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

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SUMMARY

Trichothecenes 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 trichothecene 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 trichothecene family, *F. graminearum* produces the red pigment aurofusarin. The gene regulation underlying the production of aurofusarin is not well understood. The *velvet* gene (*veA*) is conserved in various genera of filamentous fungi. Recently, 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 highly 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 trichothecenes. 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 trichothecene mycotoxins which accumulate in the infected grains, thus making them unsuitable for food and feed (Goswami and Kistler, 2004). *Fusarium graminearum* produces type B trichothecenes, including deoxynivalenol (DON), acetyldeoxynivalenol (3ADON or 15ADON), nivalenol (NIV) and acetylnivalenol (4ANIV) (Xu and Berrie, 2005). The biosynthetic pathway leading to the formation of trichothecene 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 trichothecene 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 trichothecene 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_2O_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-Gadais *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 biosynthesis (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 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%), FfVel1 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 *velB/Vel2* 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-

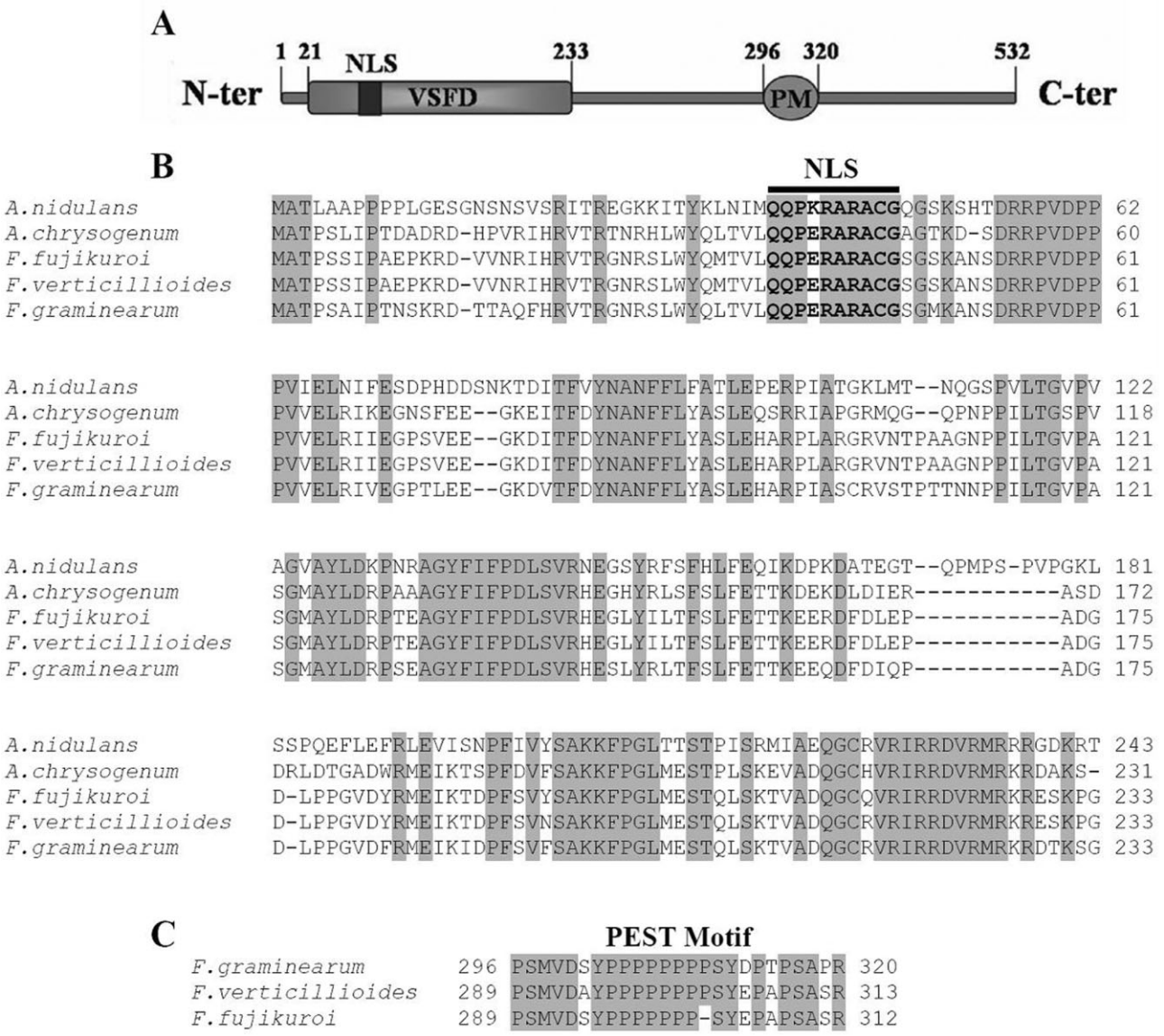

Fig. 1 Alignment of FgVe1. (A) Representation of the conserved domains in the FgVe1 protein. NLS, nuclear localization signal sequence; PM, proline-, glutamate-, serine- and threonine-rich (PEST) motif; VSFD, velvet superfamily domain. (B) Multiple sequence alignment of the first 233 amino acids from the N-terminus corresponding to the velvet superfamily domain of the predicted FgVe1 protein with VeA proteins from filamentous fungi. Species and accession numbers are as follows: *Aspergillus nidulans* (AAD42946.1); *Acremonium