FgVelB globally regulates sexual reproduction, mycotoxin production and pathogenicity in the cereal pathogen Fusarium graminearum

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The velvet genes are conserved in ascomycetous fungi and function as global regulators of differentiation and secondary metabolism. Here, we characterized one of the velvet genes, designated FgVelB, in the plant-pathogenic fungus Fusarium graminearum, which causes fusarium head blight in cereals and produces mycotoxins within plants. FgVelB-deleted (∆FgVelB) strains produced fewer aerial mycelia with less pigmentation than those of the wild-type (WT) during vegetative growth. Under sexual development conditions, the ∆FgVelB strains produced no fruiting bodies but retained male fertility, and conidiation was threefold higher compared with the WT strain. Production of trichothecene and zearalenone was dramatically reduced compared with the WT strain. In addition, the ∆FgVelB strains were incapable of colonizing host plant tissues. Transcript analyses revealed that FgVelB was highly expressed during the sexual development stage, and may be regulated by a mitogen-activated protein kinase cascade. Microarray analysis showed that FgVelB affects regulatory pathways mediated by the mating-type loci and a G-protein alpha subunit, as well as primary and secondary metabolism. These results suggest that FgVelB has diverse biological functions, probably by acting as a member of a possible velvet protein complex, although identification of the FgVelB–FgVeA complex and the determination of its roles require further investigation.

INTRODUCTION

Fusarium graminearum (telomorph: Gibberella zeae) is a homothallic ascomycetous fungus that causes fusarium head blight (FHB) of cereal crops and produces mycotoxins such as trichothecenes and zearalenone (ZEA) in host plants, threatening human and animal health (Leslie & Summerell, 2006). Trichothecenes are potent inhibitors of eukaryotic protein biosynthesis and are a virulence factor in plants (Desjardins, 2006; Proctor et al., 1995), and ZEA causes oestrogenic disorders in laboratory rats, mice and farm-raised swine (Desjardins, 2006). Fungal propagules enter plant florets through the anthesis during the soft stage of kernel development, and the infected florets become necrotic and bleached. Ascospores within perithecia produced on crop debris are forcibly discharged into the air to initiate the infection, and conidia are produced on the infected heads, playing a role in disease dispersal (Parry et al., 1995). Therefore, the sexual and asexual development of this fungus is very important for initiation and dispersal of the disease in fields.

To identify genes related to pathogenicity, sexual/asexual development and toxin production in F. graminearum, forward and reverse genetics approaches have been extensively performed by many research groups worldwide. A recent study that systematically deleted all putative transcription factors (TFs) in this fungus found that many TFs are required for these primary fungal traits (Son et al., 2011b). In addition to the specific TFs, putative global regulators that are involved in the regulation of more diverse functions have also received attention. Recently, the velvet family proteins, which are global regulators

Abbreviations: COG, Clusters of Orthologous Groups; DON, deoxynivalenol; FDR, false discovery rate; FHB, fusarium head blight; GO, gene ontology; MAP kinase, mitogen-activated protein kinase; TF, transcription factor; WT, wild-type; ZEA, zearalenone.

The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE36638.

Supplementary material, with supplementary references, and 10 supplementary tables are available with the online version of this paper.
conserved in filamentous fungi (Bayram & Braus, 2012; Jiang et al., 2011; Kato et al., 2003; Kim et al., 2002; Merhej et al., 2012; Wiemann et al., 2010), have been identified in *F. graminearum*. In *Aspergillus nidulans*, the velvet protein complex forms a heterotrimeric complex consisting of VelB–VeA–LaeA proteins, and regulates diverse cellular functions such as sexual development and secondary metabolism by participating in chromatin remodelling. The *veA* gene is a positive regulator of sexual development but also represses asexual development (Kim et al., 2002). It is also required for biosynthesis of the mycotoxin sterigmatocystin and the antibiotic penicillin (Bok et al., 2006; Keller et al., 2005). *VelB* represses asexual development and is essential for asexual and sexual spore maturation (Bayram et al., 2010). In *F. graminearum*, disruption of the *veA* orthologue, designated *FgVeA*, suppresses aerial hyphae formation, sporulation, production of several metabolites, such as aurofusarin pigment and trichothecene, and virulence towards the host plant (Jiang et al., 2011; Merhej et al., 2012). These results indicate that the *F. graminearum* velvet complex also plays a pivotal role in the regulation of sexual development and secondary metabolites, similar to its function in *A. nidulans* and *Fusarium fujikuroi* (Wiemann et al., 2010). However, the function of an orthologue of *VelB*, designated *FgVelB*, has not been intensively studied in *F. graminearum*. Therefore, in the present study, we investigated the biological functions of *FgVelB* and identified putative regulatory networks controlled by *FgVelB* under various conditions. Our results provide insight into the global regulatory pathway that controls the major traits of *F. graminearum* through *FgVelB* itself or a possible velvet complex.

