61604, USA*

§*Crop Genetics and Production Research Unit, US Department of Agriculture-ARS, CG&PRU, P.O. Box 345 Stoneville, Mississippi 38776, USA.*

Abstract

The *veA* gene positively regulates sterigmatocystin production in *Aspergillus nidulans* and aflatoxin production in *A. parasiticus* and *A. flavus*. Whether *veA* homologs have a role in regulating secondary metabolism in other fungal genera is unknown. In this study, we examined the role of the *veA* homolog, *FvVE1*, on production of two mycotoxin families, fumonisins and fusarins, in the important corn pathogen *F. verticillioides*. We found that *FvVE1* deletion completely suppressed fumonisin production on two natural substrates, corn and rice. Furthermore, our results revealed that *FvVE1* is necessary for the expression of the pathway-specific regulatory gene *FUM21* and structural genes in the fumonisin biosynthetic gene (*FUM*) cluster. *FvVE1* deletion also blocked production of fusarins. The effects of *FvVE1* deletion on the production of these toxins were found to be the same in two separate mating types. Our results strongly suggest that *FvVE1* play an important role in regulating mycotoxin production in *F. verticillioides*.

Keywords

Fusarium verticillioides; fumonisin; fusarins; *FvVE1*; *veA*; secondary metabolism

INTRODUCTION

The filamentous fungus *Fusarium verticillioides* (syn. *F. moniliforme*, teleomorph *Gibberella moniliformis*) is one of the most common causes of corn ear rot worldwide and can produce multiple families of mycotoxins (1,2). Consequently, *F. verticillioides* mycotoxins are commonly detected in corn (maize, *Zea mays* L.) and often contaminate corn-based human food and animal feed (1,3). Fumonisins are currently considered the most agriculturally significant *F. verticillioides* mycotoxins because they can cause several animal diseases and are epidemiologically associated with some human diseases (4,5). Fumonisins are polyketide-derived metabolites that can inhibit ceramide synthase, a key enzyme in sphingolipid metabolism, and induce apoptosis (1,5). Fumonisin B_1 (FB_1) is typically the most abundant fumonisin in contaminated corn and accounts for approximately 70% of the total fumonisin

*Author to whom correspondence should be addressed [telephone: (815) 753−0451; fax (815) 753−0461; e-mail E-mail: amcalvo@niu.edu].

content. Fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) are also common in corn but typically comprise 10−20% of the total fumonisin content (1).

In *F. verticillioides*, fumonisin biosynthetic genes (*FUM*) are clustered. The cluster consists of seventeen genes, designated as *FUM1* through *FUM3* and *FUM6* through *FUM21* (6,7,8). Disruption of *FUM1*, *FUM6*, and *FUM8* has been shown to abolish fumonisin production (7, 8). In many cases, genes responsible for the synthesis of fungal secondary metabolites such as sterigmatocystin, gibberellins, aurofusarin, trichothecenes, and lovastatin, are also found clustered and specific regulatory genes are located within these gene clusters (9-11). *FUM21*, a predicted a Zn(II)2Cys6 DNA-binding transcription factor located in the *FUM* cluster, positively regulates *FUM* gene expression and is required for fumonisin synthesis (12). Nevertheless, the regulatory mechanism controlling fumonisin biosynthesis is poorly understood. Among the genes involved in fumonisin gene regulation are *FCC1*, *PAC1*, and *ZFR1* (13-15). *FCC1* encodes a cyclin-like protein (Fcc1) that positively regulates fumonisin biosynthesis and conidiation (13) and interacts with *FCK1*, a cyclin-dependent kinase (Fck1) (16). *PAC1* is required for growth at alkaline pH and may act as a repressor of fumonisin biosynthesis (14). *ZFR1* encodes a zinc binuclear cluster-type protein (Zfr1) which functions as a positive regulator of fumonisin biosynthesis (15). Studies by Flaherty and Woloshuk (15) indicated that Fcc1 is required for Zfr1 function. On the other hand, Pac1 and Fcc1 seem to act independently of each other in regulating fumonisin biosynthesis (15).

In addition to fumonisins, *F verticillioides* produces other mycotoxins. Among them are the polyketide compounds fusarins (17-19). Fusarins have been reported to induce mutagenesis in mammalian cells *in vitro* (17) and to cause immunosupression (18). Although a polyketide synthase gene required for fusarin biosynthesis has been identified in several *Fusarium* species (20,21), nothing is known about how fusarin biosynthesis is regulated.

