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FfVel1 and FfLae1, components of a *velvet*-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence

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Summary

Besides industrially produced gibberellins (GAs), *Fusarium fujikuroi* is able to produce additional secondary metabolites such as the pigments bikaverin and neurosporaxanthin and the mycotoxins fumonisins and fusarin C. The global regulation of these biosynthetic pathways is only poorly understood. Recently, the *velvet* complex containing VeA and several other regulatory proteins was shown to be involved in global regulation of secondary metabolism and differentiation in *Aspergillus nidulans*. Here we report on the characterization of two components of the *F. fujikuroi velvet*-like complex, FfVel1 and FfLae1. The gene encoding this first reported LaeA ortholog outside the class of *Eurotiomycetidae* is upregulated in Δ*Ffvel1* microarray-studies and FfLae1 interacts with FfVel1 in the nucleus. Deletion of *Ffvel1* and *Fflae1* revealed for the first time that *velvet* can simultaneously act as positive (GAs, fumonisins and fusarin C) and negative (bikaverin) regulator of secondary metabolism, and that both components affect conidiation and virulence of *F. fujikuroi*. Furthermore, the *velvet*-like protein FfVel2 revealed similar functions regarding conidiation, secondary metabolism and virulence as FfVel1. Cross genus complementation studies of *velvet* complex component mutants between *Fusarium*, *Aspergillus* and *Penicillium* support an ancient origin for this complex which has undergone a divergence in specific functions mediating development and secondary metabolism.

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Keywords

Fusarium fujikuroi; *velvet* complex; LaeA; gibberellin; bikaverin

Introduction

Fusarium fujikuroi Nirenberg (teleomorph: *Gibberella fujikuroi* mating population C [MP-C]) belongs to the *G. fujikuroi* (Sawada) Wollenweber species complex. It predominantly occurs on rice (*Oryza sativa* Linné) where it causes the *bakanae* (e.g. foolish seedling) disease, resulting in a variety of symptoms such as abnormal elongation of plants, yellowish leaves, reduced tillering and sterile or empty grains at late infection (Desjardins *et al*., 2000; Sun and Snyder 1981). Many of these symptoms are due to exposure to gibberellins (GAs), a group of diterpenoid compounds originally characterized as plant hormones, which are produced by the fungus (Tudzynski and Hölter 1998; Yabuta, 1935). The growth promoting function of GAs has been exploited extensively by the agri- and horticulture biotechnology industries and GAs are routinely produced in mass quantities by *F. fujikuroi* fermentation (Rademacher 1997).

In addition to GAs, *F. fujikuroi* can produce a variety of other secondary metabolites including the mycotoxins fumonisins (Proctor *et al*., 2004) and fusarin C (Barrero *et al*., 1991), as well as the pigment bikaverin (Kjær *et al*., 1971). The genes involved in the production of secondary metabolite are typically clustered in the fungal genome (reviewed in Hoffmeister and Keller 2007). For example, the seven genes involved in GA synthesis (reviewed in Tudzynski 2005), the six genes involved in bikaverin synthesis (Wiemann *et al*., 2009) and the ~10 genes involved in fusarin C synthesis (D. Brown, unpublished results) are located adjacent to each other at separate loci. Although not examined in *F. fujikuroi*, a cluster of 21 genes in *F. verticillioides* has been associated with fumonisin synthesis (Brown *et al*., 2007; Proctor *et al*., 2003).

Little is known about environmental conditions that affect the transcriptional regulation of most secondary metabolite gene clusters in *Fusarium*. We do know that the GA cluster genes in *F. fujikuroi* are repressed by high amounts of nitrogen in an AreA-dependent manner (Mihlan *et al*., 2003; Tudzynski *et al*., 1999). We also know that fumonisin gene expression in *F. verticillioides* is repressed in a similar AreA-dependent manner (Kim and Woloshuk 2008) as well as repressed by PacC at alkaline ambient pH (Flaherty et al., 2003). Bikaverin cluster genes in *F. fujikuroi* are also repressed by nitrogen but independently of AreA, are repressed by PacC at alkaline ambient pH and are only expressed in liquid culture at early time points (Wiemann *et al*., 2009).

In addition to these environmental attuned systems, recent work has described tremendous progress towards understanding the role global regulators play in controlling secondary metabolism pathways and/or developmental processes. The best characterized global regulator to date is *velvet* (VeA), originally shown in *Aspergillus* spp. to activate production of a variety of secondary metabolites including sterigmatocystin (ST) in *A. nidulans* (Kato *et al*., 2003), aflatoxin (AF), cyclopiazonic acid and aflatrem in *A. flavus* (Amaike and Keller,

2009; Duran *et al*., 2007) as well as AF in *A. parasiticus* (Calvo *et al*., 2004). In other fungal genera, orthologs of VeA have a similar influence on species specific metabolites including positive regulation of both cephalosporin in *Acremonium chrysogenum* (Dreyer *et al*., 2007) and fumonisins and fusarins in *F. verticillioides* (Myung *et al*., 2009). So far there is no secondary metabolite that is clearly repressed by VeA or one of its orthologs although the influence of VeA on penicillin (PN) production in *A. nidulans* is ambiguous (Kato *et al*., 2003; Spröte and Brakhage 2007).

In addition to its effect on secondary metabolism, VeA also influences development and morphology. In *A. nidulans* the light mediated localization of VeA to the nucleus initiates the positive regulation of sexual development (Kim *et al*., 2002; Stinnett *et al*., 2007), most likely by activating the early sexual development gene *esdC* (Han *et al*., 2008). In contrast, VeA negatively affects asexual development by limiting transcription of *brlA* the central regulator of conidiophore development (Kato *et al*., 2003). In *Neurospora crassa*, deletion of the *veA* ortholog *ve-1* leads to a similar increase in conidiation (Bayram *et al*., 2008a). In other fungi, the effect of *veA* deletion on development varies. In *A. parasiticus* and *A. fumigatus* sporulation is nutrient-dependent in Δ*veA* strains (Calvo *et al*., 2004; Krappmann *et al*., 2005), in *A. flavus* conidiation is decreased in *veA* deletions (Amaike and Keller, 2009) while in *F. verticillioides*, the ratio of microconidia-to-macroconidia produced is altered in the *Fvve1* deletion mutant (Li *et al*., 2006).

Recent work in *A. nidulans* indicates that the positive regulation of secondary metabolite production observed is most likely achieved through the physical interaction of VeA with the *velvet*-like protein VelB and LaeA in the nucleus (Bayram *et al*., 2008b). LaeA, so far only identified in the fungal class of *Eurotiomycetidae*, is a putative methyltransferase that modulates heterochromatin structure thereby regulating transcription of specific DNA regions (Bok and Keller, 2004; Bok *et al*., 2006a; b; Reyes-Dominguez *et al*., 2010). Deletion of *laeA* in *A. nidulans* results in repression of transcription of the terrequinone A, lovastatin, ST and PN gene clusters with a concomitant loss of their respective products (Bok and Keller 2004; Bouhired *et al*., 2006). A similar effect was observed in *A. flavus* on AF (Amaike and Keller, 2009; Kale *et al*. 2008) and in *A. fumigatus* on helvolic acid and gliotoxin production (Bok *et al*., 2005; Lodeiro *et al*., 2009). In contrast, in *Penicillium chrysogenum*, although a decrease in PN production was seen, roquefortin C synthesis was not affected in the Δ*laeA* strain (Kosalková *et al*., 2009). Microarray analysis of wild-type and Δ*laeA* strains of *A. fumigatus* indicated that LaeA controls up to 9.5 % of the transcriptome and up to 13 of its 22 secondary metabolite gene clusters (Perrin *et al*., 2007).

In this study, we explore the influence of *velvet* on secondary metabolites produced by the biotechnologically important fungus *F. fujikuroi*. We show for the first time that FfVel1 can act as a positive (GAs, fumonisins and fusarin C) as well as a negative (bikaverin) regulator. In addition, FfVel1 dramatically affects asexual and sexual development and morphogenesis. The homolog of the *A. nidulans velvet*-like protein VelB, FfVel2, seems to execute similar functions as FfVel1 regarding GA and bikaverin production as well as sporulation in *F. fujikuroi*. Differential expression studies by microarray with the Δ*Ffvel1* mutant led to the first identification of a LaeA ortholog outside the class of *Eurotiomycetidae*. We show that both *velvet* family proteins, FfVel1 and FfVel2, as well as

FfLae1 affect virulence of *F. fujikuroi* on rice. Bimolecular fluorescence complementation (BiFC) and yeast two hybrid (Y2H) analysis show that FfVel1 and FfLae1 interact with each other as well as with an orthologous component from *A. nidulans*. Furthermore, VeA and LaeA orthologs from *P. chrysogenum* can restore certain wild-type phenotypes of knock-out mutants in *F. fujikuroi*, and FfLae1 can rescue secondary metabolism in a Δ*laeA* strain of *A. nidulans*.

Results

F. fujikuroi velvet displays common and distinct features to other velvet proteins

F. fujikuroi veA (*Ffvel1*) was obtained by heterologous PCR. Primers specific to the *F. verticillioides ve1* (*Fvve1*) sequence (Li *et al*., 2006) were designed and used with *F. fujikuroi* genomic DNA as a template. The resulting amplicon was cloned and sequence analysis indicated that it shared significant identity with *Fvve1*. RT-PCR analysis revealed an ORF of 1593 bp interrupted by one intron of 94 bp. The gene encodes a predicted protein of 531 amino acids and was designated FfVel1 (FN548142). BLASTp analysis indicates that FfVel1 shares identity to *velvet* proteins from different ascomycetes: 98 % to FvVe1 (ABC02879) from *F. verticillioides*, 55 % to AcVeA (CAL68582) from *A. chrysogenum*, 42 % to Ve-1 (XP_957154) from *N. crassa*, 38 % to PcVeA (CAP92389) from *P. chrysogenum* and 36 % to VeA (AAD42946) from *A. nidulans*.

Analysis of the predicted protein and comparison to other *velvet* proteins identified a number of interesting characteristics. First, a pairwise alignment using the algorithm ClustalW of the above mentioned sequences identified several conserved regions between the proteins (Fig. 1). Using WolF PSORT (www.wolfpsort.org; Horton *et al*., 2007) to predict protein subcellular localization, VeA from *A. nidulans* contains a putative pat7 nuclear localization signal (NLS) and a second, overlaid bipartite NLS, at the N-terminus (Kim *et al*., 2002; Stinnett *et al*., 2007). In contrast, FfVel1 contains a pat4 NLS ($K^{471}RKH^{474}$) at the C-terminus which is highly conserved in FvVe1 and AcVeA but not present in the other *velvet* proteins (Fig. 1). The EMBOSS program “epestfind” (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind) identified several proline, glutamate, serine and threonine (PEST) rich regions. PEST motifs are associated with proteins having a short intracellular half-life, where the PEST motif is thought to act as a signal peptide for protein degradation (Rogers *et al*., 1986). While the PEST region in FfVel1 (R^{288}PSMVDAYPPPPPPPPSYEPAPSASR^{312}) is highly conserved in FvVe1, the PEST motifs in the *A. nidulans* (VeA) and *N. crassa* (Ve-1) proteins are predicted at different positions, respectively (Fig. 1).

FfVel1 regulates morphology and development

In order to examine the role FfVel1 plays in *F. fujikuroi*, *Ffvel1* knock-out and *Ffvel1* addback strains were created. In the first case, a DNA construct containing a hygromycin resistance cassette flanked by 1 kb upstream of the predicted *Ffvel1* start codon and 1 kb downstream from the predicted *Ffvel1* stop codon was transformed into *F. fujikuroi* strain IMI58289. Transformants whereby a homologous gene replacement event had occurred were identified by PCR (data not shown) and ectopic integration events were identified by

Southern hybridization (Fig. S1A). Transformants Δ*Ffvel1*-T3, -T4 and -T11 were found to have lost the target *Ffvel1* gene and did not contain an ectopic integration of the gene replacement cassette. As all three transformants exhibited the same phenotype (data not shown), Δ*Ffvel1*-T3 was arbitrarily chosen for further work. Two *Ffvel1* addback strains were created by transforming Δ*Ffvel1*-T3 with constructs containing a nourseothricin resistance cassette and a wild-type copy of *Ffvel1* or a copy where the putative pat4 NLS was changed to $A^{471}AAH^{474}$ creating $Ffvel1^{C}$ and $Ffvel1^{C\Delta pat4}$, respectively.