**METHODS**

**Fungal strains and culture conditions.** The *F. graminearum* strain used was GZ3639 (Bowden et al., 2008) belonging to lineage 7 of the *F. graminearum* species complex (O’Donnell et al., 2000). This self-fertile wild-type (WT) strain is highly virulent in wheat and barley, and produces mycotoxins such as deoxynivalenol (DON), its 15 acetyl-deoxynivalenol derivative, and ZEA at relatively high concentrations. These WT and mutant strains were stored in a 20% (v/v) glycerol solution at −70°C. Conidiation and sexual reproduction were induced on carrot agar (Leslie & Summerell, 2006). For genomic DNA extraction, each strain was grown in 50 ml complete medium (CM) (Leslie & Summerell, 2006) at 25°C for 72 h on a rotary shaker (150 r.p.m.). The number of conidia produced on carrot agar after removal of aerial mycelia was counted as follows. An agar block (4 mm in diameter) was taken from carrot agar and resuspended in a microcentrifuge tube with 1 ml distilled water by vortexing for several seconds. Then the numbers of conidia were counted using a haemocytometer. For mycotoxin production and DNA gel blot hybridization of genes related to mycotoxin biosynthesis, 1 ml conidial suspension (10⁶ conidia ml⁻¹) of each strain was inoculated into rice substrate covered with three layers of cheesecloth. The mycelia on the cheesecloth and rice substrate were used to extract RNA and mycotoxins, respectively.

**Nucleic acid manipulations and primers.** Fungal genomic DNA was prepared as described previously (Leslie & Summerell, 2006), and total RNA was extracted from mycelia using an Easy-Spin Total RNA Extraction kit (Intron Biotech) according to the manufacturer’s instructions. Restriction endonuclease digestion, agarose gel electrophoresis, and DNA and RNA blot hybridization were performed following standard techniques (Sambrook & Russell, 2001). The PCR primers (Table 1) used in this study were synthesized by the Bioneer Corporation and those for quantitative real-time PCR are listed in our previous study (Lee et al., 2010). For cDNA synthesis and microarray analysis, total RNA was isolated from 3-day-old carrot agar culture after sexual induction.

**Targeted gene deletions and complementation.** A DNA construct for deleting *FgVelB* from the *F. graminearum* genome was created using the double-joint PCR method, as described previously (Yu et al., 2004). The 5’ and 3’ flanking regions of *FgVelB* were amplified from the WT strain GZ3639 with *VelB*-p4/*VelB*-p2 and *VelB*-p3/*VelB*-p1 primer sets, respectively (Table 1), and fused to 1.8 kb of the geneticin-resistance gene cassette (*gen*) as a fungal selectable marker amplified from vector pH99 (Namiki et al., 2001) using primers GenFor and GenRev. Primers *VelB*-p1 and *VelB*-p2 carried 19 bp of nucleotide sequence corresponding to GenFor and GenRev, respectively. The final fusion PCR product, amplified using

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’–3’)</th>
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<tr>
<td>GenFor</td>
<td>CTCTAACACAGTGATACCTCTGC</td>
</tr>
<tr>
<td>GenRev</td>
<td>CGACAGAAGATGATATTTGAAAGG</td>
</tr>
<tr>
<td>VelB-p1</td>
<td>GAGGTACACTTTGTTAGACGGCGACGAAGATGATGTA</td>
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</tr>
<tr>
<td>FgVelB-p2</td>
<td>CCCCCGGGTCCTCTTCTACC</td>
</tr>
<tr>
<td>FgVelB-p3</td>
<td>AAGCGGGGAGAGATGGATTTGAGC</td>
</tr>
<tr>
<td>FgVelB-p4</td>
<td>CGAACGGGGGAAGGCGAGATGAGC</td>
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*Underlined sequences for VelB-p1 and VelB-p2 complementary to those of GenFor and GenRev, respectively.*
the nested primers VelB-p3 and VelB-p4, was added to fungal protoplasts for transformation, as described previously (Lee et al., 2002). Fungal transformants were selected on regeneration medium containing geneticin (75 µg ml\(^{-1}\)). For genetic complementation, the FgVelB gene (including its native promoter and terminator regions) was amplified from the GZ3639 strain using primers VelB-p3 and VelB-p4. This amplicon was cloned into a pGEM-T Easy vector (Promega) to generate plasmid pFgVelB, which was introduced into the ΔFgVelB strain protoplasts with pUCH1 (Turgeon et al., 1987), which carries a hygromycin-resistance gene cassette (hph), and fungal transformants were selected on regeneration medium containing hygromycin (75 µg ml\(^{-1}\)).

Sexual reproduction. Sexual reproduction of F. graminearum was induced on both carrot agar and carnation leaf agar, as described elsewhere (Leslie & Summerrell, 2006). For outcrosses, the mycelial plug of a self-sterile strain that carries a deletion of the MAT1-1 gene (Lee et al., 2003) was placed on carrot agar and incubated at 25 °C for 7 days. Conidial suspension (10\(^5\) conidia ml\(^{-1}\)) of each fungal strain was dropped onto mycelia of the ΔMAT1-1 strain to induce outcresses, as described previously (Lee et al., 2003). The fertilized plates were incubated for an additional 5–10 days under a mixture of white and blue light with a 12 h photoperiod (Leslie & Summerrell, 2006).

