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The velvet gene, FgVe1, affects fungal development and positively regulates trichotheccene biosynthesis and pathogenicity in Fusarium graminearum

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SUMMARY

Trichotheccenes are a group of toxic secondary metabolites produced mainly by Fusarium graminearum (teleomorph: Gibberella zeae) during the infection of crop plants, including wheat, maize, barley, oats, rye and rice. Some fungal genes involved in trichotheccene biosynthesis have been shown to encode regulatory proteins. However, the global regulation of toxin biosynthesis is still enigmatic. In addition to the production of secondary metabolites belonging to the trichotheccene family, Fusarium graminearum produces the red pigment aurofusarin. The gene regulation underlying the production of aurofusarin is not well understood. The veA gene from Aspergillus nidulans has been shown to be the key component of the velvet complex regulating development and secondary metabolism. Using BLAST analyses, we identified the velvet gene from F. graminearum, FgVe1. Disruption of FgVe1 causes several phenotypic effects. However, the complementation of this mutant with the FgVe1 gene restores the wild-type phenotypes. The in vitro phenotypes include hyperbranching of the mycelium, suppression of aerial hyphae formation, reduced hydrophobicity of the mycelium and greatly reduced sporulation. Our data also show that FgVe1 modulates the production of the aurofusarin pigment and is essential for the expression of Tri genes and the production of trichotheccenes. Pathogenicity studies performed on flowering wheat plants indicate that FgVe1 is a positive regulator of virulence in F. graminearum.

INTRODUCTION

The filamentous fungus Fusarium graminearum (teleomorph: Gibberella zeae Schwabe) is a ubiquitous plant pathogen in cereal-growing areas worldwide, which causes Fusarium head blight (FHB) disease on wheat ears (Parry et al., 1995). The growth of this fungus on potato dextrose agar (PDA) is rapid, resulting in dense aerial mycelia that vary in colour from white to pale orange or red. The bottom of the plate is usually carmine red. The colour is produced by two pigments: aurofusarin and rubrofusarin (Booth, 1971; Kim et al., 2005). The biosynthesis pathway of these two secondary metabolites has been well characterized and the genes implicated in this pathway have been identified and sequenced (Frandsen et al., 2006, 2011).

The infection of wheat plants with F. graminearum, leading to the devastating disease FHB, occurs during favourable weather conditions and poses a threat to human and animal health. In addition to the reduction in grain yield, F. graminearum produces trichotheccene mycotoxins which accumulate in the infected grains, thus making them unsuitable for food and feed (Goswami and Kistler, 2004). Fusarium graminearum produces type B trichotheccenes, including deoxynivalenol (DON), acetyldeoxynivalenol (3ADON or 15ADON), nivalenol (NIV) and acetylnivalenol (4ANIV) (Xu and Berrie, 2005). The biosynthetic pathway leading to the formation of trichotheccenes initiates from farnesyl pyrophosphate, a precursor of the sterol pathway, and involves at least 15 different biochemical steps in Fusarium spp. (Alexander et al., 2009). Fifteen Tri genes encoding proteins involved in trichotheccene biosynthesis and regulation are located in three different loci in the genome of F. graminearum: 12 genes are clustered and form the core ‘Tri cluster’ locus, a two-gene cluster ‘Tri1–Tri16’ is located at another locus and the unique gene ‘Tri101’ has been identified at a third location (Alexander et al., 2009; Brown et al., 2003; Hohn et al., 1993; Kimura et al., 1998, 2003, 2007). Two Tri genes have been shown to be pathway-specific regulators. Tri6, located in the core ‘Tri cluster’, encodes a zinc finger transcription factor and regulates positively the other Tri genes and genes implicated in the isoprenoid biosynthetic pathway, upstream of trichotheccene biosynthesis (Proctor et al., 1995; Seong et al., 2009). Tri10, also located in the core ‘Tri cluster’, is known to regulate the Tri biosynthetic genes (Seong et al., 2009; Tag et al., 2001). Two other Tri genes, Tri14 and Tri15, have been suggested to play a regulatory function (Alexander et al., 2004; Dyer et al., 2005), but this has not been proven experimentally.

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The expression of *Tri* genes and the production of trichothecene are induced *in planta* or *in vitro* under very specific conditions. Various studies aiming to investigate their regulation have been reported (Merhej et al., 2011a). However, the results are still not sufficient to understand the whole mechanism underlying the regulation of biosynthesis during infection in the field. In addition to the role of the pathway-specific regulators, many 'external' factors, such as carbon and nitrogen sources, oxidative stress by H$_2$O$_2$, phenolic acids, fungicides, temperature and magnesium, have been shown to modulate the expression of *Tri* genes and the production of trichothecenes (Boutigny et al., 2010; Covarelli et al., 2004; Jiao et al., 2008; Miller and Greenhalgh, 1985; Pinson-Gadis et al., 2008; Ponts et al., 2007; Schmidt-Heydt et al., 2008).