The Δ*Ffvel1*, $Ffvel1^{C}$, $Ffvel1^{C\Delta pat4}$ and the wild-type strains were grown on different solidified media under constantly dark conditions and photographed after ten days. The Δ*Ffvel1* mutant exhibited several growths defects including reduced aerial hyphae and increased pigmentation. In contrast, the addback strains ($Ffvel1^{C}$ and $Ffvel1^{C\Delta pat4}$) displayed wild-type-like fluffy growth with white aerial hyphae (Fig. 2A). The similar growth of the $Ffvel1^{C\Delta pat4}$ and $Ffvel1^{C}$ strains suggest that the putative pat4 NLS does not play a major role in the nuclear localization of FfVel1. When grown in submerged cultures, there was no visible difference in hyphal morphology between the different strains (data not shown). When the strains were grown on solidified V8 medium under constant light conditions for 14 days, the wild type and both *Ffvel1* addback strains produced almost identical amounts of microconidia, while the Δ*Ffvel1* strain generated significantly fewer microconidia (approximately 0.5% of the wild type) (Fig. 2B). To determine if FfVel1 plays a role in perithecial formation, the Δ*Ffvel1* mutant and its progenitor strain IMI58289 were crossed with the *MAT-2* standard tester strain *F. fujikuroi* C-1995 and the *MAT-1* standard tester strain C-1993. The observation that perithecia were only formed by the IMI58289/C-1995 cross indicated that IMI58289 contains the *MAT-1* locus. It was interesting to note that the Δ*Ffvel1*/C-1995 cross generated significantly more perithecia as compared to the wild type (IMI58289/C-1995 cross) (Fig. 2C).

Microarray analysis reveals distinct functional sets of FfVel1 target genes

To gain broader insight into the function of FfVel1 as well as identify genes that it may regulate, a heterologous microarray experiment was performed. The *F. fujikuroi* wild-type and *Ffvel1* deletion strains were grown under nitrogen-limiting conditions in liquid media, in duplicate, and sampled after 24 h, 72 h and 120 h. Isolated RNA was labelled and hybridized to *F. verticillioides* microarrays (Roche-NimbleGen) containing oligos representing over 12,500 genes or about 88% of the current predicted gene models.

We found that 233 genes were downregulated while 187 genes were upregulated in Δ*Ffvel1* compared to the wild type. We then subdivided these two sets according to the time-point of up- and downregulation (Fig. 3A and B) and grouped them into several functional categories using the Munich Information Center for Protein Sequences (MIPS) functional database catalogue (http://mips.gsf.de) (Fig. 3C).

Among the 233 genes downregulated in the Δ*Ffvel1* mutant, 16 % (37 genes) grouped into the functional category of transport, comprising the largest group apart from the unclassified genes (Fig. 3C). Of these transport-associated genes 65 % (24 genes) were exclusively found to be downregulated at the 24 h time-point and include the L-α-amino acid transporter Dip5p homolog (Regenberg *et al*., 1998) and the peptide transporter Ptr2p homolog (Perry *et*

al., 1994) (Table. S1). Another category of interest was the group of 14 genes (6 %) assigned to the developmental function group. Seven of the identified genes have been previously identified as regulators of development in different ascomycetes. These include a homolog of the *Sordaria macrospora* transcriptional regulatory protein Pro1 (Masloff *et al*., 2002) and the *A. nidulans* dioxygenase PpoC which affects the ratio of asexual to sexual spores (Tsitsigiannis *et al*., 2004), FluG which is responsible for production of an extracellular diffusible conidiation factor, and the C2H2-type transcriptional activator FlbC (Fig. 3D and Table S1). FluG functions most upstream of FlbC which has been shown to be essential for *brlA* expression and subsequent conidiation in *A. nidulans* (reviewed in Adams *et al*., 1998).

Consistent with findings in other fungi, we found 11 secondary metabolite genes exclusively downregulated at 120 h. These include *Fffum1* and *Fffum2*, encoding a polyketide synthase and P450 monooxygenase, respectively, required for fumonisin production (Proctor *et al*., 2003) and *des*, required for gibberellin synthesis (Tudzynski *et al*., 2003). *des* is the only gene of the *F. fujikuroi* GA cluster present in *F. verticillioides* (Bömke *et al*., 2008) (Table S1).

Of the 187 upregulated genes, we were surprised to note 6 (3 %) that are likely associated with secondary metabolism including *bik3* encoding the *O*-methyltransferase needed for bikaverin production (Wiemann *et al*., 2009) as well as two other genes encoding putative methyltransferases (FVEG_02919 and FVEG_06165), and three genes encoding putative oxygenases (FVEG_09388, FVEG_04071 and FVEG_08860) (Fig. 3D and Table S1). This group also contains a set of 19 genes (10 %) that were upregulated at all time-points sampled (Fig. 3C). The presence of several genes from the heat shock protein (Hsp) families (Table S1) suggests that stress perception is more pronounced in the Δ*Ffvel1* strain. We were most intrigued by the presence of a putative *laeA* homolog (FVEG_00539 and designated *Fflae1*) which shared 32% identity to LaeA from *A. nidulans* (AAQ95166; Bok and Keller 2004) (Table S1). Notably, *laeA* is also upregulated in the *A. nidulans veA* knock-out strain (Bayram *et al*., 2008b).

The differential expression of some FfVel1 target genes from different functional groups was confirmed by Northern blot analysis (Fig. S2). Additionally, expression of the *flbC* homolog (*FfflbC*) was also analyzed under conidiation-inducing conditions (solidified V8 medium) and also found to be significantly downregulated in the Δ*Ffvel1* mutant. These expression data fit very well with the significant reduction of microconidia formation by the mutant.

FfVel1 plays both positive and negative roles in regulating secondary metabolism

As genes from several secondary metabolite gene clusters (GA, bikaverin and fumonisin) were found to be deregulated in the Δ*Ffvel1* mutant, a more thorough chemical analysis of secondary metabolite production by the *Ffvel1* knock-out strain was performed. At this time, we also created an *Ffvel1* over-expressing mutant (designated OE::*Ffvel1*) by transforming a vector containing *Ffvel1* fused to the *F. fujikuroi glnA* promoter (from the glutamine synthase gene *glnA*; Teichert *et al*., 2004) into the wild-type IMI58289 strain. The *glnA* promoter provides constitutive high expression under nitrogen limiting conditions.

Therefore, all strains were grown under the same nitrogen limiting conditions used for the microarray analysis.

The culture broth of the Δ*Ffvel1* mutant appeared significantly more red-pigmented at all time-points than the wild type, while the OE::*Ffvel1* strain showed significantly less coloration (Fig. 4A). Previously, we determined that the pigment responsible for this red color is the polyketide bikaverin (Wiemann *et al*., 2009). The deregulation of bikaverin production in the *Ffvel1* knock-out and over-expressing strains was also detected in studies of the bikaverin cluster genes (shown for *bik1* and *bik2*) which were significantly upregulated at all time-points in the Δ*Ffvel1* mutant and downregulated in the OE::*Ffvel1* strain (Fig. 4B). In contrast to the bikaverin biosynthesis genes, expression of the GA cluster genes (shown for *ggs2*, *cps/ks*, and *P450-3*; Tudzynski, 2005) was not detected in the Δ*Ffvel1* mutant compared to the wild type (Fig. 4B) with a concomitant loss of $GA_{4/7}$ and GA_3 production (Fig. 4C).

In comparison to GA and bikaverin biosynthesis, little is known about fusarin C and fumonisin biosynthesis in *F. fujikuroi*. In order to elucidate the influence of FfVel1 on fumonisin production, the wild type and the Δ*Ffvel1* mutant were grown on cracked corn media for 10 days, extracted and analyzed by liquid chromatography-mass spectrometry (LC-MS). Consistent with the microarray data, the Δ*Ffvel1* mutant accumulated significantly less (2 %) fumonisin B_2 (FB_2) as compared to the wild type and did not produce any detectable fumonisin B_1 (FB_1) (Table 1). For fusarin C analysis, the wild type and the Δ*Ffvel1* mutant were cultivated under restrictive (10 % ICI) and excessive (100 % ICI) nitrogen conditions, before extraction and LC-MS/MS analysis. Like the major effect of FfVel1 on GAs, bikaverin and fumonisin, there was a significant difference between relative amounts of fusarin C produced by the wild type and the Δ*Ffvel1* mutant under nitrogen sufficient conditions (100 % ICI medium) that paralleled with the expression patterns of three putative fusarin C cluster genes (FVEG_11079, FVEG_11080 and FVEG_11082) (Table 1 and Fig. S3). We noted, with interest, that fusarin C production was drastically reduced when the fungus was grown under nitrogen-limiting (10 % ICI) conditions (Table 1). This is in contrast to the production of GAs, bikaverin and fumonisin for which nitrogen-limiting conditions are essential for biosynthesis (reviewed in Tudzynski 1999; Wiemann *et al*., 2009; Kim and Woloshuk, 2008).

Does FfVel1 interfere with environmentally attuned regulation mechanisms?

For GA gene expression, the GATA-type transcription factor AreA is known to be a key activator upon nitrogen starvation in *F. fujikuroi* (Mihlan *et al*., 2003) whereas bikaverin gene expression is dependent on the positively acting pathway-specific $Zn(II)_2Cys_6$ transcription factor Bik5 (Wiemann *et al*., 2009). Bikaverin gene expression is repressed under nitrogen sufficient conditions by an enigmatic mechanism which is different from the canonical AreA-dependent nitrogen regulation and by the C2H2 transcription factor PacC in response to ambient pH (Wiemann *et al*., 2009). To test whether deletion of *Ffvel1* would affect the environmentally attuned specific regulation of both secondary metabolite gene clusters in *F. fujikuroi*, expression studies varying nitrogen concentration and ambient pH were performed.

First, we tested whether expression of *Ffvel1* was affected in the *areA* deletion mutant. We found that *Ffvel1* transcript levels did not differ significantly between the wild type and the *areA* mutant and was dependent on nitrogen concentration. A significantly weaker signal for *Ffvel1* was observed when both strains were shifted into excessive nitrogen conditions (100 % ICI) compared to nitrogen-free conditions (0 % ICI) indicating that *Ffvel1* itself is subject to nitrogen regulation in an AreA-independent manner (Fig. 5A). The loss of *Ffvel1* or *areA* affected GA and bikaverin synthesis differently. As expected, expression of the GA biosynthetic gene *ggs2* was significantly downregulated in both the *Ffvel1* and *areA* deletion mutants compared to the wild type (Fig. 5A and B). Interestingly, no significant alteration in gene expression of key components of the nitrogen regulation network (*areA*, *areB* and *nmrA*) were observed in the Δ*Ffvel1* mutant (Fig. 5B). In contrast to GA gene expression, signal intensities of the bikaverin cluster genes *bik1-3* were significantly stronger in the Δ*Ffvel1* mutant compared to the wild type and the *areA* deletion strain (Fig. 5A). It is worth noting that the expression of these *bik* genes was still detected in the Δ*Ffvel1* mutant with excessive nitrogen (100 % ICI) in contrast to the wild type where no signal was observed (Fig. 5A and B). Even more surprising, in the Δ*Ffvel1* mutant, *bik2* expression was detected in neutral to alkaline media containing $NaNO_3$, although expression of *pacC* was not changed compared to the wild type (Fig. 5B). Taken together, these data indicate that FfVel1 may partially overcome nitrogen and pH repression of bikaverin genes and modulate the AreA-dependent activation of GA genes.

Expression of the *F. fujikuroi laeA*-like gene is regulated by FfVel1

One of the most interesting differentially expressed genes found by microarray analysis shares significant similarity to the *A. nidulans laeA* gene, encoding a key regulator of secondary metabolism (Bok and Keller, 2004). So far, no *laeA* homolog has been identified for fungi outside the class of *Eurotiomycetidae*. The genomic fragment of the *F. fujikuroi laeA* homolog was cloned and sequenced. RT-PCR analysis revealed an ORF of 1266 bp. The ORF is interrupted by eight introns of 34 bp, 60 bp, 51 bp, 49 bp, 56 bp, 50 bp, 47 bp and 57 bp, respectively. The gene encodes a predicted protein of 421 amino acids and was designated FfLael1 (FN548141). BLASTp analysis indicates that FfLael1 shares 33 % amino acid identity to both LaeA (AAQ95166) from *A. nidulans* and PcLaeA (ACD50375) from *P. chrysogenum*. A pairwise alignment using ClustalW of these three LaeA homologs showed a highly conserved region which has been described as a HemK motif and is associated with proteins of the *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase superfamily (Fig. 6A).

In order to confirm that FfLae1 is an ortholog of *A. nidulans* LaeA we complemented an *A. nidulans laeA* deletion mutant with a *Fflae1* gene copy. Transformants containing the entire *F. fujikuroi* gene were confirmed by PCR (Fig. 6B) and checked for restoration of norsolorinic acid (NOR, a visible orange precursor of sterigmatocystin) biosynthesis. As shown in Figure 6C, NOR production was clearly restored in the *Fflae1* transformant.