Virulence test. The virulence of fungal strains was determined on wheat and barley, as described previously (Son et al., 2011a). Briefly, conidia of each strain were harvested from carrot agar and suspended in sterile water at a concentration of 1 × 10\(^5\) conidia ml\(^{-1}\). This suspension was injected into a centre spikelet of wheat (cv. Eunpamil) head at mid-anthesis, or sprayed onto the barley (cv. Sangrok) spikes, and the plants were placed in a greenhouse after a 3 day incubation in a humidity chamber. Spikelets with FHB symptoms were counted 14 days after inoculation.

Mycotoxin analysis. Each F. graminearum strain was screened for ZEA and trichothecene production on rice medium. Rice cultures were harvested 3 weeks after incubation at 25 °C and extracted, as described previously (Seo et al., 1996). A Shimadzu LC-6A HPLC equipped with an RF-10A XL fluorescence detector (Shimadzu) was used to analyse ZEA, and a Shimadzu QP-5000 GC-MS in full scan mode was used for trichothecene analysis, as described previously (Seo et al., 1996).

Microarray analysis and quantitative real-time PCR. Microarray analysis was performed as described previously (Lee et al., 2010). Expression profiling was conducted with the Fusarium_tiling_300k MicroArray Analysis designed from the F. graminearum sequencing assembly containing 13,382 transcripts (http://www.broadinstitute.org/annotation/genome/fusarium-group/MultiHome.html), which was manufactured at NimbleGen (http://www.nimblegen.com/). In total, 133,612 probes were designed with an average size of 60 nt. Experiments were repeated two times with independently prepared total RNA samples. The data were normalized and processed with cubic spline normalization using quantiles to adjust signal variations between chips. Probe-level summarization by Robust Multi-Chip Analysis (RMA) using a median polish algorithm implemented in NimbleScan was used to produce call files. Multiple analyses were performed with the limma package in the R computing environment. Linear models and empirical Bayes methods were used for assessing differential expression in microarray experiments. Complete datasets of the microarray experiments can be found in Tables S1–S10 available with the online version of this paper. For quantitative real-time PCR, total RNA was isolated from vegetative cultures (3-, 5- and 7-day carrot agar cultures) and cultures at various sexual stages (carrot agar cultures 3, 5 and 7 days after induction of sexual reproduction), and the amount of each gene transcript from a 3 day-old vegetative sample was used as a reference; other procedures were performed as previously described (Lee et al., 2010; Kim & Yun, 2011).

Gene ontology (GO) analysis. GO term enrichments were calculated using GOMiner (http://www.geneontology.org/; http://discover.nci.nih.gov/gominer/index.jsp/). A total of 8338 of 13,382 genes from the F. graminearum PH-1 genome were matched to the Magnaporthe grisea sequencing assembly SCS (http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/) with scores of 100 or greater based on BLASTP analysis, and were used as a total gene set for GOMiner analysis. False discovery rate (FDR) values were obtained from 100 randomizations. GO terms with FDRs of less than 0.05 were collected. To further characterize each protein, the up-to-date Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases were used for pathway analysis (Kanehisa et al., 2006). All proteins were searched against the Clusters of Orthologous Groups (COG) database (Tatusov et al., 2000).

RESULTS

Identification of FgVelB in F. graminearum

The A. nidulans velB gene (GenBank accession no. ABQ17967, 1369 aa) was searched against the F. graminearum genome databases to find its orthologues in F. graminearum. The F. graminearum gene showing the highest similarity (with 64 % over 134 aa) was FGSG_01362.3, which contains a 2389 bp gene encoding a 136 aa protein annotated as a hypothetical protein. FgVeA (FGSG_11955.3), encoding a velvet protein (Jiang et al., 2011), showed the second highest similarity (40 % over a 104 aa region). For verification of the FgVelB transcript, we amplified cDNA clones of FgVelB from total RNA of fungal mycelia grown in potato dextrose broth (PDB) for 3 days using the primer pairs FgVelB-p1/FgVelB-p2 and FgVelB-p3/FgVelB-p4 by RT-PCR. Sequencing of the amplified cDNA fragments confirmed the presence of five introns in FgVelB, and identified a new 471 aa protein that is longer than the original protein (136 aa) predicted in the genome database. The new FgVelB, which carries two velvet factor conserved domains, showed high sequence similarity to VelB orthologues from other filamentous fungi: 77 % to F. fujikuroi velvet 2 (CBK25977), 52 % to VelB of Metarhizium anisopliae (EFZ03170) and 46 % to VelB of Magnaporthe oryzae (EHA54799).

Targeted deletion of the FgVelB gene and complementation

To determine the function of FgVelB in F. graminearum, the entire FgVelB gene (474 aa) was replaced with a fungal selectable marker gen in the genome of GZ3639 using a targeted gene-replacement strategy. A PCR fragment (4.5 kb) that carries gen fused to both the 5'-flanking and 3'-flanking region of FgVelB was added to fungal protoplasts of GZ3639, and putative transformants were selected on geneticin-amended medium. Southern analysis identified fungal transformants with a FgVelB deletion generated by homologous recombination (Fig. 1). Ten transformants were confirmed to contain a FgVelB deletion.