It has also been reported that various amines are potent inducers of trichothecene production, and the early activation of polyamine biosynthesis during FHB implicates putrescine as an inducer of trichothecene production, and the early activation of polyamine biosynthesis during FHB implicates putrescine as an inducer of trichothecene production (Gardiner et al., 2009a, 2010). Global transcriptional analysis of *F. graminearum*, when grown in the presence of agmatine, has identified two novel genes that negatively regulate trichothecene production (Gardiner et al., 2009b). In addition, *Tri* gene expression is now known to be negatively regulated by neutral and alkaline pH (Merhej et al., 2010). The pH regulatory factor *FgPac1* has been shown to be a negative regulator of *Tri* genes (Merhej et al., 2011b).

In many species of filamentous fungi, light has been reported to influence development and to regulate various metabolic pathways (Tisch and Schmoll, 2010). The most important light regulatory protein VeA, encoded by the *velvet* gene, was first identified in *Aspergillus nidulans* (Käfer, 1965). This protein does not carry a photoreceptor domain but interacts with the photoreceptor domain of a *velvet* protein LreB, a member of the white collar complex regulating the circadian clock, via the red-light sensor protein FphA (Purschwitz et al., 2008). Initially, VeA was found to be implicated in the regulation of conidiation, the activation of sexual development and the inhibition of asexual development in *A. nidulans* (Champe et al., 1981; Yager, 1992). A second role was then assigned to VeA in the regulation of secondary metabolites (Calvo et al., 2004; Cary et al., 2007; Duran et al., 2007; Kato et al., 2003). Recently, the presence of a *velvet* complex, integrating light signals with fungal development and secondary metabolism, has been demonstrated (Bayram et al., 2008). In this complex, VeA acts as a bridge between VelB, a protein implicated in asexual development, and LaeA, a general regulator of secondary metabolites in *Aspergillus* spp. (Bayram et al., 2008; Bok and Keller, 2004). In the dark, the VeA/VelB proteins are transported to the nucleus by KapA and interact with LaeA to form the complex supporting sexual development and secondary metabolism production (Bayram et al., 2008).

The VeA protein and the velvet complex have been identified in various fungi. It has been demonstrated that VeA affects various aspects of fungal development and differentiation. In particular, VeA acts as a positive regulator of the production of several metabolites, including sterigmatocystin and penicillin in *A. nidulans* and *Penicillium chrysogenum* (Hoff et al., 2010; Kato et al., 2003), aflatoxin in *Aspergillus parasiticus* (Calvo et al., 2004), cyclopiazonic acid, aflatrem and aflatoxin in *Aspergillus flavus* (Duran et al., 2007), cephalosporin C in *Acremonium chrysogenum* (Dreyer et al., 2007), fumonisins and fusarins in *Fusarium verticillioides* (Myung et al., 2009), gibberellins, bikaverin, fumonisins and fusarin C in *Fusarium fujikuroi* (Wiemann et al., 2010) and melanin in *Mycosphaerella graminicola* (Choi and Goodwin, 2011).

In this study, we identified the homologue of the *velvet* gene in *F. graminearum*, *FgVe1*. Using *FgVe1D18*, a strain disrupted for the *FgVe1* gene, and *FgVe1D18-C*, a complemented strain, we investigated the role of the Ve1 protein in fungal development, pathogenicity, and aurofusarin and trichothecene production.

**RESULTS**

**Characterization of the FgVe1 gene and corresponding protein**

Using the protein sequence of FvVe1 from *F. verticillioides*, the genomic sequence FGSG_11955 corresponding to *FgVe1* was identified in the genome of *F. graminearum* at the Munich Information Centre of Protein Sequences (MIPS) and the *Fusarium* comparative database from the Broad Institute (http://www.broadinstitute.org). However, the nucleotide sequence FGSG_11955 encoded only a truncated velvet protein of 399 amino acids which was lacking more than 100 amino acids when compared with *FvVe1* from *F. verticillioides*. We amplified and re-sequenced a polymerase chain reaction (PCR) fragment of the locus from *F. graminearum* strain PH-1 (see 'Experimental procedures') and deposited the sequence in GenBank under accession number HQ436464. The comparison of this FgVe1 polypeptide complete sequence with the protein sequence of FvVe1 from *F. verticillioides* revealed the presence of an open reading frame (ORF) of 1656 nucleotides encoding a predicted protein of 532 amino acids.

Multiple sequence alignment of the predicted FgVe1 protein with known VeA proteins using CLUSTALW 2.0.12 software indicated that FgVe1 shares identity to FvVe1 from *F. verticillioides* (77%), FvVe1 from *F. fujikuroi* (77%), AcVeA from *Ac. chrysogenum* (54%) and VeA from *A. nidulans* (32%). A phylogenetic analysis confirmed that the FgVe1 gene identified in this study is closely related to the veA/ve1 group and more distantly related to the ve1B/ve12 group and other members of the *velvet* family genes (results not shown). The highest identity of the proteins was found in the N-terminal region (Fig. 1). This region comprises the *velvet* superfamily domain and was identified using the Conserved Domain Architecture Retrieval Tool (CDART) avail-
From the National Center of Biotechnology Information (Bethesda, MD, USA). Using the 'epestfind' tool from the EMBOSS package (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind), several proline-, glutamate-, serine- and threonine-rich (PEST) regions were identified. One is a potential PEST motif conserved in FvVe1 from *F. verticillioides* and FfVel1 from *F. fujikuroi* (Fig. 1C). PEST motifs of Ve1 from *Fusarium* spp. are predicted at different positions relative to the PEST motif position in VeA from *Aspergillus* spp.