FfVel1 and FfLae1 are localized in the nucleus and physically interact

In order to investigate subcellular localization of FfLae1 and FfVel1, we constructed strains *Ffvel1gfp* and *Fflae1gfp*, harbouring a wild-type copy of *Ffvel1* or *Fflae1* fused to *gfp*,

respectively. Three independent transformants containing either construct were verified by PCR (data not shown) and termed *Ffvel1gfp*-T4, -T14 and -T15 and *Fflae1gfp*-T1, -T6 and -T8. By using an epifluorescence microscope we showed that FfVel1 and FfLae1 were localized in the nucleus (Fig. 7A). We looked for evidence of a physical interaction between FfVel1 and FfLae1 using BiFC, recently established for filamentous fungi (Hoff and Kück, 2005). Our microscopic analysis in three independent transformants (e.g. *Ffvlyfp*-T22, -T26 and -T33), carrying FfVel1 tagged with C-terminal YFP and FfLae1 tagged with N-terminal YFP, showed that the two proteins interact in the nucleus in *F. fujikuroi* (Fig. 7B). Complementation of the *Ffvel1* and *Fflae1* deletion mutants with the plasmids containing the respective gene fusions restored wild-type features regarding sporulation (data not shown), indicating that the tagged gene products remain functional.

Does the *laeA*-like target gene of FfVel1 have similar functions as LaeA in *A. nidulans*?

To examine if the newly identified *F. fujikuroi laeA*-like gene is similar in function to LaeA in *A. nidulans*, we generated a knock-out mutant by transforming a double stranded fragment of DNA, containing a hygromycin (hyg) resistance cassette flanked by 1 kb non-coding regions of *Fflae1*, into *F. fujikuroi* strain IMI58289. Double homologous gene replacement events were identified by PCR (data not shown) and ectopic integration events were excluded by Southern hybridization (Fig. S1B) leaving strains Δ*Fflae1*-T48, -T53 and -T55. As all mutants exhibited the same phenotype, strain Δ*Fflae1*-T48 was arbitrarily chosen for further work.

GA and bikaverin production by the Δ*Fflae1* and Δ*Ffvel1* mutants and the wild type were examined after growth in 10 % ICI media for 3 days. GA_3 and $GA_{4/7}$ production by the Δ *Fflae1* mutant was significantly decreased compared to the wild type and was similar to the Δ*Ffvel1* mutant (Fig. 8A). The quantity of GAs produced by each strain roughly paralleled the expression levels of the GA cluster genes examined (Fig. 8B) suggesting that FfLae1 and FfVel1 are activators of GA production. In contrast, the pigmentation of the Δ*Fflae1* mutant was slightly reduced compared to the wild type while it was significantly increased in the Δ*Ffvel1* mutant (Fig. 8C). Expression analysis of *bik1* paralleled pigmentation and further confirmed that FfLae1 does not affect bikaverin production as severely as GA production and in opposite manner to FfVel1. We also observed a reduction in microconidia formation by the *Fflae1* knock-out mutant compared to wild type of about 30 % (Fig. 8D).

In order to investigate if FfLae1 affects chromatin modification as suggested for *A. nidulans* (Reyes-Dominguez *et al*., 2010), a plasmid containing the GA cluster gene *ggs2* under the control of the strong *glnA* promoter ($pglnA_{prom}$::*ggs2*) was integrated into the *Fflae1* deletion strain either ectopically or at the GA cluster locus (confirmed by PCR, data not shown). As FfLae1 and FfVel1 were shown to interact in the nucleus and similarly affect GA production, plasmid $pglnA_{prom}$::*ggs2* was also integrated into the Δ*Ffvel1* mutant in a similar manner. Northern blot analyses revealed that integration of the plasmid into the original GA cluster locus resulted in a much weaker expression of *ggs2* compared to the signal obtained from ectopically integrated plasmids (Fig. 8E). Taken together, our data support a mechanism in *F. fujikuroi* where the *velvet*-like complex controls gene expression of defined regions in the fungal genome.

Are *velvet* and LaeA proteins of different ascomycetes functionally conserved?

In order to further investigate whether the distinct roles of FfVel1 and FfLae1 on secondary metabolism and fungal morphology in *F. fujikuroi* can also be executed by homologs from other ascomycetes, we performed heterologous complementation of *Ffvel1* and *Fflae1* knock-out strains with constructs containing *PcvelA* and *PclaeA* from *P. chrysogenum*, respectively. Transformants were selected using nourseothricin and integration of the heterologous genes was confirmed by PCR yielding strains $Ffvel1^{CPcvelA}$-T1 and -T2 as well as $FflaeA^{CPclaeA}$-T1, -T2 and -T3 (data not shown).

Transformants containing the *P. chrysogenum* genes were grown on solidified V8 medium. PcVelA almost fully restored the ability to produce aerial hyphae in the *Ffvel1* mutant (Fig. S4A). In order to investigate if the drastic reduction of microconidia formation observed in *Ffvel1* and *Fflae1* knock-out mutants could be rescued by the *P. chrysogenum velA* and *laeA* constructs, the strains were grown for 10 days under microconidia inducing conditions. Interestingly, *PcvelA* enabled the *Ffvel1* mutants to form approximately 50% of the amount of microconidia produced by the wild type (Fig. S4B), while *PclaeA* restored microcondia formation to wild-type level in the *FflaeA* mutants (Fig. S4B). Despite the morphological rescue achieved by PcVelA and the restoration of microconidia formation achieved by PcVelA and PcLaeA, the production of GAs could not be restored, neither by PcVelA nor by PcLaeA (Fig. S4C).

Finally, a Y2H experiment was executed to assess the *velvet* complex formation of heterologous proteins. Results from β-galactosidase tests showed a strong interaction of FfVel1 with AnLaeA (Fig. S5).

The *velvet*-like protein FfVel2 controls processes of development and secondary metabolism similar to FfVel1

In *A. nidulans* a second *velvet*-like protein, VelB, is also part of the *velvet* complex (Bayram et al., 2008b). Therefore, we identified the *F. fujikuroi* homolog and characterized the *FfvelB* mutants with respect to morphology and secondary metabolism. Sequencing of the genomic fragment and cDNA clones of the *F. fujikuroi velB* homolog revealed an ORF of 1389 bp. The ORF is interrupted by three introns of 61 bp, 57 bp and 69 bp, respectively. The gene encodes a predicted protein of 482 amino acids and was designated FfVel2 (FN675836). BLASTp analysis indicates that FfVel2 shares 49 % amino acid identity to VelB (ABQ17967) from *A. nidulans*. Knock-out mutants were obtained by transforming a fragment of DNA, containing a hygromycin (hyg) resistance cassette flanked by 1 kb non-coding regions of *Ffvel2*, into *F. fujikuroi* strain IMI58289. Homologous integration events were identified by PCR (data not shown) and additional ectopic integration of the replacement cassette was excluded by Southern hybridization (Fig. S6) leaving strains Δ*Ffvel2*-T3, and –T7.

Comparing growth of *Ffvel1* and *Ffvel2* deletion strains with the wild type and the *Ffvel1* over-expressing mutant OE:*Ffvel1* on different media, both deletion mutants exhibited similar defects of aerial hyphae formation (Fig. 9A) and significantly reduced microconidia formation compared to the wild type, while OE::*Ffvel1* produced significantly more

microconidia (Fig. 9B). Northern analyses revealed similar effects on bikaverin and GA gene expression in the *Ffvel1* and *Ffvel2* deletion mutants compared to the wild type. In the Δ*Ffvel2* mutants, the first gene of the bikaverin pathway, *bik1*, was upregulated similar to the situation in the *Ffvel1* mutant with concomitant darker coloration of the culture fluid (Fig. 9C and D). Regarding GA production, deletion of *Ffvel2* resembled the restricted GA_3 production and gene expression of the Δ*Ffvel1* mutant (Fig. 9C and E). These data suggest that FfVel1 and FfVel2 coordinate similar processes regarding sporulation and secondary metabolite production in *F. fujikuroi*.

FfVel1, Ffvel2 and FfLae1 are virulence factors

To test whether the deletions of *Ffvel1* and/or *Fflae1* have an influence on virulence of *F. fujikuroi* on rice, virulence assays were performed with 7 day-old seedlings. The seedlings, grown in glass tubes filled with Vermiculite, were inoculated with agar plugs from plates containing the wild-type, Δ*Ffvel1*, *Ffvel1*^{C} or Δ*Fflae1* strains. Seedlings inoculated with the GA-deficient strain Δ*cps/ks* or with sterile water served as negative controls while seedlings inoculated with water containing GA_3 served as a positive control. After two weeks, those plants infected with the wild-type and the *Ffvel1*^{C} strains showed the typical *bakanae* symptoms like etiolation of the whole plant with chlorotic stems and leaves similar to the positive control (Fig. S7). In contrast, the water control and the plants infected with Δ*cps/ks*, Δ*Ffvel1*, Δ*Ffvel2* and Δ*Fflae1* looked similar to each other with normal height and dark green stems and leaves (Table 2 and Fig. S7). These findings clearly show that FfVel1, FfVel2 and FfLael1 are virulence factors of *F. fujikuroi* during infection, most likely due to their role as activators for GA biosynthesis.

Discussion

Recent studies in several fungi have conclusively shown that the conserved VeA protein plays a major role in fungal growth, hyphal and colony morphology, development and secondary metabolism. However, the mode of action of VeA may differ in different fungi. Thus, it can act as positive or negative regulator of sexual and asexual differentiation and mainly as positive regulator of secondary metabolism (reviewed in Calvo, 2008). In *A. nidulans*, VeA is part of a nuclear protein complex together with LaeA, a putative methyltransferase, which is proposed to affect secondary metabolism by chromatin remodelling (Bok and Keller, 2004; Bok *et al*., 2006a; b; Bayram *et al*., 2008b; Reyes-Dominguez *et al*., 2010). In addition, this complex can include the blue light (LreA and LreB) and red light (phytochrome FphA) receptor proteins leading to the hypothesis that VeA serves as a scaffold protein that senses external light by interaction with light-sensing proteins (Purschwitz *et al*., 2009) and integrates the light signals with a nuclear response by an orchestrated action with other proteins such as LaeA (Calvo, 2008).

F. fujikuroi is an industrially important fungus used for the production of GAs in large scale with a yearly market value exceeding $100 million (Rademacher, 1997). Understanding the regulatory mechanisms affecting GA production as well as co-occurring and devaluing other secondary metabolites is therefore of great importance. To clarify the involvement of FfVel1 in regulation of secondary metabolism, growth and development, we generated *Ffvel1*

knock-out and over-expression mutants and searched for *velvet* target genes by microarray analysis.

Ffvel1 expression is affected by nitrogen availability

A general tenant of *veA* is that it is constitutively expressed. For example, in *A. nidulans*, *veA* transcripts were detected in all growth stages tested (Kim *et al*., 2002), and in *N. crassa*, the *veA* transcripts were detected in all different developmental stages and light conditions tested (Bayram *et al*., 2008a). Initial evidence in *F. fujikuroi* was consistent with this tenant. We found that the expression of *Ffvel1* was not altered over a time course of 5 days (Table 1). However, we did find that expression of *Ffvel1* is significantly affected by nitrogen. Its expression was repressed in media containing high amounts of nitrogen (60 mM glutamine). It will be interesting to determine if *veA* expression is similarly affected by nitrogen in *A. nidulans* and *N. crassa*. A link between nitrogen source, conidiation and *veA* has been described in *A. fumigatus*. In this case, deletion of *veA* led to a more pronounced decrease of asexual spore formation when the fungus was grown on nitrate instead of ammonium as the sole nitrogen source (Krappmann *et al*., 2005). Taken together, these results suggest that these fungi share many aspects of the *veA* regulatory mechanism.