**Fig. 1.** Deletion and complementation of *FgVelB* from the WT strain GZ3639. *FgVelB* was replaced with a geneticin-resistance gene cassette (gen) by homologous recombination (left) and the circular plasmid pFgVeLB carrying *FgVelB* was introduced into Δ*FgVelB* (middle). The targeted gene deletion and pFgVeLB integration into Δ*FgVelB* were confirmed by Southern analysis (right). Lanes: 1, wild-type strain GZ3639; 2 and 3, *FgVelB*-deletion strains; 4, Δ*FgVelB* strain that carried pFgVeLB. Genomic DNA was digested with XhoI (indicated by X) and probed with the 5′ flanking region of *FgVelB*.

(Fig. 1) designated Δ*FgVelB*). For genetic complementation, the circular plasmid pFgVeLB carrying *FgVelB* and its flanking sequences was introduced into the Δ*FgVelB* strain derived from GZ3639 along with pUCH1 carrying a hygromycin B-resistance gene. A total of eight out of 15 transformants resistant to both geneticin and hygromycin B carried an integration of pFgVeLB, as confirmed by Southern analysis (Fig. 1).

**FgVelB** affects vegetative growth, pigmentation and conidia production

The Δ*FgVelB* strains derived from the WT strain GZ3639 produced fewer aerial mycelia under several growth conditions, such as potato dextrose agar (PDA; Difco Laboratories), minimal medium (MM) and carrot agar, while GZ3639 produced thick aerial hyphae (Fig. 2). The Δ*FgVelB* strains produced more yellowish or white mycelia on PDA and carrot agar, respectively, instead of the reddish-pink mycelia produced by the WT strain on solid media. In addition, the Δ*FgvelB* strains showed different patterns of conidiation on carrot agar compared with the WT strain. For example, conidial production in the WT strain increased until 3 days after inoculation for vegetative growth, and then remained at a similar level until 6 days after inoculation. When the aerial mycelia were removed for perithecia induction (representing the sexual development process), conidiation of the WT strain was generally repressed during sexual development. In contrast, the Δ*FgVelB* strain showed a severe reduction in conidial production until 3 days after inoculation, but almost recovered back to the WT levels on day 5 after inoculation and continued to produce large numbers of conidia after perithecial induction. Ultimately, after 10 days on carrot agar the numbers of conidia produced by the Δ*FgVelB* strains were approximately threefold higher than those of the WT strain. The conidiation phenotype during sexual development of the complementation strain (Comp), which is the Δ*FgVelB* strain carrying an intact copy of *FgVelB*, was very similar to that of the WT strain (Fig. 3).

**ΔFgVelB** affects sexual reproduction

Self-fertility of the Δ*FgVelB* strains was determined based on perithecia production on both carrot agar and carnation leaf agar. The GZ3639 strain began to produce protoperithecia 3 days after sexual induction on carrot agar or on carnation leaf agar 12 days after inoculation. After an additional 3 or 4 day incubation, mature perithecia formed; they contained asci, each with eight ascospores. In contrast, the Δ*FgVelB* strains produced only mycelia and/or conidia, and never produced fruiting bodies (Fig. 4). All sexual defects in the

![Fig. 2. Vegetative growth and pigmentation of *F. graminearum* strains on PDA and carrot agar. WT, wild-type strain GZ3639; Δ*FgVelB*, the Δ*FgVelB* strain derived from GZ3639; Comp, the Δ*FgVelB* strain carrying an intact copy of *FgVelB*.](image-url)
ΔFgVelB strains were recovered to the WT level in the complementation strain Comp.

When a conidial suspension of the ΔFgVelB strain was spermatized for the self-sterile ΔMAT1-2 strain, fertile perithecia successfully formed on carrot agar, indicating that the ΔFgVelB strain retains male fertility. We purified 61 single ascospores from the mating plate 10 days after induction and checked the self-fertility of each progeny. Of the 61 single ascospore-derived isolates, 34 and 27 were resistant and sensitive to geneticin, respectively, and all of the geneticin-resistant progenies showed the same phenotypes (on carrot agar) as the ΔFgVelB parent. When a conidial suspension of the WT strain was spermatized for the ΔFgVelB strain, neither protoperithecia nor perithecia were produced. Taken together, these results demonstrate that ΔFgVelB strains lose female (self) fertility, although the strains are male-fertile.

FgVelB affects virulence on host plants and mycotoxin production on rice substrate

In plant inoculation tests, the WT strain caused typical head blight and complete bleaching on wheat, whereas the ΔFgVelB strains caused few blight symptoms even on the inoculated spikelets (Fig. 5). In addition, the WT strain produced both DON and ZEA on rice substrates, whereas toxin production in the deletion strains was significantly reduced. HPLC analysis revealed that ZEA production in the ΔFgVelB was approximately 100-fold lower than that of the WT strain. DON was not detectable in the ΔFgVelB strains by GC/MS analysis (Fig. 6).