**FgVe1** regulates trichothecene production

After the transformation of *F. graminearum* wild-type strain CBS185.32 with the disruption vector pCB1004-D-Fg11955 (Fig. 2A), 22 fungal transformants were isolated and purified by single-spore isolation. Among these transformants, seven were analysed by PCR to determine which mutants carried a disrupted FGSG_11955 gene. Different primer combinations were used to detect the insertion of the hygromycin B resistance cassette into the *FGSG_11955* gene.
The disruption of FGSG_11955. (A) Expected result for insertion of pCB1004-D-fg11955 disruption vector into the genome of Fusarium graminearum strain CBS185.32 via a single homologous integration event. The polymerase chain reaction (PCR) fragment amplified from wild-type genomic DNA with primers P3–P4 was used to complement the disrupted strain. P1, primer D-FGSG11955-F; P2, primer FGSG11955-R; P3, primer Ve1-C-F; P4, primer Ve1-C-R; P5, primer T3; P6, primer T7. (B) PCR strategy for the screening of the transformants. A 3.7-kb fragment amplified with primers P3–P4 indicates a wild-type locus. A 1.7-kb fragment amplified with primers P3–P6 indicates vector insertion at the 5’ side. A 1.6-kb fragment amplified with primers P5–P4 indicates vector insertion at the 3’ side. (C) Reverse transcription (RT)-PCR on RNA of the indicated strains to amplify FgVe1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcripts.

Impact of FgVe1 disruption on fungal development

Fungal morphogenesis, growth and conidiation were assessed to analyse the effect of FgVe1 disruption on morphological development in F. graminearum. The FgVe1D18 strain obtained in this study exhibited various specific developmental characteristics. Measurement of the colony diameter on PDA medium during the first 4 days of culture indicated that the radial growth of the FgVe1-disrupted strains did not differ from that of the wild-type strain. During growth on PDA, the colour of the mycelium of the FgVe1D18 mutant strain was dark yellow, whereas the mycelium of the wild-type and complemented strains was red at the bottom of the plate with a white appearance at the agar surface because of the abundant aerial hyphae (Fig. 3A). Fusarium graminearum is known to produce red pigments on agar medium (Booth, 1971). This observation indicates that the disruption of FgVe1 may affect the production of pigments. The FgVe1D18 strain only grew horizontally on the agar surface and formed a viscous thin layer of mycelium. The formation of aerial mycelium was completely inhibited. However, the wild-type and complemented strains both displayed an abundance of fluffy aerial mycelium which was predominantly white in colour at the colony margin and pink in colour in the colony centre (Fig. 3B, C).

Interestingly, microscopic inspection revealed an aberrant hyphal branching pattern for the FgVe1D18 mutant when grown between two layers of cellophane on PDA (Fig. 3D). The wild-type and complemented (FgVe1D18-C) strains showed long apical extension with normal regularly spaced hyphal branching patterns. In contrast, disruption of the FgVe1 gene appeared to cause the loss of apical dominance, leading to a hyphal hyperbranching morphology with random tip branching, possibly caused by the loss of ability to form normal apical extension.

The ability to produce conidia was evaluated in two different culture conditions. Firstly, the conidia were collected from a 10-day-old agar plate culture by harvesting all spores in the plate with 5 mL of water. Under this condition, the wild-type and complemented strains produced (5.98 ± 0.59) × 104 spores and (3.48 ± 3.83) × 104 spores, respectively. However, the FgVe1D18 mutant was not able to produce conidia (data not shown). Secondly, the conidia were collected from 3-day-old shaking cultures grown in 100 mL of carboxymethyl cellulose (CMC) medium in the dark, as described previously (Merhej et al., 2010). In liquid culture, the FgVe1D18 strain produced almost 1000 times less conidia than the wild-type and complemented strains (Fig. 3E). This result indicates that the FgVe1 gene positively regulates conidiogenesis in F. graminearum. Conidial formation was also assessed on agar plates exposed to light and the results did not differ from cultures grown in the dark. The germination of conidia was also studied. The results obtained showed that the disruption of FgVe1 did not have a significant influence on germination (data not shown).
In order to evaluate fungal biomass accumulation in liquid medium, mycelia from cultures in minimum synthetic medium were collected at days 3, 4, 5 and 7 post-inoculation. Under these conditions, mycelial growth for the wild-type strain was maximal at day 3. Over the following days, the biomass was stable without significant variation (Merhej et al., 2010). Determination of the dry fungal biomass indicated that, at day 3, the growth of the FgVe1D18 strain was reduced relative to that of the wild-type and complemented strains (Table 1). Subsequently, the fungal biomass did not increase and the differences between the wild-type, FgVe1D18 and FgVe1D18-C strains were conserved.