FfVel1 affects sexual and asexual development

Although the *velvet* proteins are highly conserved throughout the ascomycetes, the effects on sporulation seem to vary significantly. In *A. nidulans* and *N. crassa*, deletion of *veA* led to a significant increase of conidia formation (Kim *et al*., 2002; Kato *et al*., 2003; Bayram *et al*., 2008a). In contrast, in *P. chrysogenum* (Hoff *et al*., 2010), *A. parasiticus* (Calvo *et al*., 2004), and *A. fumigatus* (Krappmann *et al*., 2005) deletion of *veA* led to a decrease in conidia formation. Similarly, a decrease of microconidia formation on solid V8 medium was observed in *vel1* mutants of *F. verticillioides* (Li *et al*., 2006) and *F. fujikuroi* (this paper, Fig. 2C and 9B). The link between microconidia production and *vel1* in *F. fujikuroi* may be *via* the *F. fujikuroi* homologs of the *A. nidulans* genes *fluG* and *flbC* which are known to be important for conidiation in *A. nidulans* (reviewed in Adams *et al*., 1998) and in *Podospora anserina* (Coppin *et al*., 2002). Both *F. fujikuroi* genes (designated *FffluG* and *FfflbC*) are significantly downregulated in the *Ffvel1* knock-out strain (Figs. 3D, S2 and Table S1). In *A. nidulans flbC* encodes a C2H2-type transcriptional activator that has been shown to be a central regulator of conidiophore development prior to BrlA (Adams *et al*., 1998). Since no obvious homologs of BrlA appear to exist in the genomes of the four sequenced *Fusarium* species, it would be interesting to further investigate the role of FfFluG and FfFlbC in conidiogenesis in *F. fujikuroi*. Interestingly, deletion of *Ffvel2* in *F. fujikuroi* resulted in a similar reduction of microconidia formation, suggesting that both *velvet* proteins feed into similar pathways regarding sporulation. The fact that overexpression of *Ffvel1* led to a significant higher production of microconidia (Fig. 9) indicates that FfVel1 is an activator of asexual development in *F. fujikuroi* in contrast to its function in *A. nidulans* and *N. crassa* (Kim *et al*., 2002; Kato *et al*., 2003; Bayram *et al*., 2008a). Furthermore, deletion of *Ffvel1* increased perithecia formation significantly when the mutant strain was crossed with a corresponding mating partner (Fig. 2C), suggesting that FfVel1 acts as repressor of sexual development. Sexual crosses with both mating types bearing a *Ffvel1* deletion would verify

this observation. Deletion of *veA* abolishes cleistothecial production in the homothallic genus *A. nidulans* but the effect of loss of this gene in *N. crassa* is unclear (Kim *et al*., 2002; Bayram *et al*., 2008a). Future work to understand better the role of *velvet*-family VeA proteins in asexual sporulation and sexual development may explain how VeA differentially affects those fundamental aspect processes of fungal development.

FfVel1and FfVel2 can act as positive regulators of secondary metabolism

Secondary metabolism throughout fungal genera has been shown to be positively regulated by *velvet* proteins. Here we show that the *velvet* homolog in *F. fujikuroi* positively regulates GA gene expression and product synthesis. Expression analysis, initially by microarray and confirmed by Northern blot, of a *velvet* deletion strain (Δ*Ffvel1*) showed a drastic downregulation of GA cluster genes compared to the wild type. As expected, low levels of GA_3 and $GA_{4/7}$ were detected by TLC (Figs. 3, 4, 5, 9, S2 and Table S1). Deletion of the second *velvet*-like gene, *Ffvel2*, paralleled the deficiency in GA_3 production of the *Ffvel1* deletion mutant (Fig. 9). The dramatic downregulation of secondary metabolite gene clusters is consistent with reports from *Aspergillus* spp. where VeA has been shown to activate production of ST in *A. nidulans* (Kato *et al*., 2003), AF, cyclopiazonic acid and aflatrem in *A. flavus* (Amaike and Keller, 2009; Duran *et al*., 2007) as well as AF in *A. parasiticus* (Calvo *et al*., 2004). Similar to our results, cephalosporin and PN production is impaired in *velvet* knock-out strains in *A. chrysogenum* and *P. chrysogenum*, respectively (Dreyer *et al*., 2007; Hoff *et al*., 2010).

Similar to GA production, formation of the polyketides FB_1 and FB_2 is reduced in Δ*Ffvel1* strains (Table 1). The limited fumonisin production was also observed recently by *velvet* knock-out strains in the related fungus *F. verticillioides* (Myung *et al*., 2009). This is the first report of a *velvet* protein to positively affect production of a terpene and a polyketide-derived secondary metabolite in one organism.

Myung *et al*., (2009) also reported that biosynthesis of fusarin C, a PKS/NRPS-derived metabolite in *F. verticillioides*, is completely blocked when grown on cracked corn. In *F. fujikuroi*, we detected a significant reduction (about 50 %) of fusarin C as well as a slight downregulation of four putative fusarin C biosynthesis genes under high nitrogen conditions in submerged medium (Table 1, Fig. S3).

FfVel1and FfVel2 can act as negative regulators of secondary metabolism

We were most intrigued to observe an increase in expression of the bikaverin biosynthesis genes and bikaverin production by the Δ*Ffvel1* strain compared to the wild type (Figs. 3 4, 5 and 9). Bikaverin formation and *bik* gene expression is known to be subject to a complex regulatory network and is affected by nitrogen source, pH and age of culture (Wiemann *et al*., 2009). Deletion of *Ffvel1* not only led to an upregulation of bikaverin genes at early time points, but also to a much longer duration of expression as compared to the wild type (Table 1, Figs. 3 and 4B). Previously, we found that nitrogen and a neutral to alkaline ambient pH represses bikaverin gene expression. Interestingly, in Δ*Ffvel1* mutants *bik* gene expression is still detectable under unfavored pH conditions despite an unchanged expression of *pacC* (Fig. 5), which was shown to encode for a repressor of *bik* genes (Wiemann et al., 2009).

Furthermore, *bik* gene expression was also observed under nitrogen repressing conditions in the Δ*Ffvel1* mutant although the expression of genes coding for key components of the nitrogen regulation network (*areA*, *areB*, and *nmr*) was unchanged (Fig. 5). However, the partial de-repression of bikaverin gene expression in the *Ffvel1* knock-out is much weaker in cultures that are amply supplied with nitrogen, indicating that nitrogen repression on one hand, and overexpression of *bik* genes in the *Ffvel1* mutant on the other hand, act independently of each other (Fig. 5). Interestingly, overexpression of *Ffvel1* does not lead to an enhanced GA gene expression (Fig. 4).

The complexity of the bikaverin regulation network regarding external signals suggests that de-repression of bikaverin genes is a secondary effect of *Ffvel1* deletion, due to a defective perception of environmental signals. The large group of FfVel1 target genes whose products are probably involved in transport supports this possibility. Furthermore, the evidence that FfVel1 seems to be involved in regulating only certain chromosomal regions (Fig. 8E), suggests a role of the *velvet*-like complex in chromatin remodelling as recently shown in *A. nidulans* (Bayram *et al*., 2008b; Reyes-Dominguez *et al*., 2010). Apart from global regulators, environmentally attuned regulators like AreA in *A. nidulans* were found to be involved in chromatin restructuring as well (Berger *et al*., 2008). In the future, we will elucidate the interconnection of the global regulator FfVel1 and repressors of bikaverin genes (e.g. PacC and a yet unknown nitrogen regulator) in order to see if restricted accessibility of repressors to the bikaverin gene cluster due to *Ffvel1* deletion might be a reason for the observed de-repression in unfavored conditions.

Beside bikaverin biosynthesis in *F. fujikuroi*, a repressing effect of *velvet* on putative secondary metabolite gene clusters is also reported in *P. chrysogenum* (Hoff *et al*., 2010) as well as in *A. nidulans*, where *veA* deletion strains exhibit an increased production of a yet uncharacterized brown pigment (Bayram *et al*., 2008a) and therefore might be a common phenomenon in fungi.

Identification of a LaeA homolog in *F. fujikuroi*

Initial analysis of the microarray data identified an upregulated gene in the *Ffvel1* knockout strain with significant similarity to LaeA, a global transcriptional regulator of secondary metabolism in *A. nidulans*. Recent reports that expression of *laeA* in *A. nidulans*, *A. flavus* and *P. chrysogenum* is negatively affected by *velvet* (Amaike and Keller, 2009; Bayram *et al*., 2008b; Hoff *et al*., 2009) support our hypothesis that we have identified the *F. fujikuroi laeA* homolog. Conclusive evidence came from complementation experiments, where the *A. nidulans laeA* deletion mutant was restored in NOR production by transformation with *F. fujikuroi Fflae1* (Fig. 6C). Localization studies in *F. fujikuroi* also revealed a nuclear localization of a FfLae1-GFP fusion protein (Fig. 7A) and, even more interesting, evidence for a physical interaction with FfVel1 in the nucleus (Fig. 7B). This is consistent with the report that VeA and LaeA can form a complex in both *A. nidulans* and in *P. chrysogenum* (Bayram *et al*., 2008b; Hoff *et al*., 2009).

Deletion of *Fflae1* resulted in significant reduction of GA and bikaverin gene expression and product formation (Fig. 8). This is consistent with reports from *Aspergillus* spp. where numerous secondary metabolic pathways are negatively affected in *laeA* knock-out mutants

(Bok and Keller, 2004; Bouhired *et al*., 2006; Amaike and Keller, 2009; Kale *et al*. 2008; Lodeiro *et al*., 2009). The same effect on PN production was observed in the *P. chrysogenum laeA* deletion mutant (Hoff *et al*., 2009). In the future, it will be interesting to see if the *velvet*-like complex in *Fusarium* spp. is involved in chromatin remodelling of secondary metabolite cluster regions as recently shown in *A. nidulans* (Reyes-Dominguez *et al*., 2010).

velvet and LaeA proteins have common and distinct functions in heterologous systems

Homologs of *veA* and *laeA* in different ascomycetes generally control development and secondary metabolism (reviewed in Calvo, 2008; Hoff *et al*., 2010). This study presents evidence for a *velvet*-like complex in *F. fujikuroi* having influence on morphogenesis, virulence and secondary metabolism. To investigate whether these features are functionally conserved in ascomycetes, we performed complementation experiments transforming heterologous genes from *P. chrysogenum* into the *F. fujikuroi* mutants, and the *F. fujikuroi lae1* gene into the *A. nidulans* mutant. The *PcvelA* gene was able to restore aerial hyphae formation and microconidia production in *F. fujikuroi vel1* knock-out strains. Additionally, addback of *laeA* orthologs of *P. chrysogenum* into the *F. fujikuroi lae1* deletion mutant and *vice versa* restored microconida production in *F. fujikuroi* and partial conidiation in *P. chrysogenum* (Hoff *et al*., 2010), proving a conservation of function in this aspect. Remarkably, *F. fujikuroi* Lae1 could also rescue production of PN when transformed into the *P. chrysogenum laeA* knock-out mutant (Hoff *et al*., 2010) as well as NOR production in the *A. nidulans* Δ*laeA* (Fig. 6C). Our data also suggest that *P. chrysogenum* and *F. fujikuroi* homologs of VeA and LaeA are able to form heterologous complexes with each other similar to the complexes formed between VeA and LaeA homologs from *A. nidulans* and *F. fujikuroi* as shown by Y2H (Fig. S5). These heterologous complexes are able to restore morphological features in *P. chrysogenum*, *A. nidulans* and *F. fujikuroi* (Hoff *et al*., 2010 and this study). Additionally, *F. fujikuroi* Lae1 and Vel1 are able to restore secondary metabolism in *P. chrysogenum* (Hoff *et al*., 2010) and *F. fujikuroi* Lae1 can rescue NOR production in *A. nidulans* (Fig. 6C). However, restoration of secondary metabolism was not achieved when *PcvelA* and *PclaeA* were used to complement the *F. fujikuroi* deletion mutants, respectively. Therefore, it is likely that *velvet* complex homologs included species specific features that allow full functionality only in certain ascomycetes. This specificity is also observed for the VeA homolog Ve1 from *F. verticillioides*, which is not able to rescue the *A. nidulans veA* knock-out strain's phenotype (Calvo, 2008), while Ve-1 from *N. crassa* can restore wild type sporulation processes (Bayram *et al*., 2008a).

In summary, we studied the influence of the *velvet* protein on secondary metabolism and development in the biotechnologically important fungus *F. fujikuroi* by microarray and chemical analytical methods and show an eclectic effect of FfVel1 on important secondary metabolites produced. Furthermore, the array data enabled us to identify an important component of the *velvet*-like complex, the putative methyltransferase FfLae1. Like FfVel1, FfLae1 has a major influence on both secondary metabolism and conidiation in *F. fujikuroi*. This is the first evidence of a *velvet*-like complex in the genus *Fusarium* that establishes novel features of its role in secondary metabolism, differentiation and virulence that will aid to the understanding of this fungal-specific protein complex in general. Significantly, the

cross species complementation experiments described in this work argue for an ancient ancestry of the *velvet* complex in the fungal kingdom which has undergone a divergence in specific functions mediating development and secondary metabolism.