Transcript analyses of FgVelB

FgVelB transcript was weakly detected in the WT strain GZ3639 during vegetative growth on carrot agar, and accumulated at higher levels throughout the entire perithecial stage than during vegetative growth (Fig. 7). No FgVelB transcript was detected in GZ3639 when grown in nutrient-rich CM liquid medium or in the ΔFgVelB strain on carrot agar (data not shown). To explore FgVelB function during the perithecial stage, the transcript levels of FgVelB were analysed in the F. graminearum strains lacking either sexual reproduction ability or a component in the signal transduction pathway, such as mating type genes (MAT1-1 and MAT1-2) (Lee et al., 2003), G-protein α subunits (GzGPA1, GzGPA2 and GzGPA3) (Yu et al., 2008), mitogen-activated protein (MAP) kinase GPMK1 (Jenczmionka et al., 2003), MAP kinase kinase (FgSTE7) (Ramamoorthy et al., 2007) and the STE12-like TF FST12. The FgVelB transcript was dramatically reduced only in

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**Fig. 3.** Conidia production of F. graminearum strains on carrot agar. The numbers on the x axis and y axis indicate the days after inoculation on carrot agar and the number of conidia produced, respectively. WT, wild-type strain GZ3639; ΔFgVelB, the ΔFgVelB strain derived from GZ3639; Comp, the ΔFgVelB strain carrying an intact copy of FgVelB.

**Fig. 4.** Self-fertility of F. graminearum strains on carrot agar (top) and carnation leaf agar (bottom). White clumpy structures shown in the ΔFgVelB strain on carrot agar consist of conidia. WT, wild-type strain GZ3639; ΔFgVelB, the ΔFgVelB strain derived from GZ3639; Comp, the ΔFgVelB strain carrying an intact copy of FgVelB. The photographs were taken 10 days after sexual induction.

**Fig. 5.** Virulence of F. graminearum strains on wheat heads. Conidia suspensions of each strain were injected into a centre spikelet of wheat and the photographs were taken 14 days after inoculation. WT, wild-type strain GZ3639; ΔFgVelB, the ΔFgVelB strain derived from GZ3639; Comp, the ΔFgVelB strain carrying an intact copy of FgVelB.

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strains in which components of an MAP kinase signalling cascade, such as GPMK1, FgSTE7 and FST12, were deleted (Fig. 8).

In the ΔFgVelB strain grown on rice substrate for 20 days, the transcript levels of the ZEB2 and TRIS genes [which are a transcriptional activator for a ZEA biosynthesis gene cluster (Kim et al., 2005b) and encode trichodiene synthase (the enzyme for the first step in the trichothecene biosynthetic pathway), respectively], were significantly reduced compared with the WT strain (Fig. 6).

**Microarray analyses**

1. **Functional categorization of differentially regulated genes in the ΔFgVelB strain.** A comparison of the microarray expression profiles identified a total of 2038 genes differentially regulated with more than twofold changes ($P<0.05$) in the ΔFgVelB strain during the perithecial induction stage compared with its WT progenitor GZ3639; 1480 and 558 genes were significantly downregulated and upregulated, respectively (Tables S1 and S2). For functional categorization of these genes, searches against the COG databases identified 331 and 136 putative proteins orthologous to the *F. graminearum* MAT1-1, MAT1-2, GPMK1 and FST12, respectively (Table S3). Among the genes classified into the cellular processes and metabolism were enriched in a set of downregulated genes.

For further functional categorization, GO term enrichments were calculated using the 8338 genes of *F. graminearum* orthologous to the *M. grisea* genome. A total of 658 and 262 genes were downregulated and upregulated, respectively, in the ΔFgVelB strain. GO term enrichment analysis showed that 15 biological processes, four cellular components and 31 molecular functions were enriched with FDRs of $<0.05$ (Table S4). The biological processes that were enriched were nitrogen metabolism, carbohydrate metabolism, transport, localization and signalling.

2. **Differentially regulated genes related to sexual reproduction and secondary metabolism.** Comparison of the differentially regulated genes in the ΔFgVelB strain with those in the ΔMAT1-2 (i.e. MAT1-1; ΔMAT1-2) strain, which were obtained using a genome-wide microarray

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**Fig. 6.** Transcription profiles of the ZEB2 and TRIS genes (left) and toxin production (right) in *F. graminearum* strains grown on rice medium. The numbers above the RNA gel indicate the days post-inoculation into rice medium. The probes (ZEB2 or TRIS) used in RNA blot hybridization are indicated on the left side of the gel. rRNA was used as a loading control. The levels of ZEA and trichothecenes produced are indicated by grey and white bars, respectively. WT, wild-type strain GZ3639; ΔFgVelB, the ΔFgVelB strain derived from GZ3639; Comp, the ΔFgVelB strain carrying an intact copy of FgVelB.

**Fig. 7.** RNA blot hybridization of FgVelB in the WT GZ3639 strain on carrot agar before and after sexual induction. The numbers above the gel indicate the days of cultivation in each condition.