The re-introduction of a copy of FgVe1 into the disrupted strain restored the initial phenotype with regard to colour, aerial mycelium formation, biomass accumulation and conidiation. This demonstrates that all the phenotypes identified in the FgVe1D18 mutant were caused by the loss of FgVe1 function.

Disruption of FgVe1 reduces the hydrophobicity of the cell surface

Functional analysis of the velvet genes from F. verticillioides and M. graminicola revealed that these genes are essential to preserve the hydrophobic property of the hyphae (Choi and Goodwin, 2011; Li et al., 2006). In order to test the hydrophobicity of the surface hyphae in F. graminearum, 15 μL of water coloured with bromophenol blue were placed on the surface of 2-day-old colonies of wild-type, FgVe1D18 and FgVe1D18-C strains (Fig. 4). The aqueous solution applied to the wild-type and complemented strains maintained a spherical droplet form, thus revealing the strong hydrophobicity of the aerial hyphae. In contrast, water droplets applied to the FgVe1D18 mutant were absorbed by the colony and spread immediately onto the mycelium surface, indicating the loss of hydrophobicity in this mutant. These data demonstrate that FgVe1 is essential to maintain the hydrophobicity of the cell surface in F. graminearum.

The FgVe1-disrupted mutant is defective in aurofusarin biosynthesis

The velvet gene in various filamentous fungi is known to affect the production of a wide spectrum of secondary metabolites, including pigments such as bikaverin in F. fujikuroi and melanin in...
**M. graminicola** (Choi and Goodwin, 2011; Wiemann et al., 2010). The disruption of the *F. graminearum* velvet gene leads to a change in the colour of the mycelium, thus suggesting a potential perturbation in the biosynthesis of pigments. To test this hypothesis, the identification of aurofusarin was performed by mass spectrometry ([M + H]+ m/z 571, [M + H–CH3]+ m/z 556 and [M + H–2 × CH3]+ m/z 541) and the production of this pigment was measured using high-performance liquid chromatography (HPLC)-UV. The results obtained showed that the production of aurofusarin was reduced more than seven-fold in the *FgVe1D18* mutant compared with the wild-type and complemented strains (Fig. 5). We conclude that *FgVe1* positively affects aurofusarin biosynthesis in *F. graminearum*.

**Gene disruption of *FgVe1* results in a loss of *Tri* gene expression and trichothecene production**

Trichothecenes are the most important mycotoxin produced by *F. graminearum*. The alteration in trichothecene production in *vitro* following the disruption of *FgVe1* was examined. Trichothecenes were extracted from minimal synthetic liquid medium (MS) at 7 days post-inoculation (dpi) and measured by HPLC. The results showed that the disrupted strain does not produce any detectable trichothecene, whereas both the wild-type and complemented strains produce large amounts of toxin (Fig. 6A).

Next, we analysed the expression of five trichothecene biosynthesis genes using RT-PCR. *Tri5* and *Tri4* are implicated in the earlier steps of the biosynthetic pathway. *Tri6* and *Tri10* encode pathway-specific regulators. *Tri101* is located outside the core ‘Tri’ cluster and encodes a 3-O-acetyl transferase implicated in self-protection against DON (Alexander et al., 2009). Total RNA was extracted from *in vitro* cultures grown for 4 days. Expression of the

**Impact of *FgVe1* disruption on the pathogenicity of *F. graminearum***

In the field, *F. graminearum* infects the spikelets of wheat plants, grows into the rachis and proliferates throughout the floral spike. During infection, the pathogen produces various trichothecenes. The mycotoxin DON is now known to be a virulent factor aiding in the establishment and propagation of Fusarium infection within the spikes (Jansen et al., 2005; Proctor et al., 1995). The *FgVe1D18* mutant has lost the ability to produce toxin and is strongly
reduced in development and conidiation. Therefore, the capacity of the \textit{FgVe1D18} strain to infect and colonize wheat spikes was studied. Wheat ears were inoculated at the early anthesis stage using three inoculation techniques: point, plug and spray inoculation. In the point-inoculated ears, the wild-type and complemented strains were able to colonize the inoculated spikelets and to progress through the rachis and affect the spikelets beneath (Fig. 7A). In contrast, the \textit{FgVe1D18} mutant generally failed to colonize the inoculated spikelets. The quantification of disease development by counting the infected spikelets at 15 and 20 dpi below the point of inoculation revealed that the \textit{FgVe1D18} mutant (0.50 ± 0.53 bleached spikelets) was significantly reduced in virulence ($P < 0.01$) compared with the wild-type (3.25 ± 2.25 bleached spikelets) and complemented (\textit{FgVe1D18-C} 2.87 ± 1.12 bleached spikelets) strains. Inoculation with the wild-type or complemented strain also caused considerable bleaching of spikelets above the point of inoculation, whereas the ears point inoculated with the \textit{FgVe1D18} mutant remained healthy.

In the plug-inoculated ears, the results obtained were similar to those in the point inoculation test, as the \textit{FgVe1D18} mutant strain caused significantly less damage than the wild-type and complemented strains (data not shown).