In *Aspergillus* spp., the velvet gene (*veA*) regulates the biosynthesis of several secondary metabolites, including the polyketide toxins sterigmatocystin and aflatoxin (22-24). Whether *veA* homologs have a similar role in regulation of toxin production in other fungal genera has not been investigated. Previously, we identified *FvVE1*, a *veA* homolog in *F. verticillioides*, and demonstrated that it functions in regulation of morphogenesis (25). In this study, we investigate the role of *FvVE1* in secondary metabolism in *F. verticillioides*, specifically in the biosynthesis of fumonisin and fusarins. Our results suggest that *FvVE1* regulates biosynthesis of both fumonisin and fusarins in this important plant pathogenic fungus.

MATERIALS AND METHODS

Strains and media

The strains used in this study are: M-3125 (*MAT1−1*, *FvVE1*); M-3120 (*MAT1−2*, *FvVE1*); M312501 (*MAT1−1*, Δ*Fvve1::HygB*); M31206 (*MAT1−2*, Δ*Fvve1::HygB*); M312501C1 (*MAT1−1*, Δ*Fvve1::HygB*, *FvVE1::GenR*); M31206C5 (*MAT1−2*, Δ*Fvve1::HygB*, *FvVE1::GenR*). *MAT1−1* and *MAT1−2* are the two different mating type idiomorphs (alleles) in *F. verticillioides*. M-3120 and M-3125 are strain designations from the Fusarium Research Center culture collection (Pennsylvania State University, University Park, PA). The *FvVE1* deletion strains and complementation strains were generated in both mating types as described by Li *et al* (25). In brief, the Δ*Fvve1* mutant strains were generated by gene replacement via double homologous recombination events using the hygromycin B resistance gene (*HygB*) as selectable marker (25). Complementation strains were obtained by transformation of the Δ*Fvve1* mutants with wild-type *FvVE1* allele using the geneticin resistant gene, *GenR*, as a selectable marker (25).

V8 agar medium (10% V8 juice, 0.1% $CaCO_3$, and 1.5% agar) was used for production of conidia. Corn medium and rice medium were prepared as previously described (26,27) with some modifications. In this study we mixed 25 g of corn kernels and 40 mL distilled water in 250 mL flasks and 50 g of long-grain rice and 60 mL distilled water in 250 mL flasks. For RNA experiments, cracked corn kernel cultures were prepared by thoroughly mixing 250 g cracked corn kernels and 100 mL water and autoclaving. After cooling, the moistened kernels were combined with 25 ml of a suspension of *F. verticillioides* conidia (1×10^7 conidia per ml water) prepared from seven-day-old, V8 agar cultures of the fungus. This mixture was then distributed among eight 100- mm plastic Petri plates and incubated in the dark at 22 °C.

RNA Preparation and Northern Blots

At 36, 48 and 72 hours of incubation, 10 g of cracked corn culture was frozen in liquid nitrogen, placed at −80 °C until the nitrogen evaporated, and then lyophilized. Total RNA was isolated from the lyophilized material with TRIzol (Invitrogen Life Technologies, Carlsbad, California) using the protocol for samples with high polysaccharide content as described by the manufacturer. For Northern blot analysis, 5 μg of total RNA for each sample was subjected to electrophoresis in a 1.3% agarose gel containing 1.8% formaldehyde and then transferred to nylon membrane following standard protocols (28). ^{32}P-labaled hybridization probes were prepared with the Ready-to Go DNA labeling kit (Amersham Biosciences, Little Chalfont, Buckinghamshire), and the hybridization, wash, and autoradiography procedures followed standard protocols (28). Templates for hybridization probes corresponding to *FUM1*, *FUM8*, and *TEF1* were prepared by PCR that employed genomic DNA from wild-type *F. verticillioides* strain M-3120 and following primer pairs: The primers used to amplify DNA templates for Northern blot hybridization probes were: for *FUM,1* rp405 (5'-TGGGACACAGTTCTCAAGGAGA-3') and rp408 (5'-CAAGCTCCTGTGACAGAGATAC-3'); for *FUM8*, rp679 (5'-CGTAGTAGGAATGAGAAGGATG-3') and rp680 (5'-GCAAGCTTTGTGGCTGATTGTC-3'); and for *TEF1*, rp992 (5'-ATGGGTAAGGARGACAAGAC-3') and rp993 (5'-GGARGTACCAGTSATCATGTT-3'). *TEF1*, encoding the transcription elongation factor 1α, was used as loading control.