Experimental Procedures

Fungal strains and culture conditions

The wild-type strain *F. fujikuroi* IMI58289 (Commonwealth Mycological Institute, Kew, UK) was used as the parent strain for all knock-out experiments. The GA deficient strain Δ*cps/ks* (Tudzynski et al., 1998) was used for rice infection experiments. *F. fujikuroi* strains C-1993 and C-1995 were obtained from J.F. Leslie (Kansas State University). For all cultures, *F. fujikuroi* was preincubated at 28°C for 48 h in 300 ml Erlenmeyer flasks with 100 ml Darken medium (DVK) (Darken *et al*., 1959) on a rotary shaker at 190 rpm. For RNA isolation, gibberellin bikaverin and fusarin C analysis, 0.5 ml DVK were used to inoculate ICI (Imperial Chemical Industries Ltd., UK) media (Geissman *et al*., 1966) containing 6 mM glutamine (10 % ICI medium) or 60 mM glutamine (100 % ICI medium). These cultures were incubated at 28°C on a rotary shaker at 190 rpm for 1, 2, 3, 5 or 10 days. For protoplasting, 0.5 ml of the starter culture was transferred into Erlenmeyer flasks with 100 ml complete medium (CM) (Pontecorvo *et al*., 1953) and incubated at 28°C on a rotary shaker at 190 rpm for 18 h. For DNA extraction, fungal strains were grown for 3 days at 28°C on cellophane sheets (Alba Gewürze, Bielefeld, Germany) placed on solidified CM. Additional solidified media used for plate assays of fungal strains were 20 % (v/v) vegetable juice (V8) (Campbell Foods, Puurs, Belgium) containing 30 mM $CaCO_3$, 3.9 % (w/v), Potato Dextrose Agar (PDA) and 5 % (w/v) Czapek Dox (CD) Agar (both Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Sexual crossings were performed on carrot agar as described by Klittich and Leslie (1988). For fumonisin analysis, the fungal strains were grown on cracked maize medium as previously described (Brown et al. 2007). *A. nidulans* strains were grown on solid glucose minimal media (GMM) at 37°C as previously described (Bok and Keller, 2004).

Standard molecular methods

DNA and RNA analysis used standard techniques (Sambrook *et al*., 1989). Fungal DNA or RNA was prepared by first grinding lyophilized mycelium into a fine powder with a mortar and pestle and then dispersing it in extraction buffer as described by Cenis (1992). DNA for Southern hybridization experiments was prepared following the protocol of Doyle and Doyle (1990). For Southern blot analysis, genomic DNA was digested with the indicated restriction enzymes (Fermentas GmbH, St. Leon-Rot, Germany), fractionated in 1 % (w/v) agarose gels, and transferred to Nytran® nylon transfer membranes (Whatman Inc., Sanford, ME, USA) by downward blotting (Asubel *et al*., 1987). ^{32}P-labelled probes were prepared using the random oligomer-primer method and membranes were hybridized according to the protocol of Sambrook *et al*., 1989.

Total *F. fujikuroi* RNA was isolated using the RNAgents total RNA isolation kit (Promega GmbH, Mannheim, Germany). Samples of 15 μg of total RNA were transferred to Hybond-N^+ membranes after electrophoresis on a 1 % (w/v) agarose gel containing formaldehyde,

according to Sambrook *et al*. (1989). Northern blot hybridizations were accomplished by the method of Church and Gilbert (1984). cDNA was synthesized from 1 μg of total RNA and the SuperScript II reverse transcriptase (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions.

All primers used for PCR were obtained from Eurofins GmbH (Ebersberg, Germany) (Table S1). PCR reactions contained 25 ng DNA, 5 pmol of each primer, 200 nM dNTPs, and 1 unit of BioTherm™ DNA polymerase (GeneCraft GmbH, Lüdinghausen, Germany) and were initiated with a 4 min soak at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 56 to 65 °C, 1–3 min at 70 °C, and a final soak for 10 min at 70 °C. PCR products were cloned into pCR®2.1-TOPO® vector using the TOPO TA Cloning® kit (Invitrogen, Groningen, The Netherlands) and transformed into *Escherichia coli* (Invitrogen).

Plasmid DNA from *E. coli* was extracted using the GeneJET™ Plasmid Miniprep Kit (Fermentas GmbH, St. Leon-Rot, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI Prism® 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according the manufacture's instructions. DNA and protein sequence alignments were done with DNA STAR (Madison, WI, USA). Sequence homology searches were performed using the NCBI database server. Protein homology was based on BlastX searches (Altschul *et al*., 1990).

Plasmid construction

The putative *F. fujikuroi veA*, *velB* and *laeA* genes were amplified using the primer pairs veA-F1/-R1 velB-F1/-R1 and laeA-F1/-R1 which were based on the putative *F. verticillioides veA*, *velB* and *laeA* sequences, respectively. The *F. fujikuroi veA* knock-out plasmid pΔveA was created by sequentially cloning the 0.8 kb 5′ *Ffvel1* flank (generated with primers veA-5′-F1/-R1) and the 0.5 kb 3′ *Ffvel1* flank (generated with primers veA-3′-F1/-R1) into pUCH2-8 (Alexander *et al*., 1999) such that the hygromycin resistance cassette was flanked by *F. fujikuroi* genomic sequence. The *Ffvel1* over-expression plasmid pOE::veA was created by cloning, via a triple ligation, a 2.2 kb NotI/SacI fragment containing the *Ffvel1* coding sequence and 0.5 kb terminator region (generated with primers veA-OE-F1 and veA-3′-R1 and digested with NotI and SacI) and the 0.5 XbaI/NotI fragment containing the *F. fujikuroi glnA* promoter (Teichert *et al*. 2004) (generated with primers glnA-XbaI-F1/glnA-NotI-R1 and digested with XbaI and NotI) into XbaI/SacI restricted pUCH2-8.

The plasmids pΔlaeA and pΔvelB were assembled using yeast recombinational cloning essentially as described (Colot *et al*., 2006). Briefly, the 5′ and 3′ flanks of *Fflae1* and *Ffvel2* were amplified using primer pairs "gene"-5′-F1/-R1 and "gene"-3′-F1/-R1, respectively. The hygromycin resistance cassette, consisting of the hygromycin B phosphotransferase gene *hph* (Gritz and Davies, 1983) driven by the *trpC* promoter, was amplified using the primer pair hph-F/-R and the plasmid pCSN44 (Staben *et al*., 1989) as a template. All three PCR fragments were cloned into *Sacharomyces cerevisiae* strain FY834 (Winston *et al*., 1995) together with the EcoRI/XbaI restricted plasmid pRS426 (Christianson *et al*., 1992) as essentially described (Gietz and Schiestl, 2007). Plasmid DNA from *S. cerevisiae* was extracted using the GeneJET™ Plasmid Miniprep Kit (Fermentas GmbH, St. Leon-Rot,

Germany) with slight modifications: cells were resuspended in 300 µl Resuspension Solution plus 100 µl glass beads, lysed by addition of 600 µl Lysis Solution and neutralized with 450 µl Neutralization Solution. Transforming DNA to delete *Fflae1* and *Ffvel2* were prepared by PCR using primers "gene"-5′-F1 and laeA-3′-R1 and 1µl of pΔlaeA or pΔvelB, respectively, as template.

The plasmids $pveA^{C}$ and $pveA_{\Delta pat4}{}^{C}$ were constructed using $pNglnA_{Prom}$ (C. Bömke, personal communication) which is a derivate of pNR1 (Malonek et al. 2004). The plasmids pveAgfp and plaeAgfp were constructed using pIGI1783 which contains an *egfp* coding region driven by the *A. nidulans gpd* promoter region (Pöggeler *et al*., 2003). Using the two primer pairs veA-NcoI-F1/-R1 and Pgpd-laeA-F1/laeA-GFP-R1, the coding regions of *Ffvel1* and *Fflae1* were amplified, respectively, thereby changing the stop codons TAG and TAA, respectively, to TCC. The *Ffvel1* NcoI fragment was cloned into NcoI restricted $pNglnA_{Prom}$, pIGI1783 and pEYFPC (Hoff and Kück, 2005) yielding $pveA^{C}$, pveAgfp and pFfvel1-C-YFP, respectively. For creating plaeAgfp the *Fflae1* fragment was cloned into NcoI restricted pIGI1783 using the In-Fusion™ CF Dry-Down PCR Cloning Kit (Clontech, Mountain View, CA, USA) after the manufacturer's instructions. For creating pFflae1-N-YFP, the coding region of *Fflae1* was amplified using the primers Pgpd-laeA-F1 and laeA-YFP-R1 (thereby exchanging the stop codon TAA for TCC) and ligated into NcoI restricted pEYFPN (Hoff and Kück, 2005) using In-Fusion™ cloning as described above. For creating a *Ffvel1* sequence which did not include the pat4 NLS motif, two primer pairs (veA-NcoI-F1/veA-NotI-R1 and veA-NotI-F1/veA-Nco-R1) were used to create two NcoI/NotI fragments that were simultaneously cloned into NcoI restricted $pNglnA_{Prom}$, yielding $pveA_{\Delta pat4}{}^{C}$. The predicted VeA protein, encoded on $pveA_{\Delta pat4}{}^{C}$ has a modified sequence where the endogenous $Lys^{471}Arg^{472}Lys^{473}His^{474}$ is changed to $Ala^{471}Ala^{472}Ala^{473}His^{474}$.

The overexpression plasmid $pglnA_{prom}$::*ggs2* was constructed using pUCH-N-glnA (Wiemann *et al*., 2009). The *F. fujikuroi ggs2* gene was amplified using the primer pair ggs2-F1-Sal/ggs2-R1-Apa and subsequently ligated into Sal/Apa digested pUCH-N-glnA.

Fungal transformations

Preparation of protoplasts from *F. fujikuroi* mycelium was carried out as described (Tudzynski *et al*., 1999). Briefly, 10^{7} protoplasts of strains IMI58289 were transformed with 10 µg of the replacement cassettes of the vectors pΔveA, pΔvelB and pΔlaeA, respectively, or with 10 µg of the over expression plasmid pOE::veA, or with 10 µg of the GFP fusion plasmids pveAgfp and plaeAgfp, respectively. For gene replacement of *Ffvel1*, *Ffvel2* and *Fflae1*, over expression of *Ffvel1*, *Ffvel1*- and *Fflae1-gfp* fusion strains, transformed protoplasts were regenerated at 28°C in a complete regeneration agar (0.7 M sucrose, 0.05 % yeast extract, 0.1 % casamino acids) containing 120 µg/ml hygromycin B (Calbiochem, Darmstadt, Germany) for 6–7 days. For complementation of Δ*Ffvel1* strains, 10 µg of the plasmids $pveA^{C}$ and $pveA_{\Delta pat4}{}^{C}$ were used for transformation as described above, but with 100 µg/ml nourseothricin (Werner-Bioagents, Jena, Germany) instead of hygromycin B. For BiFC strain construction, 10^{7} protoplasts from the wild-type strain IMI58289 were transformed with pFfvel1-C-YFP/pFflae1-N-YFP or pFfvel1-C-YFP/pEYFPN and pFflae1-N-YFP/pEYFPC as negative controls and selected on 120 µg/ml hygromycin B and 100

μg/ml nourseothricin as described above. For complementation of Δ*Ffvel1* and Δ*Fflae1* strains with the corresponding genes from *P. chrysogenum*, 10 μg of pYNvelA and pYClaeA (Hoff *et al*., 2010) or with the *F. fujikuroi* gene containing plasmids pFfvel1-C-YFP or pFflae1-N-YFP were used, respectively, following the transformation described above using 100 μg/ml nourseothricin or 120 μg/ml hygromycin B, respectively, for selection. For over-expressing *ggs2* in the Δ*Ffvel1* and Δ*Fflae1* strains, respectively, pglnA$_{prom}$::*ggs2* was used for transformation following the procedure described above using 100 μg/ml nourseothricin as selection marker.

Co-transformation with plasmids pFflae1-N-YFP and p14 (containing the *pyroA* allele, May *et al*., 1989) was performed to complement the *A. nidulans laeA* deletion strain RJW33.2 following previously described procedures (Bok and Keller, 2004).

The homologous integration events in transformants targeting replacement of *Ffvel1*, *Ffvel2* or *Fflae1* with a selectable marker were verified by PCR using a primer targeting the replaced coding region ("gene"-WT-F1/-R1) or outside the replacement fragment (veA-F1/pUCH-P and veA-R1/pUCH-T in case of Δ*Ffvel1* and "gene"-F1/pCSN44-trpC-T and "gene"-R1/pCSN44-trpC-P in case of Δ*Fflae1* and Δ*Ffvel2*) and another primer located within the hygromycin resistance cassette. Homologous integration of pglnA$_{prom}$::*ggs2* in strains Δ*Ffvel1* and Δ*Fflae1*, respectively, was verified by PCR using the primer pair GS-M/ggs2-D-F1 and were named Δ*Ffvel1*+OE::*ggs2*^{loc} and Δ*Fflae1*+OE::*ggs2*^{loc}, respectively. Mutants containing an ectopic integration of the plasmid did not yield a PCR signal using the primer pair GS-M/ggs2-D-F1 but revealed a signal using the primer pair GS-M/ggs2-R1-Apa and were named Δ*Ffvel1*+OE::*ggs2*^{ect} and Δ*Fflae1*+OE::*ggs2*^{ect}, respectively.