In the spray inoculation test (Fig. 7B), the wild-type and complemented strains caused severe bleaching of the whole spikelets of the ears. In addition, the fungus reached the rachis and this tissue turned brown. The wheat ears inoculated with the \textit{FgVe1D18} strain did not exhibit any visual symptoms; the spikelets remained green, continued to flower and to develop. The measurement of DON produced \textit{in planta} 10 days after spray inoculation showed that DON biosynthesis by the \textit{FgVe1D18} mutant strain (1.04 ± 0.87 ppm) was much lower than the level of production by the wild-type (66.14 ± 33.98 ppm) and complemented (30.97 ± 12.97 ppm) strains. Although a low level of \textit{in planta} DON production by the \textit{FgVe1D18} mutant strain was detectable, these wheat results were broadly in agreement with the trichothecene production levels detected \textit{in vitro} for the three strains.
DISCUSSION

To date, plant breeding and chemical control strategies aimed at reducing *F. graminearum* development and trichothecene accumulation in wheat ears within field crops have failed to provide effective control. The targeting of regulatory pathways implicated in both the development of the fungus and mycotoxin biosynthesis may lead to novel ways to control FHB disease in crops and the production of trichothecene in grains, which pose a real threat to human and animal health. In this study, we demonstrated that the velvet protein potentially plays this advantageous dual role for *F. graminearum*. The velvet gene, identified in various *Aspergillus* spp., *Penicillium* spp., *Acremonium* spp., in *F. verticillioides* and, very recently, in *F. fujikuroi* and *M. graminicola*, is known to control fungal growth and morphogenesis and to regulate mycotoxin biosynthesis. The velvet gene encodes VeA, the key component of the velvet complex. By interacting with blue light-sensing (LreA and LreB) and red light-sensing (FphA) proteins, VeA coordinates the perception of the light signal to fungal development (VeIB and VosA) and secondary metabolism (LaeA) (Bayram et al., 2008; Calvo, 2008). Recently, it has been demonstrated that VeA modification and protein levels in *A. nidulans* are regulated by LaeA. In this species, the LaeA protein appears to play a dual role in inhibiting sexual development, as well as regulating secondary metabolite cluster genes (Sarikaya-Bayram et al., 2010).

The conservation of the velvet gene sequence across several fungal genera has facilitated the identification of the homologous gene in *F. graminearum*, which was designated *FgVe1*. This gene encodes a highly conserved protein, especially in the velvet domain, and shows an identity of up to 77% with *FvVe1* from *F. verticillioides* and *FfVel1* from *F. fujikuroi*. The analysis of the *FgVe1* protein sequence showed the presence of the velvet superfamily domain, including the nuclear localization signal (NLS) sequence in the N-terminus. The NLS sequence in *A. nidulans* and *A. chrysogenum* is implicated in the subcellular localization of VeA in response to light (Dreyer et al., 2007; Stinnett et al., 2007). The NLS sequence in *FgVe1* might play a similar role. VeA proteins from various fungi contain, in their sequence, a PEST motif. The *FgVe1* sequence also encodes a putative PEST domain located on the N-terminal side of the protein. PEST motifs are proteolytic signals located at the N- or C-terminus of the protein and lead to rapid protein destruction by caspase cleavage and proteasome degradation (Belizario et al., 2008; Rechsteiner and Rogers, 1996). It has been suggested that VeA might be subjected to degradation in a PEST-dependent manner (Calvo, 2008). The PEST motif in the VeA homologues from various fungi, including the PEST motif of *FgVe1*, might be implicated in the rapid turnover of the protein. Further studies are required to fully understand its role.

The analysis of velvet mutants in various fungi has identified its role in the regulation of development; velvet contributes to conidiation efficiency and to the switch towards sexual reproduction. However, its mode of action differs between species. In *Aspergillus* spp., deletion of veA increases conidiation and reduces sexual development (Calvo, 2008). In *F. verticillioides* and *F. fujikuroi*, the deletion of Ve1 suppresses aerial hyphal growth, increases the ratio of macroconidia to microconidia and reduces the hydrophobicity of the cell surface (Li et al., 2006; Wiemann et al., 2010). In this study, the disruption of *FgVe1* from *F. graminearum* suppressed aerial hyphal growth, reduced conidiation and reduced the hydrophobicity of the vegetative mycelium. These observations suggest that *velvet* homologues from *Fusarium* spp. may assume very similar functions related to development. It has been demonstrated that the deletion of the velvet gene from various fungi affects sexual development and impairs perithecial formation (Calvo, 2008; Wiemann et al., 2010). During this study, the CBS185.32 wild-type strain used was found not to form perithecia during culture in inductive carrot agar medium (data not shown). Therefore, unfortunately, the role of *FgVe1* in the induction of perithecia formation could not be explored.