Reverse transcription PCR

Total RNA was treated with Turbo DNA-free DNase (Applied Biosystems, Carlsbad, California) following manufacturers recommended protocol. RNA from DNase treated samples was quantified on a Nanodrop spectrophotometer (Thermo Scientific, Delaware) and diluted to a concentration of 40 ng/μL. A total of 60 ng RNA was used per reverse transcription (RT) PCR reaction. RT-PCR was accomplished with the Easy-A One-tube RT-PCR System (Stratagene, La Jolla, California) following manufacturers recommended protocol. Primers used are: for *TEF1*, rb291 (5'-ATGGGTAAGGAGGACAAGAC-3') and rb292 (5'-GGAAGTACCAGTGATCATGTT-3'); for *FUM21*, rb373 (5'-TAAATGCGAGACAGCATTTGCGGG-3') and rb374 (5'-TGCATCTTGCCCTACTCAATCGGA-3'); for *FUM8*, rb379 (5'-TCCATGTTTACGGGCGCATTTGTC-3') and rb380 (5'-TCGTGAAACCTAGACGCTTGCTGA-3'); for *ZFR1*, rb384 (5'-ATCCACGAAGGAGGCATGTTGGTA-3' and rb385 (5'-AGGCGGATACAAAGAACGACAGGT-3'); and for *FCC1*, rb391 (5'-AATGTTTCCGCTTCCGCA-3') and rb394 (5'-TGCCGCTTCTCCTTAGGTTCT-3'). When possible, primers were designed to amplify different size fragments from genomic DNA and cDNA. *TEF1* was used as control reference to indicate amounts of total RNA. Primers for *TEF1* amplify a 771-bp fragment from genomic DNA and a 324-bp fragment from cDNA. *FUM21* primers amplify 920-bp and 707-bp fragments from genomic DNA and cDNA respectively. *FUM8* primers amplify 789-bp and 638-bp fragments from genomic DNA and

cDNA respectively. Primer pairs for *ZFR1* and *FCC1* amplify the same size fragments from genomic DNA and cDNA (566 bp and 725 bp, respectively due to the absence of introns in these genes).

Fumonisin analysis

A plug (1.6 cm diameter) containing mycelia and conidia from a 7-day old V8 agar culture was used as inoculum. In each case, the cultures were mixed twice during the first three days of incubation by shaking for 30 sec to ensure homogenization. Cultures were incubated for a total period of 2 weeks. After that time, the samples were lyophilized and ground to powder. Fumonisin analysis was performed as previously described by Abbas *et al.* (29). Samples were analyzed by liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS). Samples (10 g) from the ground corn and rice cultures were extracted with 50 mL of 70% methanol and filtered through No. 1 Whatman filter paper. An aliquot (10 mL) was applied to a SAX clean-up column (Varian, Harbor City, CA). The sample was reconstituted in 1 mL of acetonitrile:water (1:1) and diluted if necessary. The LC/ESI/MS analysis was performed on a Thermo Finnigan LCQ Advantage, coupled to a Thermo Finnigan Surveyor MS, and a Thermo Finnigan Surveyor MS Pump (Thermo Electron Corp., West Palm Beach, FL). A 10 μL aliquot was injected and each sample was evaluated in full-scan mode, using the appropriate mass ranges, fumonisin B_1; 722 (M+H), fumonisin B_2 and B_3; 706 (M+H), fumonisin B_4; 690 (M+H), fumonisin (FA_1, FA_2, FA_3); 764, 748, 748 (M+H), and fumonisin C_1; 708 (M+H). MS/MS was performed on 722 (M+H) for further confirmation of FB_1. The column used for fumonisin analysis was a 3.0 mm × 150 mm i.d, 5 μm, Intersil ODS-3 column (MetaChem Technologies Inc., Torrance, CA). The mobile phase at initial elution starting condition consisted of water: 1% acetic acid in methanol (65:35) at 300 μL per min, followed by water: 1% acetic acid in methanol:methanol (5:35:65) at 10 min. The gradient was held constant for 10 min, and returned to the initial starting conditions for 4 min for column equilibration. Quantitation of FB_1, FB_2, and FB_3 was carried out by the external standard method where FB_4 was calculated as a percentage of FB_1. Other derivatives of fumonisin were monitored for qualitative purposes only.

Fumonisin and fusarins analysis was also carried out on crack-corn cultures used for RNA analysis to further investigate the correlation between fumonisin production and gene expression levels. Ten grams of cracked corn kernel culture were extracted in 25 ml acetonitrile:water (1:1, v/v) on a rotary shaker set at 250 rpm. After 2.5 hours of shaking the mixture was centrifuged at 500 *g* for 5 minutes and the supernatant was recovered for analysis by reversed phase liquid chromatography-mass spectrometry (LC-MS) in electrospray mode as previously described (30). Briefly, 10 grams of cracked corn kernel culture were extracted in 25 ml acetonitrile:water (1:1, v/v) on a rotary shaker set at 250 rpm. After 2.5 hours of shaking the mixture was centrifuged at 500 g for 5 minutes and the supernatant was recovered for analysis by reversed phase liquid chromatography-mass spectrometry (LC-MS) in electrospray mode. The LC-MS system consisted of a ThermoFinnigan LCQ Deca mass spectrometer coupled to a ThermoSpectraPhysics HPLC with a C18 column. The column used for fumonisin analysis was the same as the one described above (Intersil ODS-3 column MetaChem Technologies Inc., Torrance, CA). Samples were run on a gradient of 35 to 95% (v/v) methanol over 35 minutes at a flow rate of 0.3 ml per minute. Fumonisins and fusarins were detected by monitoring masses of 240 through 1000. The identities of fumonisins and fusarins were confirmed by retention time and the presence of appropriate [M+H]+ ions. Quantification was accomplished by comparison of the integrated intensity of ions corresponding to fumonisin and fusarin standards (30)