Random integration of pveA^{C} and pveA$_{\Delta pat4}^{C}$, creating rescue strains *Ffvel1*^{C} and *Ffvel1*$^{C\Delta pat4}$, respectively, were verified using the primers veA-NcoI-F1 and mrfp-R1. To confirm integration of pveAgfp and plaeAgfp the primers gpd-F1 and gfp-R1 were used. Integration of pOE::veA was confirmed using primers GS-M and veA-3′-R1. The BiFC strains were screened using primers gdp-F1/EYFP-N-MCS and gdpd-F1/EYFP-C-MCS to confirm integration of both plasmids. Integration of pYNvelA and pYClaeA into the rescue strains *Ffvel1*^{CPcvelA} and *Fflae1*^{CPclaeA}, respectively, was confirmed using primers gpd-F1/EYFP-N-MCS- and gpd-F1/EYFP-C-MCS, respectively.

A. nidulans transformants, TJW97, were PCR screened for the presence of *Fflae1* using the two primers Fwdfusar and Revfusar and simultaneously grown on oatmeal medium for a visual screen to look for restoration of NOR production.

Yeast two hybrid analysis

The yeast two hybrid plasmid pTLex3 (Cho *et al*., 2003) was modified by the Quick change technique (van den Ent and Löwe, 2006) using *Pfu*Ultra II fusion HS DNA polymerase (Stratagene). The bait plasmid with *Ffvel1* cDNA was constructed using two Quick change primers (FveALexAFWD and FveALexAREV) to place *Ffvel1* into pTLex3. Ffvel1-pTLex3 was co-transformed into the *S. cerevisiae* reporter strain L40 with either pGAD424 (empty prey vector) or with *A. nidulans laeA* cDNA in pGAD424 (Bayram *et al*., 2008b). Transformants were selected on -UTL (-leu, -trp, -ura) containing 2 % (w/v) glucose (SD)

media. Ten transformants of each combination, YTJW7 (Ffvel1-pTlex3 plus pGAD424) and YTJW10 (Ffvel1-pTlex3 plus *laeA* prey), were tested for their coloration on -UTL medium containing X-Gal.

Expression microarrays and data analysis

Each Roche-NimbleGen (Madison, WI) array contained up to 13 unique 60 mer oligonucleotide probes per gene. The gene sequence dataset from which the oligos were designed included 12,514 *F. verticillioides* gene models derived from a gene call set obtained from the Broad Institute in January, 2008. Additional sequences unique to the *F. verticillioides* EST collection (Brown *et al*., 2005) were included bringing the total number of sequences represented on the array over 13,000. The microarray data is available from NCBI Gene Expression Omnibus (GEO) under the series accession number GSE21623.

F. fujikuroi mycelium was harvested from two individual cultures and RNA was extracted as described above. Cross species microarray hybridization, data acquisition and initial analysis were conducted by Roche NimbleGen, Iceland. Normalized data from the probe sets of the 12 arrays (two biological repetitions for each strain and time point) were compared using the Acuity 4.0 microarray analysis software package (Molecular Devices Corp, Sunnyvale, USA.) and the Excel® 15 macro collection FiRe (Garcion *et al*., 2006) essentially as described (Schönig *et al*., 2008). Briefly, only oligo sets with at least 2 fold up- or downregulation in both replicate hybridizations as compared between strains or time-points, were considered to be differentially regulated. The *F. verticillioides* genes corresponding to hybridizing oligo sets were analysed by BlastN against the EST database of a *F. fujikuroi* cDNA library (Teichert *et al*., 2004). BlastN matches of this cDNA library with e-value $\leq 1.00\times10^{-100}$ were regarded as homologous. Both, *F. verticillioides* gene calls and *F. fujikuroi* ESTs were examined by BlastX against the non-redundant protein sequence NCBI database. BlastP searches against the MIPS functional database catalogue (http://mips.gsf.de) were performed in order to group the proteins into several functional categories.

Virulence assays

Seeds from a single plant of *Oryza sativa* spp. *japonica* c.v. Nipponbare were prepared and surface sterilized. Briefly, the husks of the seeds were removed and seeds were submerged in 70 % (v/v) ethanol for 1 min followed by three rinses with sterile H_2O. Next, seeds were incubated in 4% (v/v) $Ca(ClO)_2$ for 10 min and again rinsed in sterile H_2O. The sterilized seeds were incubated on solidified H_2O (15 g/l Agar) at 4 °C in the dark for 3 days and subsequently at 28°C at a 12 h light – 12 h dark cycle for 3 days. A 5 mm diameter mycelial plug of a V8 media plate with *F. fujikuroi* strains grown for 3 days on V8 media was transferred to a 3×20 cm test tube filled 25 % with Vermiculite (Deutsche Vermiculite Dämmstoff-GmbH, Sprockhövel, Germany) and covered with a 1 cm layer of Vermiculite. As negative control, an agar plug from solidified H_2O was used instead of a fungal strain and 100 ppm GA_3 were used as positive control. Germinated seeds were transferred to the prepared test tubes, covered with 1cm of Vermiculite and watered with 10 ml of a 3.16 g/l Gamborg B5 Medium (Duchefa Biochemie, Haarlem, The Netherlands) solution. The test tubes were then incubated at 28°C at a 12 h light – 12 h dark cycle for 7 days. Rice plants

were cleaned from Vermiculite and measured from the base of the stem to the second nodule.

Gibberellic acid, fumonisin, fusarin C and norsolorinic acid analysis

The gibberellic acids GA_3 and $GA_{4/7}$ were extracted from 900 µl of *F. fujikuroi* culture filtrates, after acidification with 10 µl 25 % (v/v) HCl, with 910 µl ethyl acetate. The ethyl acetate fraction was then evaporated to dryness, dissolved in 20 µl 20 % (v/v) ethanol and applied to a 20×20 cm Silica gel 60 F_{254} TLC aluminium sheet (Merck KGaA, Darmstadt, Germany). Chloroform, ethyl acetate and acetic acid (57:38:5 %) (v/v/v) served as the mobile phase. The dried sheet was vaporized over 32 % (v/v) HCl, baked for 10 min at 110 °C and GAs were visualized using 254 nm UV light. For analysis of fumonisins, *F. fujikuroi* cultures on cracked maize medium were extracted as previously described (Brown *et al*., 2007) and quantified by liquid chromatography-mass spectrometry (LC-MS) as previously described (Plattner *et al*., 1996).

The fusarin C content in the wild type and the Δ*Ffvel1* mutant culture filtrates of ICI 100% N and ICI 10% N was analyzed by HPLC-MS/MS after purification on RP-18 material. After centrifugation with a Hettich-Universal 16 R centrifuge (Hettich AG, Bäch, Germany) at 3000 rpm for 10 min, 1 mL of the culture filtrate was passed through a Strata C18-E column (55 um, 70 A, 100 mg/1 mL) (Phenomenex, Aschaffenburg, Germany) and the column was washed with 3 mL H_2O before fusarin C was eluted with 3 mL MeOH. The eluate was directly used for analysis using an Agilent 1200 series HPLC System (Agilent Technologies, Waldbronn, Germany) linked to a API 3200 mass spectrometer (Applied Biosystems, Darmstadt, Germany). Data acquisition was performed with the Analyst 1.4.2 software (Applied Biosystems).

Chromatographic separation was achieved on a 150×2.00 mm i.d., 3 µm Hyperclone 3u BDS C8 130 A column (Phenomenex) at 35 °C using a linear binary gradient from 45% MeOH and 55% H_2O to 100 % MeOH in 25 min. The injection volume was 10 µL and the flow rate was set at 200 µL/min.

For electrospray ionization, the ion voltage was set at 5500 V in the positive mode. Zero-grade air served as nebulizer gas (35 psi) and turbo gas for solvent drying (50 psi) which was heated at 350 °C. Nitrogen served as curtain gas (20 psi) and as collision gas (4.5×10^{-5} Torr). The mass spectrometer was operated in multiple reaction monitoring mode (MRM) detecting the fragmentation of the $[M+H]^+$ ions into specific product ions after collision with nitrogen.

Each transition reaction was monitored for duration of 150 ms with both quadrupols set at unit resolution. The declustering potential (DP) was set to 31 V and collision cell exit potential (CXP) set to 4 V. The collision energy (CE) for respective transition is given in brackets: *m*/*z* 432.26 – *m*/*z* 115.20 (CE 127 V). *m*/*z* 432.26 – *m*/*z* 141.20 (CE 93 V).

For quantitative comparison, cultivations and analysis were preformed in duplicates. The fusarin C levels are given as area percent relative to the peak area determined for the sum of the fusarin C peaks observed for the wild type IMI58289 grown under excessive nitrogen

conditions. For quantification, the mass transition *m*/*z* 432.26 – *m*/*z* 115.20 was used. Further conformation of the identity of the fusarin C peaks was achieved by applying a second qualifier transition (*m*/*z* 432.26 – *m*/*z* 141.20).

For NOR (a sterigmatocystin precursor regulated identically to sterigmatocystin) extractions, all *A. nidulans* strains were point inoculated onto solid GMM in 10-cm-diameter petri dishes and incubated for 5 days at 37°C. For NOR visualization, dried organic extracts were resuspended in 100 μl of chloroform, and 10 μl was separated by TLC using chloroform:acetone (80:20 %) (v/v) as mobile phase.

Cytological techniques

F. fujikuroi strains grown in 10 % ICI were used for microscopy. Calcofluor White staining of hyphae was done by incubation of the whole slide for 20 min under dark conditions in Calcofluor White (0.1% in 0.1 M Tris-HCl pH 8.5). Slides were washed with distilled water twice and observed using epifluorescence microscopy. Nuclei were stained using Hoechst 33342 as described previously (Kangatharalingam and Ferguson, 1984). Epifluorescence was observed using a Leica DMRBE microscope (Leica, Wetzlar, Germany) equipped with a high-performance charge-coupled device (CDD) 12 bit SensiCam (PCO AG, Kehlheim, Germany) and filter set A (excitation band-pass filter 340–380, dichromatin mirror 400, suppression long-pass filter 425) for Hoechst 33342 fluorescence, or filter set L5 (excitation band-pass filter 480/40, dichromatin mirror 505, suppression band-pass filter 527/30) for GFP and YFP fluorescence.

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Figure 1. Multiple sequence alignment of FfVel1 with other characterized VeA homologous sequences

The alignment was generated using ClustalW. Putative nuclear localization signals (NLS) were predicted using WolF PSORT (Horton *et al*., 2007) and are marked in shades of red. Putative PEST domains were predicted using the EMBOSS program “epestfind” and marked in green. Sequences aligned are: VeA (AAD42946) from *A. nidulans*, Ve-1 (XP_957154) from *N. crassa*, AcVeA (CAL68582) from *A. chrysogenum*, PcVeA (CAP92389) from *P. chrysogenum*, FvVe1 (ABC02879) from *F. verticillioides* and FfVel1 (FN548142) from *F. fujikuroi*.