The second function assigned to velvet is the regulation of secondary metabolism. The deletion of velvet-like genes in various fungi has demonstrated its role as a positive regulator of a large spectrum of secondary metabolites. For example, the presence of the velvet protein positively regulates the production of aflatoxin, sterigmatocystin and penicillin in *A. nidulans*, cyclopiazonic acid and afлатrem in *A. flavus*, and fumonisins and fusarin C in *F. verticillioides* by regulating the gene clusters implicated in the biosynthesis of these secondary metabolites (Duran et al., 2007; Kato et al., 2003; Myung et al., 2009; Spröte and Brakhage, 2007). In this study, we showed that the deletion of the *FgVe1* gene from *F. graminearum* strongly reduces the production of aurofusarin and prevents trichothecene production by suppressing the expression of *Tri* genes. We demonstrate, therefore, a role for *FgVe1* in the regulation of secondary metabolites from *F. graminearum*, in agreement with a conserved role of velvet homologues across the fungal genera. To date, only *FvVe1* from *F. fujikuroi* has been shown to negatively regulate the red pigment bikaverin, whilst positively regulating the other secondary metabolites produced by this fungus, especially gibberellins (Wiemann et al., 2010). Surprisingly, the red pigment of *F. graminearum* seems to be regulated in the opposite manner. These data indicate that, although the role of velvet in secondary metabolism regulation is conserved throughout the *Fusarium* genus, the ability to regulate positively or negatively each secondary metabolite differs between species.

The trichothecene toxin is considered to be a key virulence factor, as it is known to play a key role during the infection and colonization of wheat plants by *F. graminearum* (Jansen et al., 2005; Proctor et al., 1995). For this reason, the pathogenicity of the *FgVe1D18* mutant strain was tested on wheat spikes. The strong reduction in virulence observed following the disruption of *FgVe1* may result from the loss of trichothecene production. However, it may also be a result of the reduced development of the
mutant, which might influence its virulence, or to the loss of expression of additional pathogenicity factor(s) in the FgVe1D18 mutant. In agreement with our results, the deletion of a velvet-like gene (not the homologue of VeA) from F. oxysporum significantly delayed its virulence on tomato plants (Lopez-Berges et al., 2009). Nevertheless, disruption of the velvet gene MVE1 from M. graminicola, although causing a similar morphological phenotype in vitro to velvet mutants generated in various Fusarium spp., did not affect the pathogenicity of this fungus towards wheat leaves (Choi and Goodwin, 2011). Overall, the results of this study suggest that the velvet gene from closely related species, especially Fusarium producers of mycotoxins, including F. graminearum, F. verticillioides and F. fujikuroi, may provide similar functions, whereas, in distantly related species, only some of these functions have evolved and/or have been retained.

In this work, we have demonstrated a dual role for FgVe1 in the regulation of fungal growth and of secondary metabolites. The velvet gene in A. nidulans has been known for many decades to assume such dual functions. Recently, the dissection of the velvet complex allowed the identification of the partners of the velvet gene product implicated in these two functions. This complex involved VelB, VelC and VosA, carrying a velvet domain in their sequence (Bayram and Braus, 2011), and LaeA, a methyl transferase protein playing a general role in the regulation of genes and clusters implicated in secondary metabolite production in A. nidulans (Bok and Keller, 2004). Recently, it has been demonstrated that this key protein controls Hülle cells, which nurse the young fruiting body during development, thus playing a pivotal role in inhibiting sexual development in response to light, and also controls modification and protein levels of the velvet regulatory proteins in A. nidulans (Sarikaya-Bayram et al., 2010). In addition to their role in the regulation of secondary metabolism, the role of both VeA and LaeA in the regulation of development seems to be common for various genera of filamentous fungi, as PcVeAa and PclaeA from P. chrysosogenum also control penicillin biosynthesis and have distinct developmental roles (Hoff et al., 2010). In F. graminearum, a LaeA-like protein might also exert multiple regulatory roles. The use of the sequence of the FfLae1 gene from F. fujikuroi (Wiemann et al., 2010) might allow the identification of the putative homologue from F. graminearum.

EXPERIMENTAL PROCEDURES

Culture conditions

The F. graminearum strains used were the wild-type CBS185.32, provided by the CentraalBureau voor Schimmelcultures (Utrecht, the Netherlands), and the mutants FgVe1D18 and FgVe1D18-C, generated in this study. The wild-type strain produces DON and its acetylated form 15ADON. Stock cultures were stored at –80 °C as spore suspensions in 30% glycerol. Spores were generated as described previously (Merhej et al., 2011b). To test fungal biomass, toxin production and gene expression in vitro, 10^6 spores were used to inoculate a Petri dish containing 8 mL of MS medium (Merhej et al., 2010). Cultures were inoculated at 25 °C in the dark without shaking. Mycelium was collected by centrifugation in sterile conditions and stored at –80 °C, either to carry out RNA extraction or for the quantification of the fungal biomass after lyophilization. Culture medium was stored at –20 °C before toxin extraction.