chrysogenum* (CAL68582.1); *Fusarium fujikuroi* (FN548142.1); *Fusarium verticillioides* (ABC02879.1). The conserved amino acids are shaded. Bold amino acids indicate the nuclear localization signal (NLS). The alignment was generated with CLUSTALW software. (C) Alignment of the potential PEST motif from the amino acid sequence of FgVe1 with the PEST motif from FvVe1 and FfVel1.

able 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 gene disruption and complementation

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

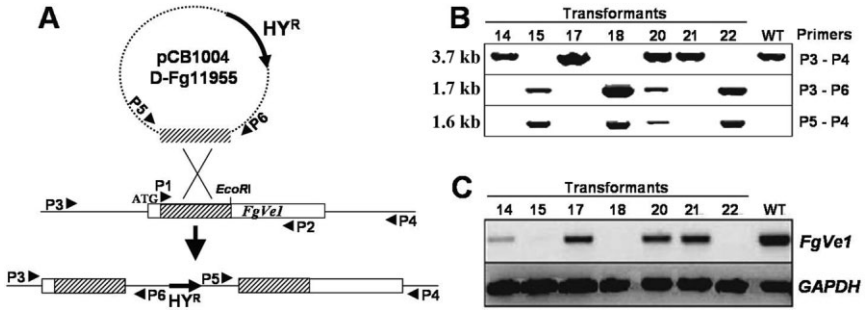

Fig. 2 Disruption of FGSG_11955. (A) Expected result for insertion of pCB1004-D-Fg11955 disruption vector into the genome of *Fusarium graminearum* strain CBS185.32 via a single homologous integration event. The polymerase chain reaction (PCR) fragment amplified from wild-type genomic DNA with primers 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.

the coding sequence and to amplify the whole locus (Fig. 2A). Figure 2B shows the diagnostic PCR analysis of these transformants. Only three (15, 18 and 22) appeared to be true gene disruptants. The remaining four transformants showed evidence of ectopic insertion events and still contained the wild-type *velvet* gene. The mycelium of the three disrupted mutants exhibited a dark yellow colour. Reverse transcription (RT)-PCR analysis of RNA extracted from the transformants revealed that these three mutants failed to produce the *FgVe1* transcript (Fig. 2C). To confirm that the phenotypes exhibited by the mutant strains were a result of the loss of *FgVe1* function, one of the three disrupted strains (transformant 18) was transformed with an intact copy of *FgVe1*. Seven transformants were obtained and analysed by PCR. One transformant showed the presence of a wild-type copy of *FgVe1* (data not shown). The disrupted strain 18 and the *FgVe1*-complemented strain were selected and designated as *FgVe1D18* and *FgVe1D18-C*, respectively, and used in further experiments.

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 $(5.98 \pm 0.59) \times 10^6$ spores and $(3.48 \pm 3.83) \times 10^6$ 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 Fg*Ve1D18* 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).