**Fig. 8.** RNA blot hybridization of FgVelB in *F. graminearum* strains deleted for GPMK1 (ΔGPMK1), FgSTE7 (ΔFgSTE7) and FST12 (ΔFST12) on carrot agar after sexual induction. The numbers above the gel indicate the days after sexual induction on carrot agar. WT, GZ3639.
H.-K. Kim and others, unpublished data), identified 309 downregulated and 57 upregulated genes, which were common to both self-sterile strains of *F. graminearum* (Tables S5 and S6, Fig. 9). In addition, 378 downregulated and 81 upregulated genes overlapped with the gene sets differentially regulated in the other self-sterile strain lacking a G-protein alpha subunit (GzGPA1) (Lee et al., 2010) under the same conditions (Tables S5 and S6, Fig. 9). Ten genes (FGSG_04480, FGSG_05239, FGSG_05325, FGSG_08320, FGSG_03673, FGSG_06484, FGSG_07578, FGSG_07590, FGSG_00348 and FGSG_01862), which were downregulated in the ΔGzGPA1 strain and are known to be directly involved in sexual reproduction (Lee et al., 2010), were also downregulated in the ΔFgVelB strain; seven genes overlapped with the gene sets in all three deletion strains (ΔMAT1-2, ΔFgVelB and ΔGzGPA1 strains) (Fig. 9). Three mating-type genes (MAT1-1-1, MAT1-1-3 and MAT1-2-1) at the MAT locus, a master regulator of sexual development in *F. graminearum* (Lee et al., 2003), were downregulated in the ΔFgVelB and ΔGzGPA1 strains (Table S1); MAT1-1-3 was also downregulated in the ΔGzGPA1 strain (Fig. 9). GzPPG1 and GzPRE1, which encode a-factor pheromone and alpha factor pheromone receptor, respectively (Kim et al., 2008), were downregulated in the ΔFgVelB strain, although the reduced hybridization signals of GzPRE1 were not statistically significant (P=0.2). These two genes were also downregulated in the ΔMAT1-2 and ΔGzGPA1 strains, respectively (Fig. 9).

Putative gene clusters for secondary metabolism, which may be controlled by FgVelB, were identified when the genes with consecutive FGSG numbers were differentially regulated. The majority of them encode probable metabolic enzymes or transporters. Thirteen and three putative gene clusters were downregulated and upregulated, respectively (Table S7). In particular, five downregulated gene clusters included PKS genes (PKS3, PKS6, PKS7, PKS11 and PKS12) (Kroken et al., 2003) and non-ribosomal peptide synthetase genes (NPS4 and NPS7) (Oide et al., 2007). Among these, PKS6, PKS12 and NPS7 were downregulated only in the ΔFgVelB strains, whereas PKS3 and PKS11 were also found in the ΔMAT1-2 strain; PKS7 overlapped in all three strains (Fig. 9). A member (FGSG_08081) of the gene cluster involved in butenolide biosynthesis in *F. graminearum* (Harris et al., 2007) was downregulated only in the ΔFgVelB strain. In addition, two ATP citrate lyase genes [ACL1 (FGSG_12857) and ACL2 (FGSG_06039)], required for production of acetyl-CoA (Son et al., 2011a), a building block for polyketide biosynthesis, were downregulated in the ΔFgVelB strain (Fig. 9).

However, genes involved in signal transduction pathways that are essential for sexual development in *F. graminearum* were not differentially regulated by ΔFgVelB. For example, members of the G protein/cAMP/PKA signal transduction pathways, such as G-protein alpha subunits (GzGPA1, GzGPA2 and GzGPA3), beta subunit (GzGBP1), gamma subunit (GzGPG1), adenyl cyclase (GzACY1), cAMP-dependent protein kinases (GzPKA1, GzPKA2 and GzPKAR) (Yu et al., 2008), MAP kinases (MGV1, Hou et al., 2002; GPMK1, Jenczmionka et al., 2003; and GzHOG1, Oide et al., 2010),

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**Fig. 9.** Number of genes expressed differentially in the *F. graminearum* strains deleted for FgVelB (ΔFgVelB), MAT1-2-1 (ΔMAT1-2-1) and GzGPA1 (ΔGzGPA1). DOWN, downregulated genes; UP, upregulated genes. The numbers of genes differentially regulated in the categories are indicated in parentheses next to the fungal strains. TF, transcription factor gene.
MAP kinase kinase (FgSTE7; Ramamoorthy et al., 2007) and STE12-like TF (FST12) were not differentially regulated. In addition, histidine kinase two-component response regulator proteins [GzSSK1, GzSKN7, GzRIM15 (FGSG_03132) and GzREC1 (FGSG_08031.3)]; Oide et al., 2010] and several other genes involved in sexual reproduction were not found in the set of differentially regulated genes in the ΔFgVelB strain; for example, genes encoding a serine/threonine protein kinase (GzSNF1; Lee et al., 2009), an F-box protein (FBP); Han et al., 2007, a TF similar to human retinoblastoma binding protein 2 (GzRUM1; Kim et al., 2011), class V chitin synthases (GzCHS3 and GzCHS7; Kim et al., 2009), syntaxin-like t-SNARE (GzSYN1 and GzSYN2; Hong et al., 2010) and acetyl-CoA synthetases (ACS1 and ACS2; Lee et al., 2011) were not identified.