RESULTS and DISCUSSION

Fumonisin production on natural substrates

Fumonisins are currently considered the most economically important mycotoxins produced by the corn pathogen *F. verticillioides* because of their widespread occurrence in corn and their potential health effects on humans and animals (1,2,4,5). Our current studies indicated that fumonisin production is affected in the Δ*Fvve1* mutants. In corn and rice cultures (Figure 1), FB_1 was produced by the wild-type strains as well as by the complemented strains (Figure 2). Trace amounts of FB_2 and FB_3 were also detected in corn and rice cultures of the wild-type and complemented strains. In contrast, neither FB_1, FB_2, nor FB_3 was detected in rice or corn cultures of the Δ*Fvve1* mutants (Figure 2). These results were consistent in both mating-type genetic backgrounds. Our findings reveled that the novel regulatory factor *Fv*Ve1 encoded by the *FvVE1* gene is required for fumonisin production when the fungus grows on the natural substrates corn and rice (Figures 1 and **2**).

Expression of fumonisin biosynthetic genes

Previous studies showed that VeA, the *Fv*Ve1 homolog in *Aspergillus* species, is required for expression of sterigmatocystin/aflatoxin biosynthetic genes and for concomitant production of the toxins (22-24). However, the possible role of VeA homologs in activation of mycotoxin biosynthetic genes in other fungal genera was not known until now. To investigate whether the expression of fumonisin biosynthetic genes is regulated by the *FvVE1* gene, transcription levels of *FUM1* and *FUM8* (essential fumonisin biosynthetic genes) were examined in the wild-type, Δ*Fvve1* mutant and complemented strains grown on cracked-corn medium. The Northern analysis in Figure 3 shows that transcription of *FUM1* was first detected at 48 h after inoculation in both wild-type and complemented strains. *FUM8* expression followed a similar pattern where transcripts started to accumulate slightly earlier in the wild-type strain. However, *FUM1* and *FUM8* transcripts were not detected in the Δ*Fvve1* mutant (Figure 3). The lack of fumonisin biosynthetic gene expression in the *FvVE1* deletion mutant is most likely responsible for the lack of fumonisin production in this strain.

Expression of *FUM21, FCC1* and *ZFR1*

With the goal of further elucidating the mechanism through which *FvVE1* regulates the expression of the *FUM* gene cluster and the concomitant fumonisin production, we investigated whether the expression of *FUM21* was altered by the *FvVE1* deletion. *FUM21* gene encodes a putative Zn(II)2Cys6 DNA-binding transcriptional activator that is likely specific for *FUM* cluster genes (12). Furthermore, a functional *FUM21* is necessary for fumonisin production in *F. verticillioides*. Genes encoding cluster-specific regulatory proteins have been previously found in other secondary metabolism gene clusters. Well known examples of these regulators are *aflR* and *aflJ*, demonstrated to govern the expression of sterigmatocystin/aflatoxin gene clusters in *Aspergillus* spp. (31-33). We have previously shown that *veA* is necessary for the expression of *aflR* and *aflJ* in *Aspergillus* (22-24). In the present study, in order to investigate whether *FvVE1* plays a role in regulating the expression of *FUM21* we chose RT-PCR analysis because of its ability to detect transcripts that are present at low levels. *FUM21* transcripts were absent in the Δ*Fvve1* mutant cultures under conditions that allow expression of this gene in the wild type and complementation strains (Figure 4). *FUM8* was also included in the RT-PCR analysis as an internal control for comparison of the RT-PCR and Northern experiments, which yielded essentially the same results. The absence of *FUM21* transcripts in the Δ*Fvve1* mutant indicates that a functional *FvVE1* is necessary for *FUM21* expression.