Identification and sequencing of FgVe1

The amino acid sequence of Fve1 from F. verticillioides (GenBank accession number ABC02879.1) was used to identify the velvet gene from F. graminearum in the F. graminearum genome database (MIPS) using the Washington University Basic Local Alignment Search Tool 2.0© (WU-BLAST 2.0, 4 May 2006). The BLAST search originally identified two highly conserved sequences (Fg13162 and Fg13163) on two different contigs. These two sequences corresponded to the N-terminal and C-terminal sides of Fve1, respectively. These two sequences have since been reassembled at the MIPS database and the Fusarium comparative database from the Broad Institute (http://www.broadinstitute.org) to form the ORF FGS11155S. However, this ORF still lacked a part of the central sequence. We designed the primers D-FGSG11955-F and FGS11955-R to amplify a 1.5-kb DNA fragment spanning the sequence gap within the reading frame of the F. graminearum Ve1 coding sequence using PH-1 (NRRL31084) genomic DNA. The resulting amplicon was sequenced (Cogenics, Genome express, Grenoble, France) and used to build the final FgVe1 gene model. The coding sequence was re-annotated based on the alignment with the Fve1 protein. Both the nucleic acid and predicted amino acid sequence were submitted to GenBank under the accession number HQ436464.

Construction of the FgVe1 disruption mutant

Following the identification of the FgVe1 gene from F. graminearum, a 1.5-kb fragment within the coding sequence of FgVe1 was amplified using the primers D-FGSG11955-F and FGS11955-R. The resulting amplicon containing two EcoRI restriction sites (one added to the primer D-FGSG11955-F and the second residing 715 bp upstream of the start codon of the FgVe1 coding sequence) was gel purified using the GFX™ PCR DNA purification kit (Amersham, France) and digested with EcoRI. The resulting 698-bp EcoRI fragment corresponding to an internal part of the FgVe1 coding sequence was cloned into the pCB1004 plasmid carrying the hygromycin B resistance cassette to generate pCB1004-D-Fg11955. The resulting vector was used to transform protoplasts of F. graminearum strain CBS185.32, as described previously (Merhej et al., 2011b). Hygromycin-resistant transformants were selected in PDA medium containing 60 μg/mL of hygromycin B. The transformants were then screened by PCR using the primers listed in Table 2. For complementation experiments, a 3.7-kb fragment encompassing 1 kb of the FgVe1 promoter, the FgVe1 coding sequence and 1 kb of the terminator was amplified from F. graminearum CBS185.32 genomic DNA by PCR using the primers Ve1-C-F and Ve1-C-R. About 3 μg of the amplicon purified using the GFX™ PCR DNA purification kit (Amersham) was mixed with 3 μg of plasmid pSM334 carrying the geneticin resistance cassette (Marek et al., 1989).
Primers for RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Forward primer sequence (5′–3′)</th>
<th>Reverse primer sequence (5′–3′)</th>
<th>( T_m ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FgVe1</em></td>
<td>FGSG11955</td>
<td>ACGTTCCACCTCTCCACCTTC</td>
<td>TCTCGTGTGTCGCTTACC</td>
<td>60</td>
</tr>
<tr>
<td><em>Tri5</em></td>
<td>FG03537</td>
<td>GACCCTAGGGACTACCTACAG</td>
<td>GCCCTGGGCTCTACCCCAAAGG</td>
<td>58</td>
</tr>
<tr>
<td><em>Tri4</em></td>
<td>FG03535</td>
<td>TATGTTGCTACCCCAAGGCCT</td>
<td>TGTCAAGATGGGCTTACCAA</td>
<td>58</td>
</tr>
<tr>
<td><em>Tri6</em></td>
<td>FG03536</td>
<td>AGCGCCAAGGCTCCCTCTCTTG</td>
<td>AGCGCCAAGGCTCCCTCTCTTG</td>
<td>58</td>
</tr>
<tr>
<td><em>Tri10</em></td>
<td>FG03538</td>
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</tr>
<tr>
<td><em>Tri101</em></td>
<td>AB011417</td>
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<td>AGCGCCAAGGCTCCCTCTCTTG</td>
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</tr>
<tr>
<td><em>G4DH</em></td>
<td>FG02657</td>
<td>CCTTCAATGAGGCTCCCTAC</td>
<td>CTGACATGGGACGCTC</td>
<td>59</td>
</tr>
</tbody>
</table>

Primers for FGSG_11955 disruption and screening

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>( T_m ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-FGSG11955-F*</td>
<td>GAGAATCCCAACAAATCAAAAGGC</td>
<td>60</td>
</tr>
<tr>
<td>FGSG11955-R</td>
<td>TCTCGTGTGTCGCTTACC</td>
<td>60</td>
</tr>
<tr>
<td>Ve1-C-F</td>
<td>TCTTGCAACCAACCATCAG</td>
<td>60</td>
</tr>
<tr>
<td>Ve1-C-R</td>
<td>TTTTACGCTCCGCCCTGTAATG</td>
<td>60</td>
</tr>
<tr>
<td><em>T3</em></td>
<td>ATTAACCTCTAAAGGGAAC</td>
<td>62</td>
</tr>
<tr>
<td><em>T7</em></td>
<td>GATATACGCTACCTAGGGGC</td>
<td>62</td>
</tr>
</tbody>
</table>

*The underlined sequence shows the placement of the EcoR1 restriction site.