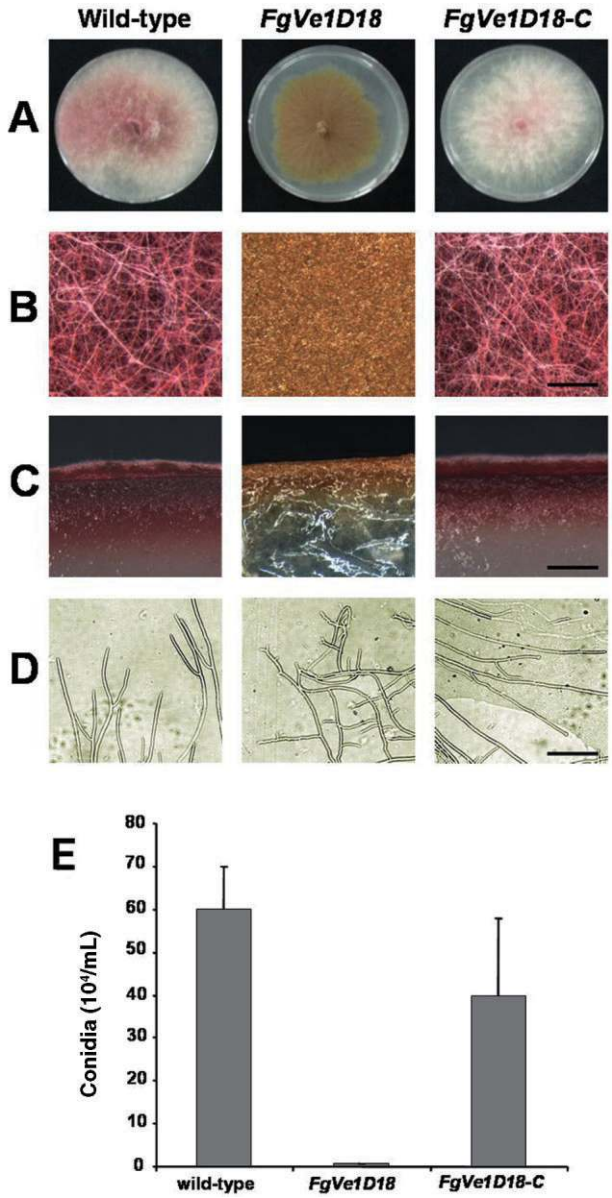

Fig. 3 Impact of *FgVe1* disruption on fungal development and conidiation. (A) Growth morphology of *Fusarium graminearum* wild-type, *FgVe1D18* and *FgVe1D18-C* strains on potato dextrose agar (PDA) medium. (B) Assessment of aerial mycelia production of the indicated strains on PDA medium. Photographs were taken using a binocular microscope. Bar, 2 mm. (C) Transverse section across the indicated strains grown on PDA medium to evaluate the thickness of the aerial mycelium. Photographs were taken using a binocular microscope. Bar, 2 mm. (D) Hyphal tip extension between two cellophane layers on a PDA plate. Bar, 100 μm. (E) Conidiation yields of *F. graminearum* wild-type, *FgVe1D18* and *FgVe1D18-C* strains after 3 days of culture in carboxymethyl cellulose (CMC) medium. Error bars represent the standard error of the mean.

Table 1 Effect of *FgVe1* disruption on *in vitro* growth*.

dpi†	Wild-type	*FgVe1D18*	*FgVe1D18-C*
3	34.45 ± 1.11	19.32 ± 1.56	32.25 ± 0.21
4	36.34 ± 2.01	21.14 ± 0.25	34.78 ± 1.52
5	36.22 ± 1.34	20.89 ± 1.48	35.26 ± 2.13
7	37.78 ± 2.45	21.37 ± 1.11	37.45 ± 4.88

*Cultures were performed in triplicate. Values are given as the weight of lyophilized mycelia in milligrams ± standard deviation.
†dpi, days post inoculation.

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

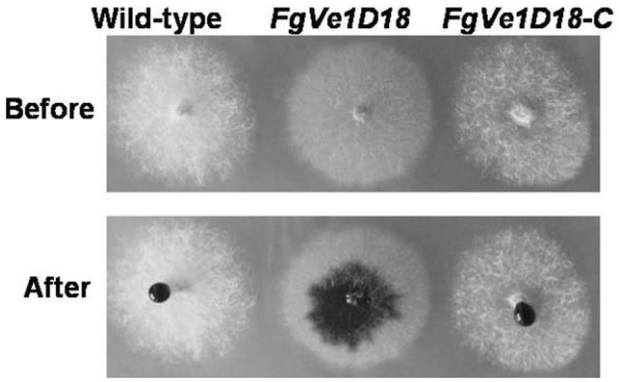

Fig. 4 Impact of FgVe1 disruption on the hydrophobicity of the surface mycelium. The wild-type, *FgVe1D18* and *FgVe1D18-C* strains were grown for 2 days on potato dextrose agar (PDA) solid medium in order to form colonies of nearly 2 cm in diameter. On top of each colony, 15 μL of water coloured with bromophenol blue were applied. Photographs were taken before and 2 min after the addition of the coloured water.