Genes outside the *FUM* cluster can also regulate fumonisin production in *F. verticillioides*. For example, deletion of the C-type cyclin-like gene, *FCC1* , abolished fumonisin production on corn kernels and in a defined medium at high pH (14). To test whether *FvVE1* controls this

regulatory gene, the transcription levels of *FCC1* were examined in wild-type, Δ*Fvve1* mutant and complemented strains grown on cracked-corn medium (Figure 4). Our results showed that *FCC1* expression was very low and only detected in the *FvVE1* deletion mutant at 48 h after inoculation. Although this result differs from those previously reported (13), where *FCC1* expression was detected at higher levels in cracked-corn cultures, under the experimental conditions assayed in our study fumonisin production was detected as well as *FUM1* and *FUM8* expression, indicating that our culture system yielded reliable results. Fumonisin production in the *fcc1* mutant is not blocked in the defined medium at low pH (14). Changes in pH did not rescue fumonisin production in the Δ*Fvve1* mutant (data not shown) as in the case of the *FCC1* mutant (14). *FCC1* is required for function of the Zfr1, a putative Zn(II) 2Cys6 transcription factor postulated to control fumonisin production by regulating genes involved in the perception or uptake of carbohydrates (15, *34*). In our study, *ZFR1* transcripts were detected in the wild type, Δ*Fvve1* mutant and complementation strain, particularly in the Δ*Fvve1* mutant where *ZFR1* transcription occurred earlier and was more abundant than in strains with a functional *FvVE1* (Figure 4). This suggests that *FvVE1* negatively influences *ZFR1* expression. Further studies will focus on elucidating possible interactions between *ZFR1* and *FvVE1*, and whether *FvVE1* has a role in carbohydrate metabolism in *F. verticillioides*.

Cultures utilized for RNA studies were also analyzed for the fumonisins. In agreement with previous experiments (Figures 1 and 2), wild-type and complementation strains produced fumonisins but the Δ*Fvve1* mutant did not (Table 1). Even when incubation time was increased to 144 hours after inoculation of the crack-corn medium, fumonisin were not detected in Δ*Fvve1* cultures.

Fusarin analysis

The VeA homologs in *Aspergillus* regulate not only production of sterigmatocystin and aflatoxin, but also production of other secondary metabolites such as aflatrem, cyclopiazonic acid, and penicillin (22-24). In addition to fumonisins, *F. verticillioides* produces an array of other secondary metabolites, including the mycotoxins fusarins. These toxins have been reported to be mutagenenic as well as immunosuppressive (17-19). In order to evaluate the role of *FvVE1* in fusarin biosynthesis, we examined the production of this compound in the wild-type, *FvVE1* deletion mutant and complementation strains on cracked-corn medium. Fusarins were detected in extracts from cultures of wild-type and complementation strains, but were not detected in extracts of Δ*Fvve1* cultures (Table 1), indicating that *FvVE1* is also necessary for fusarin biosynthesis in *F. verticillioides*. To our knowledge, this is the first report of a gene described to regulate fusarin production in *F. verticillioides*. Interestingly, a recent report by Estrada and Avalos (35) showed that the white-collar gene *wcoA* modulates fusarin production in a light-dependent manner in *F. fujikuroi*. In *Aspergillus nidulans*, VeA forms a nuclear protein complex that includes light-sensing proteins, such as the red phytochrome-like FphA and the white-collar LreA and LreB proteins responsive to blue light (36). In future studies we will investigate if a similar protein complex that includes *Fv*Ve1 also exists in *F. verticillioides*.

As in the case of *veA* regulation of secondary metabolism in *Aspergillus* (22-24), the effect of *FvVE1* on secondary metabolism in *F. verticillioides* could also be broad. The differences in pigmentation observed in the natural substrate cultures (Figure 1, and data not shown) indicate that the synthesis of other unknown metabolites is also regulated by *FvVE1*.

In conclusion, we demonstrated that *F. verticillioides FvVE1* is required for fumonisin and fusarin production on the natural substrates corn and rice. We also showed that *FvVE1* is necessary for the expression of the fumonisin biosynthetic enzyme-encoding genes *FUM1* and *FUM8* as well as the transcription factor gene *FUM21*. This study also revealed that *FvVE1*

affects production of a second family of *F. verticillioides* secondary metabolites, namely fusarins. The consistent blockage of fumonisin on natural substrates, particularly in corn, suggests that *FvVE1* could be a potential target to control fumonisin contamination of food commodities. We are currently exploring this possibility by investigating whether the *FvVE1* deletion affects the ability of *F. verticillioides* to produce fumonisins in living corn plants.