TFs differentially regulated by ΔFgVelB are described in the supplementary materials and listed in Table S8.

Real-time PCR analyses
A total of 108 genes identified as downregulated due to FgVelB deletion by microarray analyses were subjected to real-time PCR analysis for confirmation of their differential expression in ΔFgVelB on carrot agar (under both vegetative and subsequent perithecial-induction conditions) (Fig. 10, Table S10). All of the genes (except one) were downregulated at least one of the culture conditions examined in the ΔFgVelB strain (Table S10) and the expression profiles were divided into five groups (Fig. 10a–e). Most genes (92 out of 108) had increased expression under perithecial-induction conditions compared with vegetative conditions in the WT strain, among which 87 were significantly downregulated in the ΔFgVelB strain throughout the entire period on carrot agar (Fig. 10a). However, five (FGSG_03116, FGSG_04861, FGSG_06149, FGSG_07590 and FGSG_12210) of the 92 sexually specific genes came to be highly expressed during vegetative growth in the ΔFgVelB strain compared with WT (Fig. 10b). In contrast, nine genes [FGSG_02324 (PKS12), FGSG_02551, FGSG_03536 (TRI6), FGSG_03800, FGSG_04738, FGSG_07838, FGSG_08081, FGSG_08208 (PKS6) and FGSG_08795 (PKS7)] were more specific to vegetative growth in the WT strain, and their expression levels, except in the case of TRI6, were also significantly reduced under both vegetative and sexual conditions in the ΔFgVelB strain (Fig. 10c). The expression of TRI6, a TF controlling the gene cluster for trichothecene biosynthesis, was significantly increased under sexual conditions in the ΔFgVelB strain (Fig. 10e), while it was strongly repressed in the ΔFgVelB strain compared with that in the WT strain when grown in agmatine-amended liquid medium, a favourable condition for trichothecene production (Fig. 10f). The remaining six genes were not specific to either the vegetative or the sexual induction stage, but their expression levels were much lower than those of the other stage-specific genes in the WT strain (Fig. 10d).

DISCUSSION
The pleiotropic changes found in the ΔFgVelB strain indicate that FgVelB has diverse functions such as pigmentation, mycotoxin production, sexual development and pathogenicity in F. graminearum. Deletion of FgVelB reduced several secondary metabolites when compared with the WT strain. Perithecial formation was completely inhibited, while conidiation was not properly repressed on carrot agar. These results suggested that FgVelB positively regulates genes involved in secondary metabolism and sexual development. These phenotypic changes are very similar to those observed in the ΔFgVeA strain (Merhej et al., 2012; Jiang et al., 2011), as well as those in the A. nidulans veA or velBA strains (Bayram et al., 2008). This strongly suggests that FgVelB functions as a member of the velvet complex in F. graminearum, and may be a counterpart of the heterotrimeric A. nidulans velvet complex consisting of VeA, LaeA and VelB for global regulation of sexual development and secondary metabolism (Bayram et al., 2008).

Our microarray showed that 2038 of the approximately 13 000 F. graminearum genes were differentially regulated in the ΔFgVelB strain compared with the WT strain during the sexual induction stage, supporting the idea that FgVelB globally regulates gene expression in this fungus. Bayram et al. (2008) suggested that VelB/VeA in A. nidulans take part in the epigenetic control of chromatin remodelling by modulating LaeA methyltransferase activity, and recent studies in several fungal species have shown that secondary metabolism gene clusters are regulated by chromatin structure and epigenetic codes (Strauss & Reyes-Dominguez, 2011). Therefore, it is likely that the FgVelB and FgVeA genes regulate similar sets of genes throughout the F. graminearum life cycle. A comparison of the genome-wide expression profiles of the ΔFgVelB and ΔFgVeA strains would confirm this hypothesis. However, the set of genes differentially regulated in the ΔFgVeA strain, which were identified by RNA sequence analysis (Jiang et al., 2011), cannot be directly compared with those in the ΔFgVelB strain generated in this study because the complete dataset from the ΔFgVeA strain was not available and the culture conditions for total RNA extraction differed (vegetative growth in PDB and sexual development on carrot agar for ΔFgVeA and ΔFgVelB, respectively).

Transcript analyses of FgVelB in the fungal strains lacking several signal transduction-related genes provided insight into the hierarchy of FgVelB. The reduced accumulation of FgVelB transcript in F. graminearum strains lacking the genes for MAP kinase (GPMK1), MAP kinase kinase (FgSTE7) or an STE12-like TF (FST12) indicates that FgVelB may be a downstream member of the regulatory network controlled by, at least, an MAP kinase signalling cascade under sexual-induction conditions.