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, $[M + H - CH_3]^+$ *m/z* 556 and $[M + H - 2 \times CH_3]^+$ *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

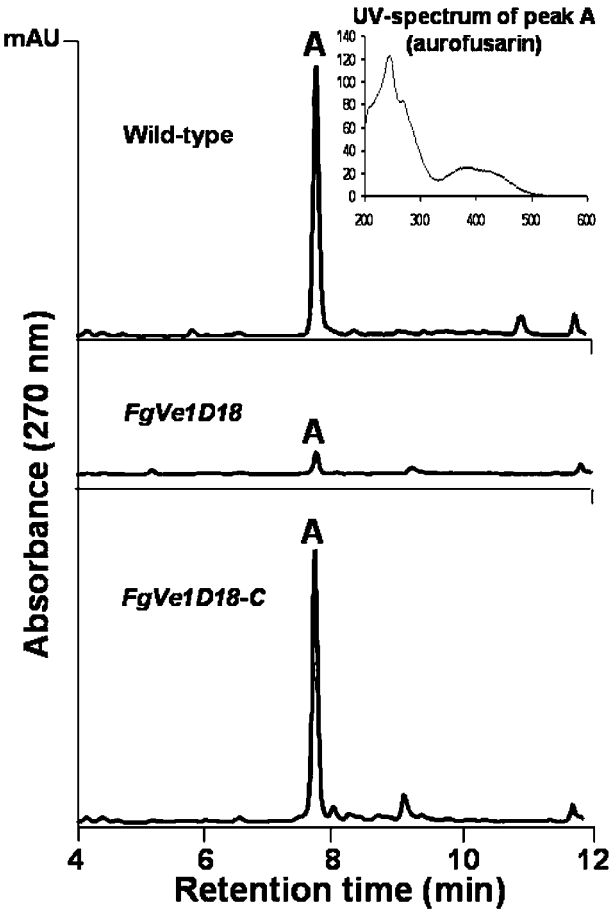

Fig. 5 *Ve1* mutation reduces aurofusarin biosynthesis. High-performance liquid chromatography (HPLC)-UV chromatograms of aurofusarin in wild-type, *FgVe1D18* and *FgVe1D18-C* strains. The UV spectrum of peak A from all strains corresponds to the typical UV spectrum of aurofusarin with a maximal absorbance at 250 nm (Malz *et al*., 2005).

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene, used as a control, showed a similar level in all three strains studied (Fig. 6B). The data obtained showed that all tested *Tri* genes are expressed in the wild-type and complemented strains at the same level, whereas no expression is detectable in the *FgVe1*-disrupted strain (Fig. 6B). These results are consistent with the profile of toxin production.

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

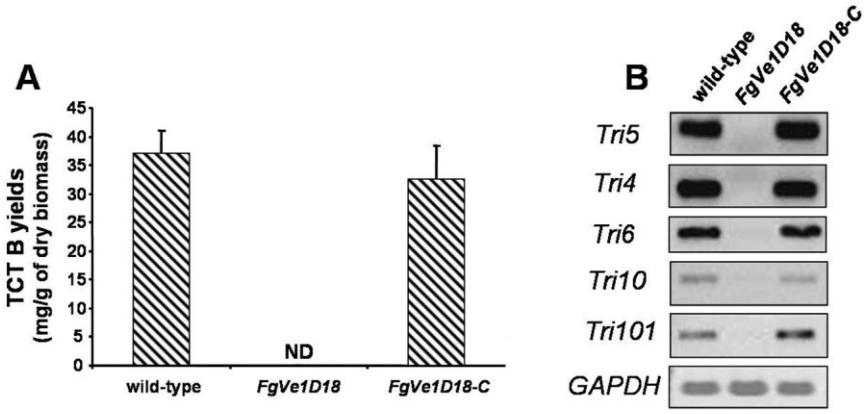

Fig. 6 Trichothecene biosynthesis and *Tri* gene expression during *in vitro* culture. (A) Amount of trichothecene B (TCT B) accumulation after 7 days of culture for wild-type, *FgVe1D18* and *FgVe1D18-C* strains. ND, not detectable. Error bars represent the standard error of the mean. (B) Expression of *Tri5*, *Tri4*, *Tri6*, *Tri10* and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) by reverse transcription-polymerase chain reaction (RT-PCR) at day 4 post-inoculation for the wild-type, *FgVe1D18* and *FgVe1D18-C* strains. The primer pairs used to perform RT-PCR are listed in Table 2.