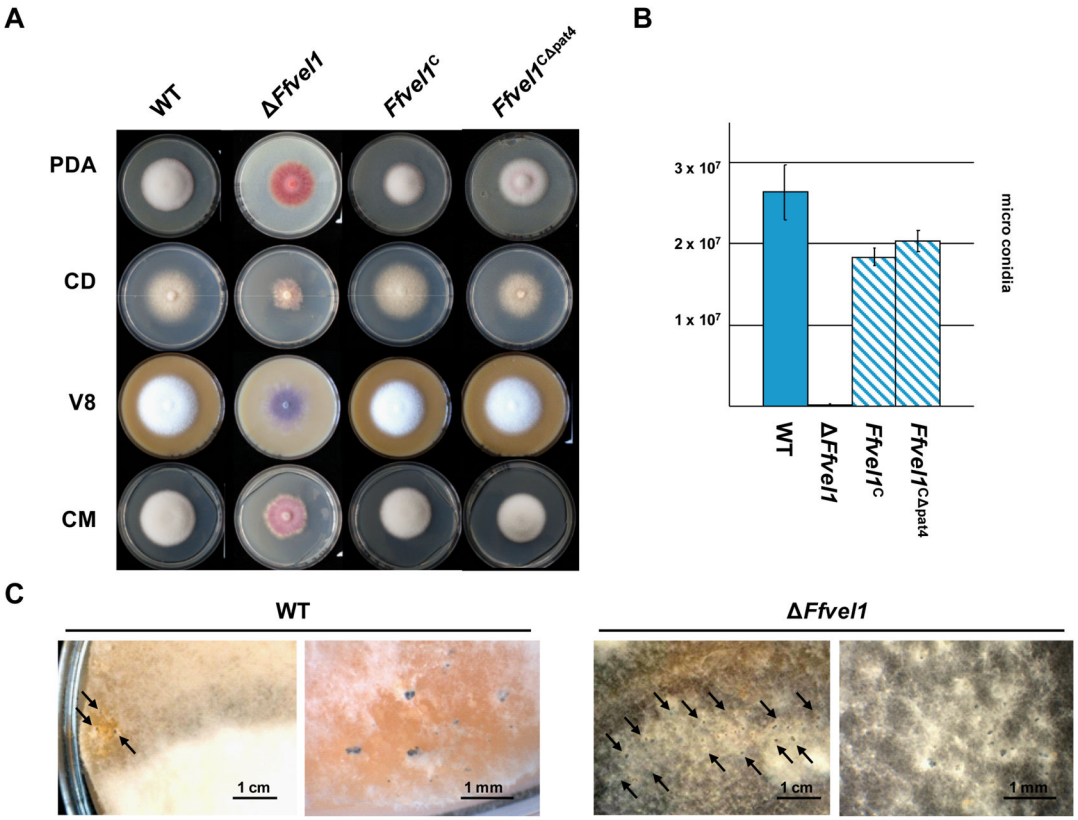

Figure 2. Phenotypical analysis of *Ffvel1* knock-out and addback strains

A Photographs of the *F. fujikuroi* wild-type strain IMI58289, the *Ffvel1* knock-out strain and the addback strains *Ffvel1*^{C} and *Ffel1*$^{C\Delta pat4}$ grown on different solidified media for 10 days under constant dark conditions. V8 = vegetable juice; CM = complete medium; PDA = Potatoe Dextrose Agar; CD = Czapek Dox (for details see *Experimental Procedures*).

B Spores produced by the *F. fujikuroi* wild type strain IMI58289, the *Ffvel1* knock-out strain and the addback strains *Ffvel1*^{C} and *Ffel1*$^{C\Delta pat4}$ grown for 10 days on V8 solidified media under constant light conditions. Experiment was carried out in triplicate; bars show standard deviations.

C Photographs of the *F. fujikuroi* wild type strain IMI58289 and the *Ffvel1* knock-out strain crossed with strain MRC1995. Strains were grown for 6 weeks on V8 solidified media as described in *Experimental Procedures*.

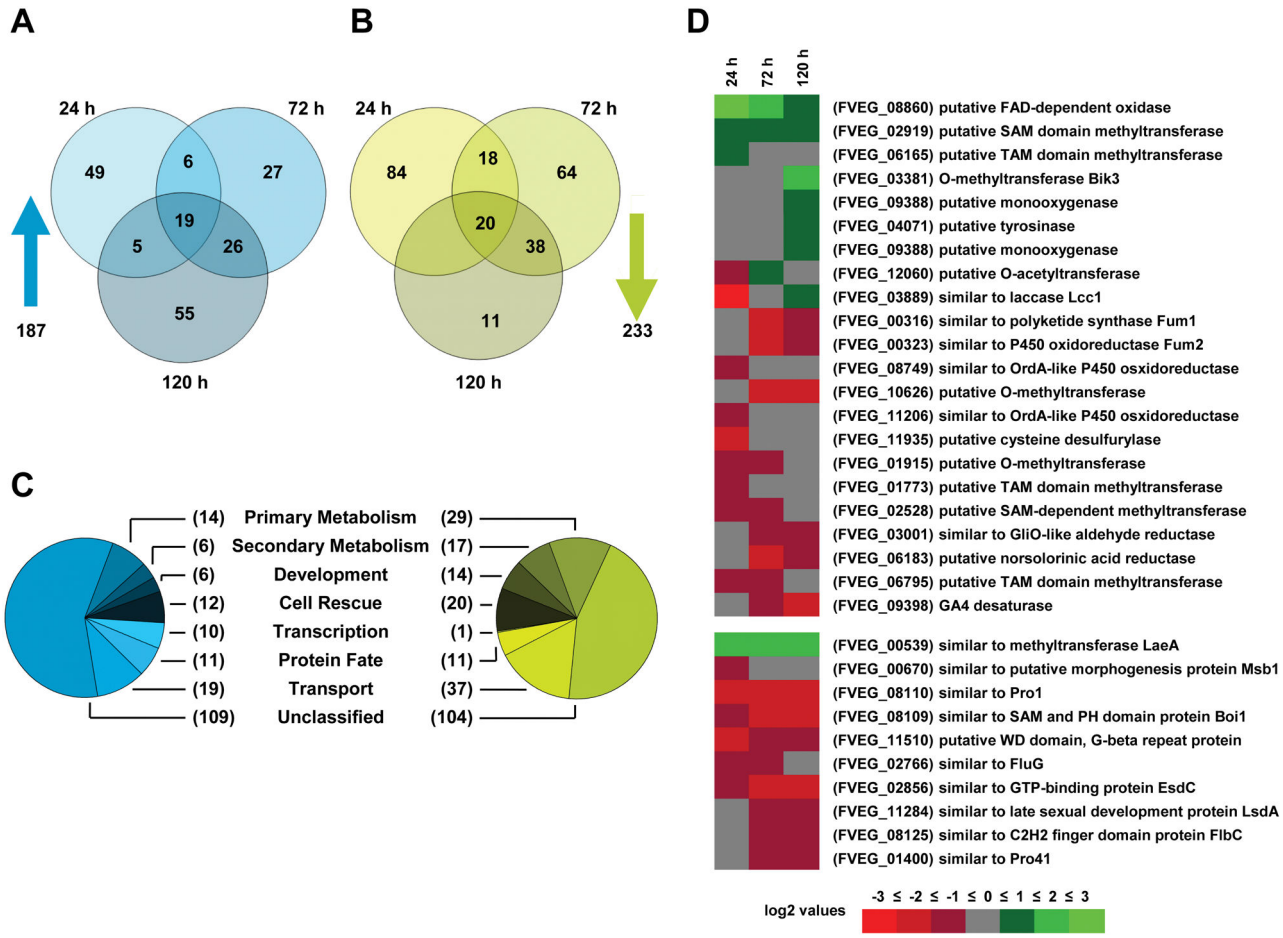

Figure 3. Microarray analysis of the *F. fujikuroi* wild-type strain IMI58289 compared to a *Ffvel1* knock-out strain

A Venn Diagram of genes upregulated in a *Ffvel1* knock-out mutant compared to the WT at 24 h, 72 h and 120 h. Only genes with at least 2 fold upregulation in both replicate hybridizations as compared between strains or time points, were considered to be differentially regulated.

B Venn Diagram of genes downregulated in a *Ffvel1* knock-out mutant compared to the WT at 24 h, 72 h and 120 h. Only genes with at least 2 fold downregulation in both replicate hybridizations as compared between strains or time points, were considered to be differentially regulated.

C Pie charts representing classification of protein sequences corresponding to differentially regulated oligo sets using the MIPS functional database catalogue (for details see *Experimental Procedures*).

D Heat map of genes encoding for proteins involved in secondary metabolism or development as classified by the MIPS functional database catalogue. Each colored square represents the mean log2-fold change of both replicate hybridizations at the given time point (green: up-regulation in the *Ffvel1* knock-out mutant compared to the wild type; red: down-regulation in the *Ffvel1* knock-out mutant compared to the wild type).

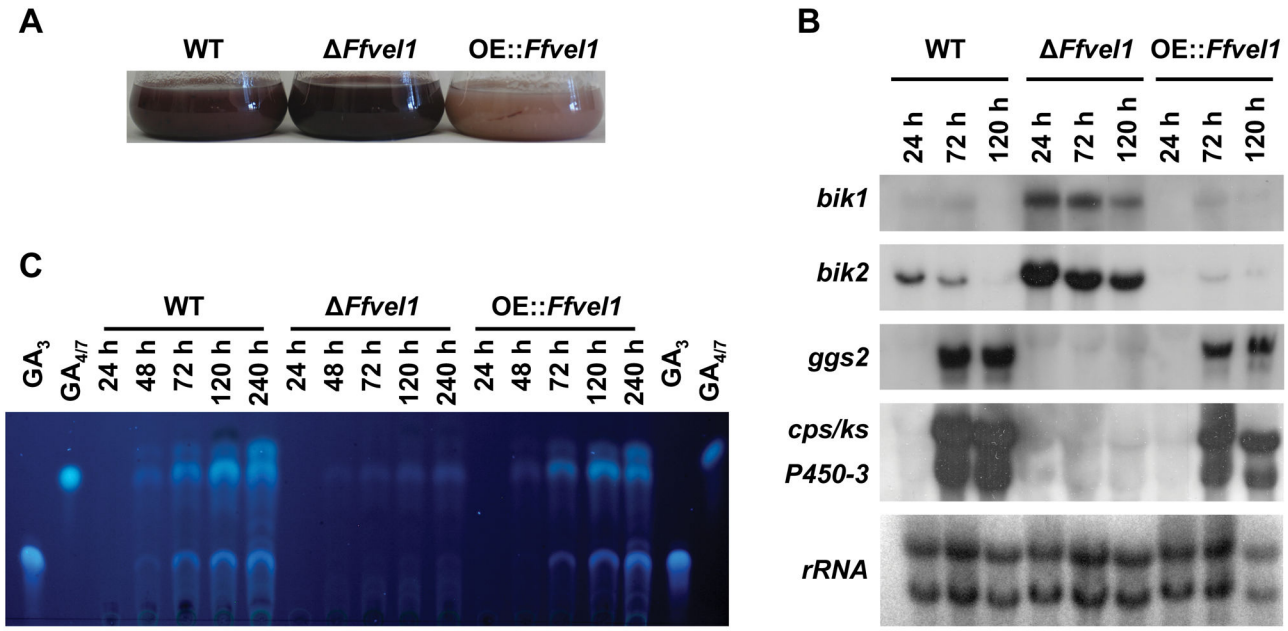

Figure 4. Gibberellin and bikaverin analysis in the *Ffvel1* knock-out and overexpression strain OE::*Ffvel1* compared to the *F. fujikuroi* wild type IMI58289

A Photographs of the *F. fujikuroi* wild-type strain IMI58289, the *Ffvel1* knock-out strain and the overexpression strain OE::*Ffvel1* grown in 10 % ICI cultures for 3 days.

B Expression of selected gibberellin and bikaverin genes in the wild type, the *Ffvel1* knockout and the OE::*Ffvel1* overexpression mutant. The strains were grown for the indicated time in 10 % ICI medium. The northern blot was hybridized with the indicated probes. 28S and 18S rRNA was visualized by EtBr staining as control.

C Thin layer chromatogram of the *F. fujikuroi* wild-type strain IMI58289, the *Ffvel1* knockout strain and the overexpression strain OE::*Ffvel1* grown in 10 % ICI cultures for the indicated time. $GA_{4/7}$ and GA_3 were used as standards (for details see *Experimental procedures*).