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Abbreviation Used

FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; veA, Velvet gene; FvVE1, *Fusarium verticillioides veA.*

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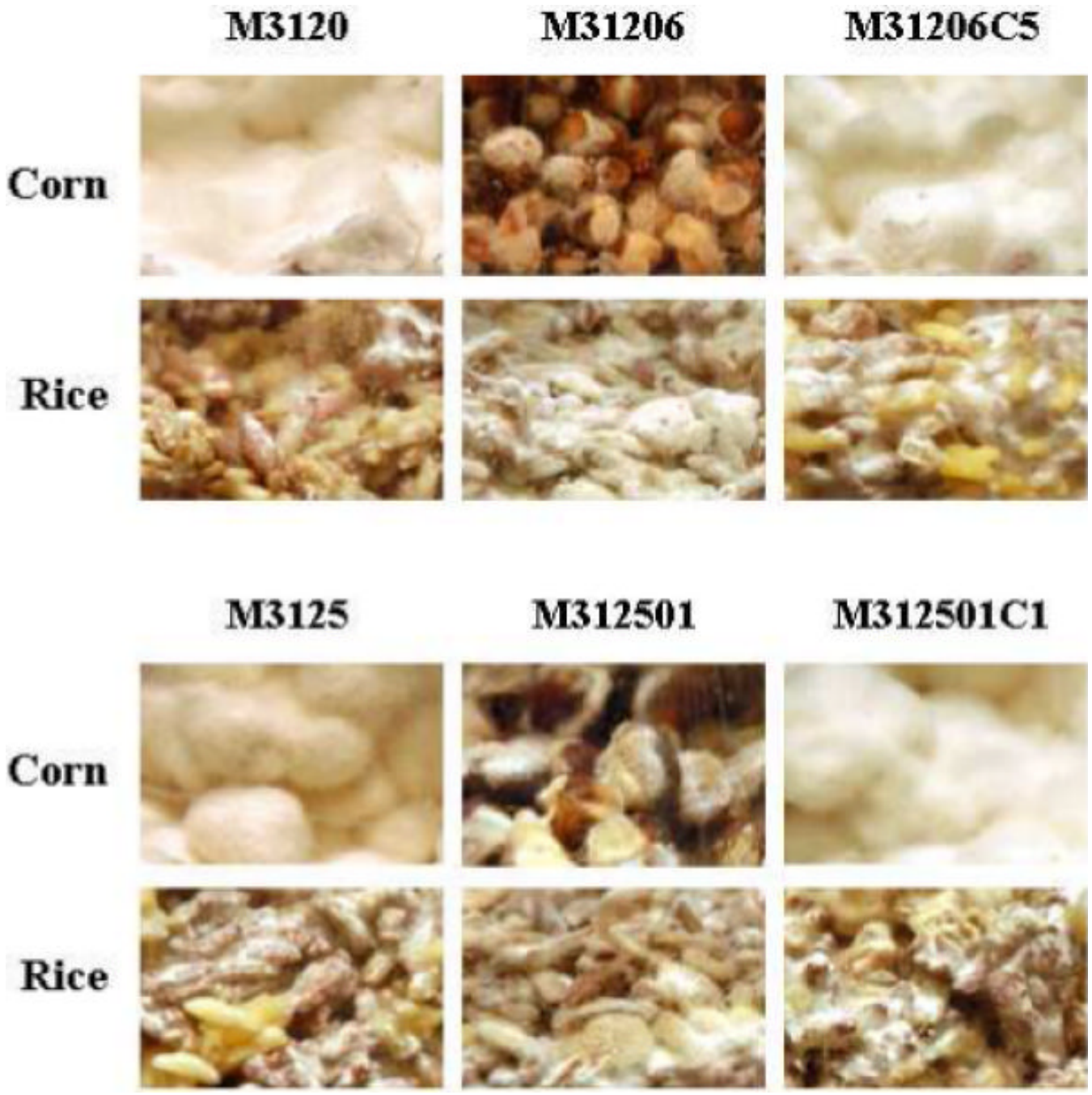

Figure 1.
Photographs of wild-type (WT, M-3120 and M-3125), *FvVE1*deletion mutant (Δ*Fvve1*, M31206 and M312501) and complemented (Com., M31206C5 and M312501C1) strains of *MAT1−1* and *MAT1−2* respectively, in corn and rice cultures.