Fig. 7 Pathogenicity phenotypes of wild-type and *FgVe1* mutant strains. (A) Wheat ears of the 'Bobwhite' cultivar were point inoculated with wild-type, *FgVe1D18* and *FgVe1D18-C* strains and incubated for 20 days. The two central inoculated spikelets are marked using a black dot. (B) Wheat ears of the 'Bobwhite' cultivar spray inoculated with wild-type, *FgVe1D18* and *FgVe1D18-C* strains and incubated for 10 days.

reduced in development and conidiation. Therefore, the capacity of the *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 *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 *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 (*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 *FgVe1D18* mutant remained healthy.

In the plug-inoculated ears, the results obtained were similar to those in the point inoculation test, as the *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 *FgVe1D18* strain did not exhibit any visual symptoms; the spikelets remained green, continued to flower and to develop. The measurement of DON produced *in planta* 10 days after spray inoculation showed that DON biosynthesis by the *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 *in planta* DON production by the *FgVe1D18* mutant strain was detectable, these wheat results were broadly in agreement with the trichothecene production levels detected *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 (VelB 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 *Ac. 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 aflatrem 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 *Ffvel1* 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 (but 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 PcVelA and PcLaeA from *P. chrysogenum* 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*, 10^5 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 *Fvve1* 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 *Fvve1*, 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 FGSG_11955. However, this ORF still lacked a part of the central sequence. We designed the primers D-FGSG11955-F and FGSG11955-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 *Fvve1* 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 FGSG11955-R. The resulting amplicon containing two *Eco*RI 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 *Eco*RI. The resulting 698-bp *Eco*RI 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).

Table 2 Primers used for reverse transcription-polymerase chain reaction (RT-PCR) and FGSG_11955 disruption.

Primers for RT-PCR analysis				
Gene	Accession no.	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	T_m (°C)
FgVe1	FGSG11955	ACGTTCCACCCTCACCTTC	TCTCGTGTTTGCGCTTACC	60
TRI5	FG03537	GACCCTAAGCGACTACAG	GTGCTACGGATAAGGTTC	58
TRI4	FG03535	TATTGTTGGCTACCCCAAGG	TGTCAGATGCGCCTTACAAA	58
TRI6	FG03536	AGCGCCTTGCCCCTCTTTG	AGCCTTTGGTGCCGACTTCTTG	58
TRI10	FG03538	TCTGAACAGGCGATGGTATGGA	CTGCGGCGAGTGAGTTTGACA	58
TRI101	AB011417	AGGGCATTAGCGAGGGAAACACA	ACGGCACGCCTTGGAGAGTAGAC	59
GAPDH	FG06257	CCTTTCATTGAGCCTCAC	CGTACATGGGAGCGTC	59

Primers for FGSG_11955 disruption and screening		
Name	Sequence (5′–3′)	T_m (°C)
*D-FGSG11955-F**	GAGAATTCCCACAAACTCAAAACGC	60
FGSG11955-R	TCTCGTGTTTGCGCTTACC	60
Ve1-C-F	TCTTGCACCAAACCATTCAG	60
Ve1-C-R	TTTCACGCTTGGCTGTAATG	60
T3	ATTAACCCTCACTAAAGGGAAC	62
T7	GTAATACGACTCACTATAGGGC	62

*The underlined sequence shows the placement of the *Eco*RI 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/m^2 at the plant surface. Once the plants entered anthesis, individual attached ears that bore two to five spikelets with extruded anthers were selected to perform infection assays.

For spray infection assays, 20 mL of spore suspension at 10^4 spores/mL was sprayed onto four ears until run-off. For point inoculation, 2 μL of the same spore suspension were placed within a floral chamber of two 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

curve were diluted 10-fold with water until values fell within the calibration curve. DON concentrations were determined using the DIAGNOSTICS data reduction program supplied by the manufacturer. Trichothecene assays were performed using three biological replicates.

Aurofusarin analysis

Aurofusarin was extracted from 7-day-old PDA cultures. Briefly, 20 g of PDA cultures from wild-type and *FgVe1D18* 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)mass spectrometry [HPLC-(ESI)MS]. HPLC-DAD analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph equipped with an autosampler system, 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.

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