The mixture was used to transform protoplasts of the *FgVe1*-disrupted strain. Geneticin-resistant transformants were selected on PDA medium containing 100 μg/mL of geneticin (Sigma Aldrich, St. Louis, MO, USA).

**Nucleic acid manipulation**

Genomic DNA was extracted from ground mycelia of *F. graminearum* as described previously (Javerzat et al., 1993). Total RNA was isolated with TRIzol® reagent (Invitrogen, Cergy-Pontoise, France) from ground mycelium following the manufacturer’s instructions. RT was performed as described previously (Merhej et al., 2011b).

RT-PCRs were performed in an iCycler™ (Bio-Rad, Marne La Coquette, France) in a 25-μl mixture containing 0.5 U of Taq DNA polymerase (Promega, Charbonnières-les-Bains, France). The amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 28 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at the appropriate primer hybridization temperature and 40 s of extension at 72 °C. The primers used for RT-PCR are listed in Table 2. Finally, 10 μL of the PCR mixture were loaded onto a 1.2% agarose gel and separated by electrophoresis.

**Wheat plant infection assays**

Seeds of spring wheat cultivar ‘Bobwhite’ were sown in Levington’s C2 coarse potting compost. Seedlings were transplanted singly into 10-cm pots and grown for an additional 2 months in a controlled environment growth room at 18 °C/16 °C during 16-h day/8-h night at 50% relative humidity. Light was supplied by a mixture of metal halide and incandescent lamps to produce a fluence level of 207 μmol at 86.2 W/m² at the plant surface. Once the plants entered anthesis, individual attached ears containing 100 spikelets midway along the ear. Four ears were inoculated for each strain. For plug inoculations, a small agar plug (1 mm in diameter) was cut from the edge of a young mycelial colony and inserted within a floral chamber of two spikelets midway along the ear. Again, four ears were inoculated for each strain. The glumes of each inoculated spikelet were marked with a waterproof pen. After inoculation, the entire plant was placed into a high-humidity chamber (>95% relative humidity) for the next 3 days. Light was excluded from the plants for 16 h post-inoculation. The plants were then returned to the standard growth room conditions. For the spray-inoculated ears, at day 10 post-inoculation, ears were separated from the whole plant, photographed, lyophilized and conserved at –20 °C for toxin analysis. The point-inoculated ears were assessed 7–20 dpi for the development of symptoms. Disease symptoms were quantified by counting the total number of spikelets at and below the point of inoculation exhibiting bleaching symptoms. Analysis of variance (ANOVA) on the results at 15 and 20 dpi was conducted with XLstat software version 2008.7.3 (Addinsoft™). At 20 dpi, the ears were separated from the plants and photographed.

**Trichothecene analysis**

For in vitro liquid culture assays, trichothecenes were extracted directly from liquid media as described previously (Merhej et al., 2010). The quantification of extracted trichothecenes was performed using high-performance liquid chromatography with diode-array detection (HPLC-DAD), as described previously (Bily et al., 2004).

For in planta assays, whole wheat ears were spray inoculated and the entire spikes were harvested at 10 dpi. The awns were removed with scissors. Spikes were freeze dried for 2 days and ground to a fine powder in the presence of liquid nitrogen using a mortar and pestle. The combined DON and 15ADON content in the wheat ear powder was analysed by competitive enzyme-linked immunosorbent analysis (ELISA) using the commercially available Diagnostix EZ-Quant vomitoxin kit (supplied by Thermo Fisher Scientific, Mississauga, ON, Canada; Cat. no. 600312), following the manufacturer’s instructions. Samples outside the calibration
Aurofusarin analysis

Aurofusarin was extracted from 7-day-old PDA cultures. Briefly, 20 g of PDA cultures from wild-type and FgVel1D18 strains were ground and mixed with 5 ml of chloroform. The mixture was shaken at 40 rpm for 1 h. The chloroform extract was concentrated to dryness at 40 °C and then dissolved in methanol–water (50%, v/v). Samples were analysed by HPLC-DAD and high-performance liquid chromatography–electrospray ionization (HPLC-ESI/MS). HPLC-DAD analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph equipped with an autosampler, an Agilent photodiode array detector (DAD), a ChemStation chromatography manager software and a Zorbax® Eclipse XDB-C8 column (150 mm £ 4.6 mm, 5 μm) (All equipment purchased from Agilent Technologies, Massy, France). HPLC analysis was performed at a wavelength of 270 nm using the conditions described previously (Kim et al., 2005).

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses were performed with a QTRAP 2000 LC-MS/MS System (Applied Biosystems, Villebon Sur Yvette, France), equipped with an ESI source and an 1100 Series HPLC System (Agilent). Chromatographic separation was achieved as described above. The electrospray interface was used in the positive ion mode at 400 °C with the following settings: curtain gas, 25 psi; nebulizer gas, 35 psi; auxiliary gas, 65 psi; ion spray voltage, +4200 V; declustering potential, 30 V; collision energy, 10 eV; entrance potential, 10 V.

ACKNOWLEDGEMENTS

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REFERENCES