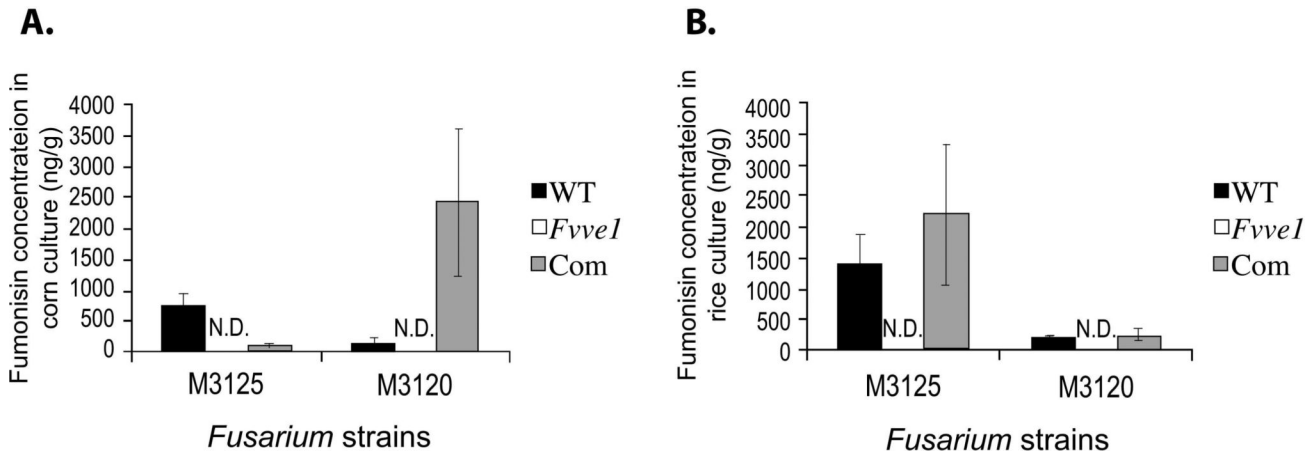

Figure 2.

Production of fumonisins in wild-type (WT), *FvVE1*deletion mutant (Δ*Fvve1*) and complemented (Com) strains of *MAT1−1* and *MAT1−2* in corn (**A**) and rice (**B**) cultures. Total fumonisins (B_1, B_2 and B_3 combined) were analyzed at 2 weeks after inoculation. Bars indicate standard errors of 3 independent cultures. N.D. = not detected.

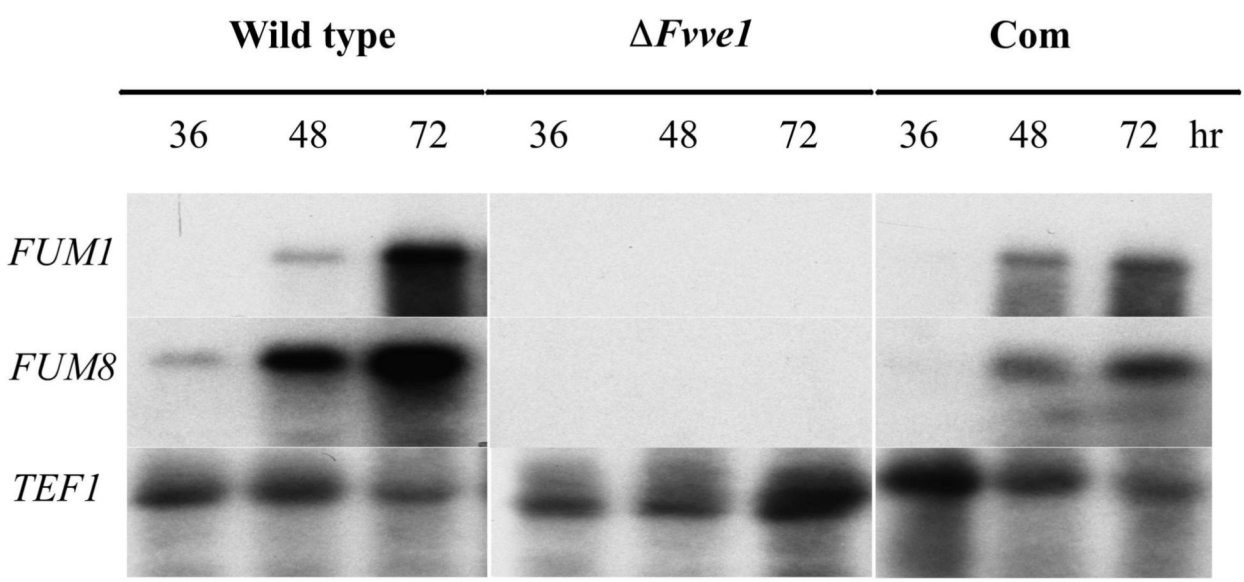

Figure 3.
Northern analysis of *FUM1*, and *FUM 8* gene expression from wild-type M-3120, *FvVE1* deletion mutant M31206 (Δ*Fvve1*), and complementation (Com) strain M31206C5. Total RNA was isolated from mycelial tissue grown on cracked-corn medium at 36, 48 and 72 h after inoculation. *TEF1* was used as loading control.