Overall, FgVelB seems to control a diverse range of genes during sexual development, including those involved in stage- or pathway-specific regulatory networks and more.
functions of FgVelB in Fusarium graminearum

Fig. 10. Expression patterns of genes downregulated in the ΔFgVelB strain on carrot agar determined using quantitative real-time PCR. The numbers on the y axis represent the relative amount of each gene transcript. The numbers on the x axis indicate the growth time points of total RNA extraction: 1, 2 and 3: days 3, 5 and 7 under vegetative growth, respectively; 4, 5 and 6: days 3, 5 and 7 after the removal of aerial mycelia for perithecia induction, respectively. AG3 and AG6: days 3 and 6 in agmatine-amended medium for trichothecene production, respectively. The asterisks on the bars for the ΔFgVelB strain indicate that the gene expression was significantly different from that of the WT according to Tukey’s test (P<0.05). FGSG_04480 is a TF gene required for sexual development; FGSG_07590 is similar to the taurine dioxygenase gene; FGSG_02551 is similar to a lignin peroxidase gene; FGSG_11145 is similar to the mucin gene.

general metabolic or regulatory pathways. First, downregulation of three transcripts at the MAT locus in the ΔFgVelB strain may be primarily attributed to the self-sterility in the ΔFgVelB strain, indicating that FgVelB controls the MAT-mediated pathway. This is supported by the observation that more than 60% of genes (307 of 509) downregulated in the ΔFgVelB strain were also downregulated in the MAT1-2 strain. Among the overlapping genes, six TFs and three PKS genes (PKS3, PKS7 and PKS11), which are known to be directly involved in or active during sexual development, were included. In addition to the MAT-mediated pathway, FgVelB appears to control the other signalling cascade, which may be mediated by a G-protein alpha subunit (GzGPA1) because 58.2% of genes (378 of 649) downregulated in the self-sterile ΔGzGPA1 strain were also downregulated in the ΔFgVelB strain under the same conditions. These results suggest that FgVelB mainly functions during sexual reproduction through pathways regulated by both the MAT and GzGPA1 genes. However, the presence of the remaining downregulated genes (>72%) in the ΔFgVelB strain that did not overlap with genes differentially regulated by both ΔMAT1-2-1 and ΔGzGPA1 indicates that FgVelB is involved in regulating other diverse cellular functions on carrot agar, such as nitrogen and carbon metabolism (likely to be required for growth under nitrogen-starvation conditions) and secondary metabolism (e.g. PKS6, PKS12 and NPS7). For example, the reduced pigmentation caused by ΔFgVelB on carrot agar can be attributed to downregulation of the PKS12 gene cluster responsible for aurofusarin production. Reduced expression of the TRI5, TRI6, FGSG_08081 and ZEB2 transcripts in the ΔFgVelB strain grown on rice substrate or agmatine-amended medium supports the idea
that *FgVelB* controls the production of diverse classes of secondary metabolites, such as polyketides (e.g. ZEA and aurofusarin), terpenes (trichotheccenes) and lactones (butenolide) under toxin-inducing conditions. The role(s) (as either an activator or repressor) of *FgVelB* or the possible *FgVelB–FgVeA* complex in the regulation of certain genes appears to be dependent on the growth or developmental stage. For example, *TRI5* and *TRI6*, which are members of the *TRI* gene cluster for triothecene biosynthesis, were down-regulated under toxin-inducing conditions, while *TRI6*, a TF regulating the gene cluster, was upregulated in the sexual condition (Figs 6 and 9, Table S10) in the *ΔFgVelB* strain. These results suggest that *FgVelB* may act as an activator for trichotheccene production under toxin-inducing conditions, but as a repressor for toxin production in the sexual condition. Six (FGSG_02210, 02287, 05140, 12573, 00809 and 04243) of the fatty acid metabolism genes upregulated under vegetative growth (in PDB) in the *ΔFgVeA* strain were down-regulated in the *ΔFgVelB* strain during sexual development (on carrot agar). In addition, two genes [FGMKK1 (FGSG_07295) and FGSLT2 (FGSG_10313)] involved in cell wall integrity that were upregulated in the *ΔFgVeA* strain were not differentially regulated in the *ΔFgVelB* strain.

In conclusion, we determined the biological functions of *FgVelB* and identified major regulatory pathways controlled by *FgVelB* in *F. graminearum*. *FgVelB* regulates mycelia growth, sexual and asexual reproduction, secondary metabolism and pathogenicity in *F. graminearum*. As proposed earlier, the similarity between the biological functions of *FgVelB* and *FgVeA* strongly suggests that the two genes function as a velvet complex, similar to that found in *A. nidulans*, by controlling a large number of genes. However, a recent study indicating that *FgVelB* does not physically interact with *FgVeA* or *FgLaA* in yeast (Jiang et al., 2011) calls into question whether *FgVelB* forms a velvet complex interact with *FgVeA* or *FgLaA* in yeast (Jiang et al., 2011) and identified major regulatory pathways controlled by *FgVelB* strain during sexual development. For example, *TRI5*, which are members of the *TRI* gene cluster for butenolide synthesis, is involved in cell wall integrity that were upregulated in the *ΔFgVeA* strain were not differentially regulated in the *ΔFgVelB* strain.

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