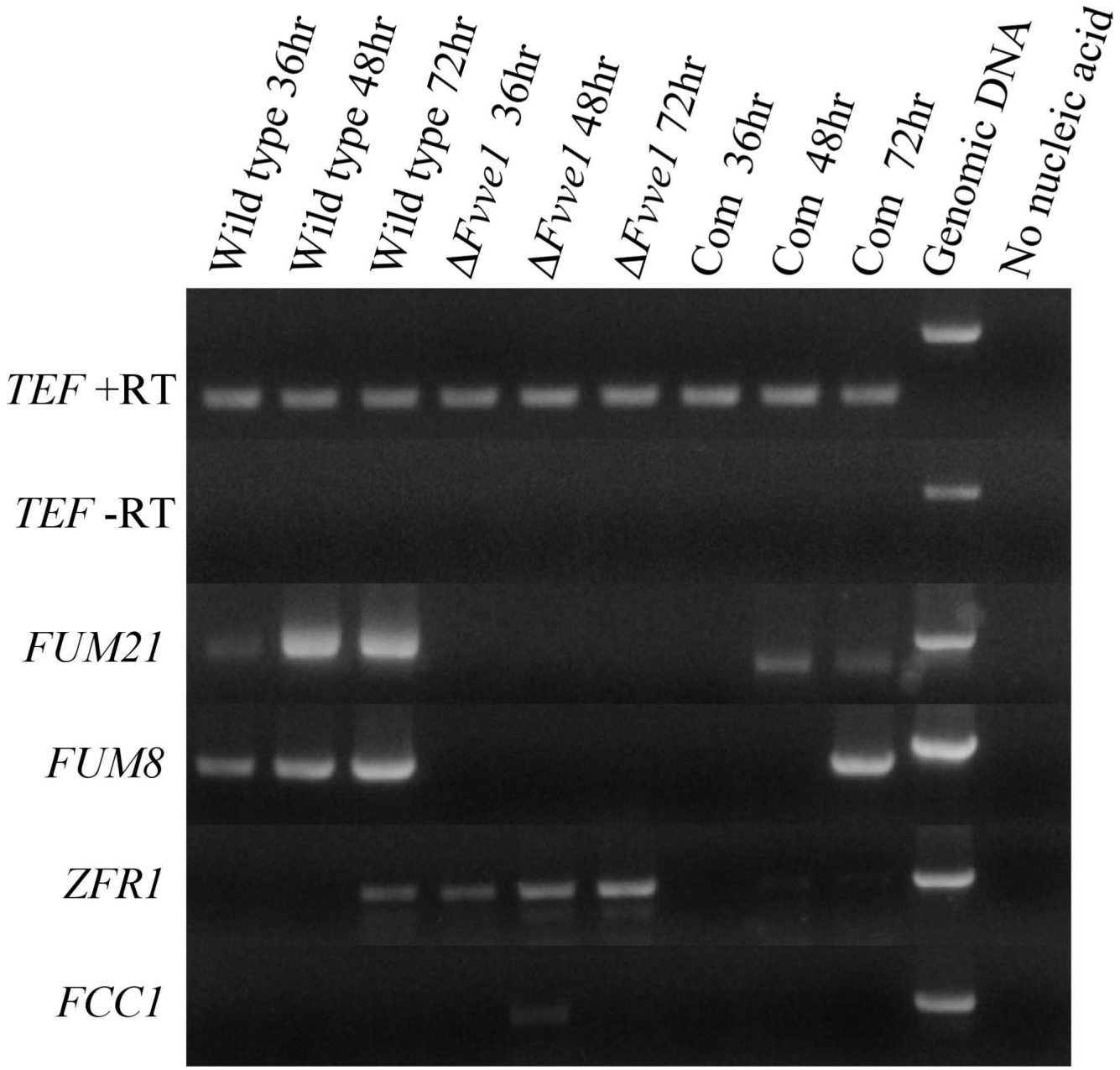

Figure 4.

RT-PCR analysis of *FUM21, FUM 8, ZFR1* and *FCC1* gene expression from wild-type M-3120, *FvVE1* deletion mutant M31206 (Δ*Fvve1*), and complementation (Com) strain M31206C5. Total RNA was isolated from mycelial tissue grown on cracked-corn medium at 36, 48 and 72 h after inoculation. *TEF1* was used as loading control. A no reverse transcriptase control reaction is shown for *TEF1* primers indicating no genomic DNA remained after DNase treatment.

Table 1

Fumonisin and Fusarin Analysis of Cracked-corn Cultures.

Time	Strain	Fumonisins[a]	Fusarins[b]
36 hours	Wild type	0	0
	Δ*Fvve1*	0	0
	Comp	0	196
48 hours	Wild type	1	0
	Δ*Fvve1*	0	0
	Comp	0	864
72 hours	Wild type	98	330
	Δ*Fvve1*	0	0
	Comp	127	1709
96 hours	Wild type	178	673
	Δ*Fvve1*	0	0
	Comp	316	2066
144 hours	Wild type	281	1945
	Δ*Fvve1*	0	0
	Comp	334	2246

[a] μg fumonisins B_1, B_2 and B_3 combined per gram of cracked corn culture

[b] μg fusarins C_1, C_2 and C_3 combined per gram of cracked corn culture