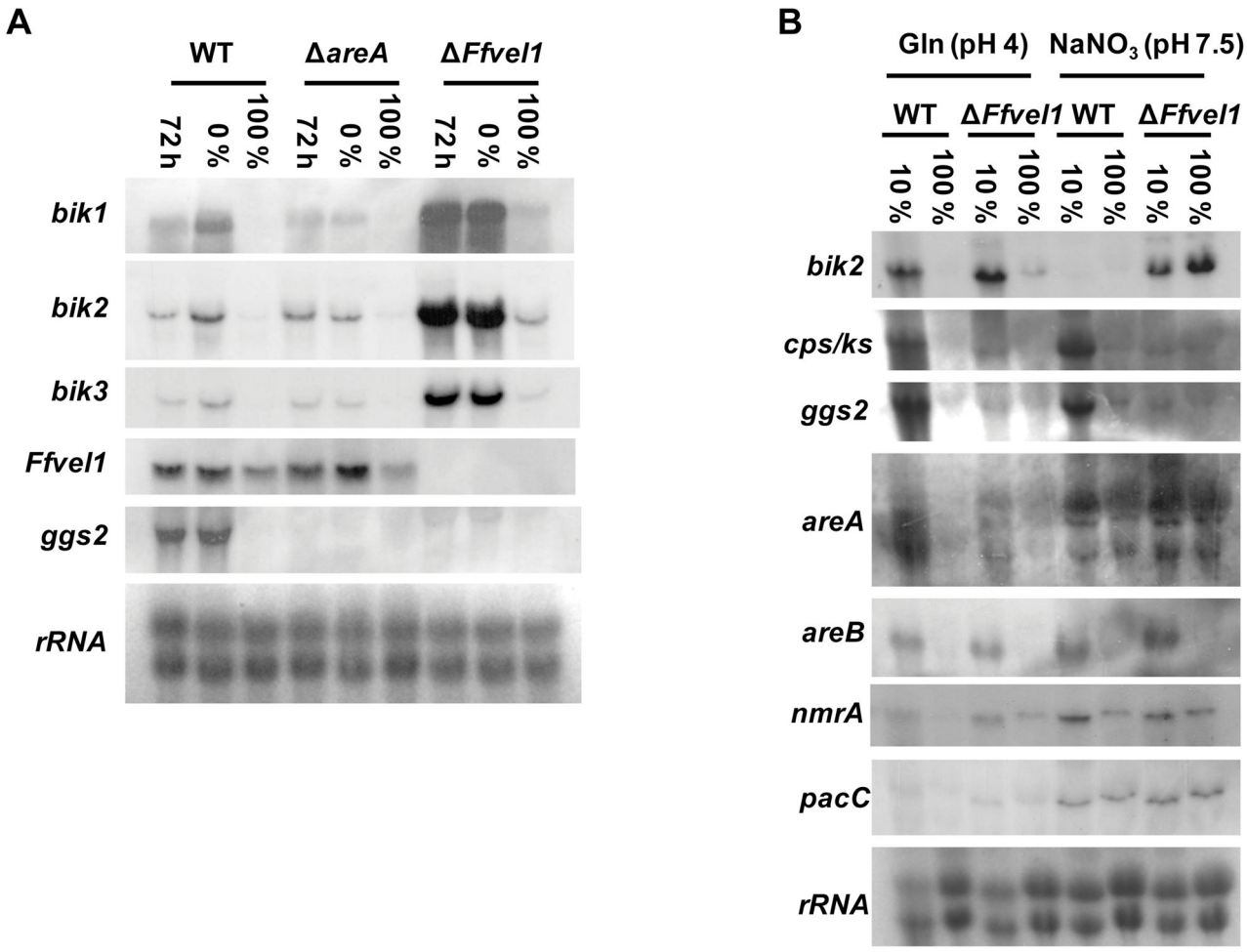

Figure 5. Influence of nitrogen availability and ambient pH on gene expression in a *Ffvel1* knock-out strain compared to the *F. fujikuroi* wild type IMI58289

A Expression of *bik1-3*, *cps/ks*, *ggs2* and *Ffvel1* in the wild type, the *Ffvel1* and *areA* knockout mutants. The strains were grown for 72 h in 10 % ICI medium, washed and shifted into 0 % ICI or 100 % ICI medium, respectively, for 2 h. 28S and 18S rRNA was visualized by EtBr staining as control.

B Expression of *bik2*, *cps/ks*, *ggs2*, *areA*, *areB*, *nmrA* and *pacC* in the wild type and the *Ffvel1* knock-out mutant. The strains were grown for 72 h in 10 % ICI or 100 % ICI medium containing glutamine or $NaNO_3$, respectively. 28S and 18S rRNA was visualized by EtBr staining as control.

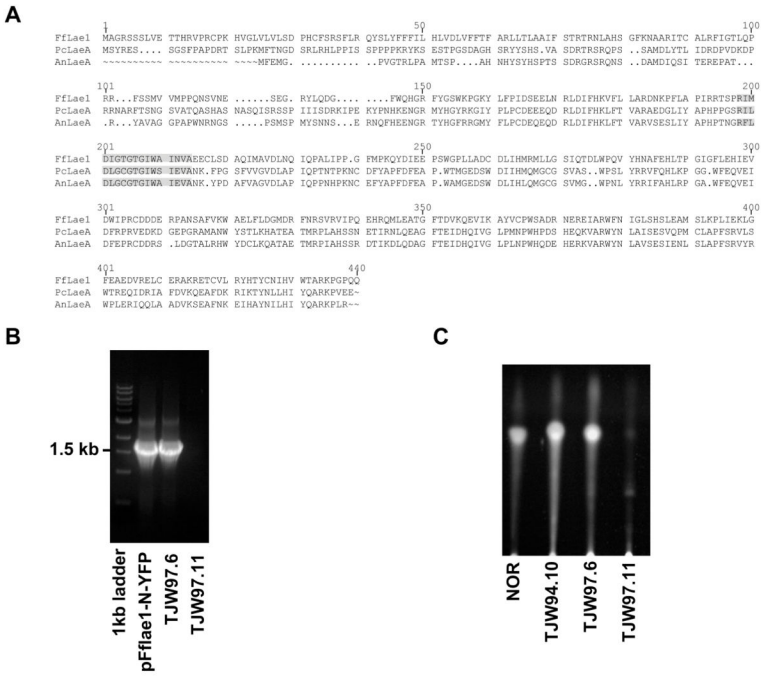

Figure 6. Multiple sequence alignment of FfLae1 with other characterized LaeA homologous sequences and restoration of secondary metabolism in *A. nidulans* Δ*laeA* by *Fflae1*

A The alignment was generated using ClustalW. Putative HemK domains are marked in gray. Sequences aligned are: LaeA (AAQ95166) from *A. nidulans*, PcLaeA (ACD50375) from *P*. chrysogenum, and FfLae1 (FN548141) from *F. fujikuroi*.

B Confirmation of heterologous complementation in *A. nidulans* by PCR. pFflae1-N-YFP: a plasmid containing the *F. fujikuroi lae1* gene used for complementation of *A. nidulans* Δ*laeA* strain RJW33.2, TJW97.6: RJW33.2 transformed with *F. fujikuroi lae1*, TJW97.11: RJW33.2 transformed with *trpC* marker gene.

C Confirmation of heterologous complementation in *A. nidulans* by TLC. NOR was used as a standard for TLC. TJW97.6 produces similar amounts of NOR as TJW94.10, an *A. nidulans laeA* over-expressed strain. Negative control TJW97.11 (Δ*laeA*) can produce a small amount of NOR.

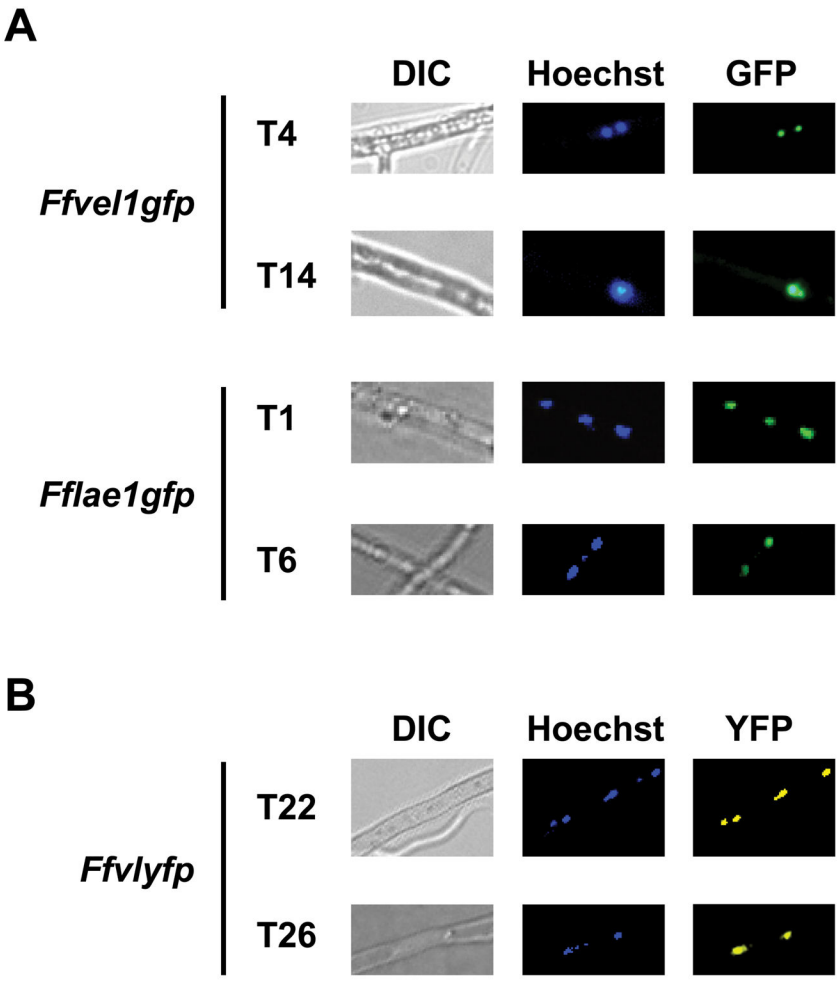

Figure 7. Localization and interaction studies of FfVel1 and FfLae1 in *F. fujikuroi*

A Microscopic analysis of strains *Ffvel1gfp* and *Fflae1gfp*. The strains were grown for 3 days in 10 % ICI in the dark. Samples were stained with Hoechst 33342 and analyzed using an epifluorescence microscope as described in *Experimental Procedures*.

B Microscopic analysis strains *Ffvlyfp* carrying *Ffvel1* fused to the C-terminal part of YFP and *Fflae1* fused to the N-terminal part of YFP. The strains were grown for 3 days in 10 % ICI in the dark. Samples were stained with Hoechst 33342 and analyzed using an epifluorescence microscope as described in *Experimental Procedures*.

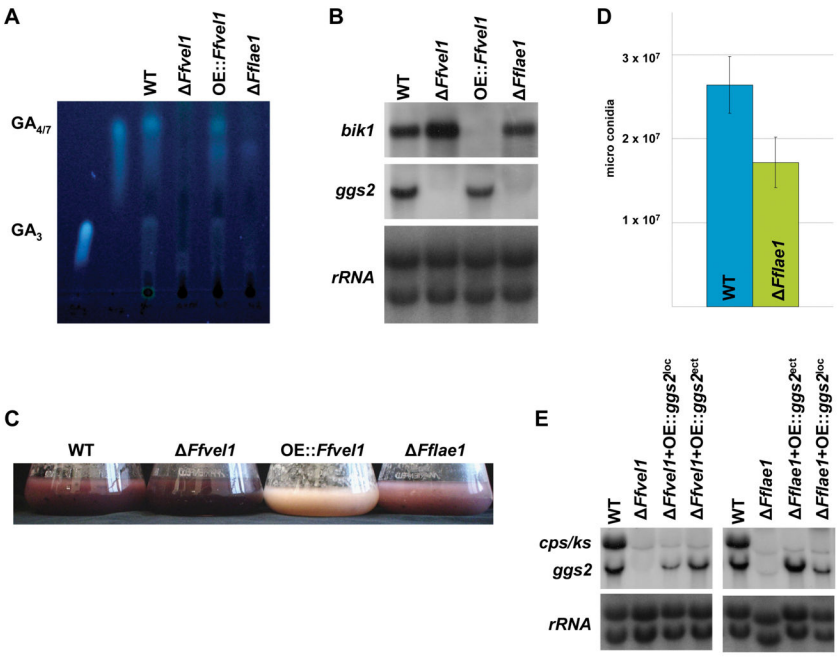

Figure 8. Analysis of the *Fflae1* knock-out strains compared to the *F. fujikuroi* wild type IMI58289 in regard to secondary metabolism and conidiation

A Thin layer chromatogram of the *F. fujikuroi* wild-type strain IMI58289, the *Ffvel1* and *Fflae1* knock-out strains as well as the overexpression strain OE::*Ffvel1* grown in 10 % ICI cultures for 7 days. $GA_{4/7}$ and GA_3 were used as standards (for details see *Experimental procedures*).

B Expression of *bik1* and *ggs2* in the wild type, the *Ffvel1* and *Fflae1* knock-out strains as well as the OE::*Ffvel1* overexpression mutant. The strains were grown for 3 days in 10 % ICI medium. 28S and 18S rRNA was visualized by EtBr staining as control.

C Photographs of the *F. fujikuroi* wild-type strain IMI58289, the *Ffvel1* and *Fflae1* knockout strains as well as the overexpression strain OE::*Ffvel1* grown in 10 % ICI cultures for 3 days.

D Spores produced by the *F. fujikuroi* wild-type strain IMI58289 and the *Fflae1* knock-out strain grown for 10 days on V8 solidified media under constant light conditions. Experiment was carried out on triplicate; bars show standard deviations.

E Expression of *ggs2* and *cps/ks* in the wild type, the *Ffvel1* and *Fflae1* knock-out strains as well as in strains expressing *ggs2* in the GA gene locus (Δ*Ffvel1*+OE::*ggs2*^{loc} and Δ*Fflae1*+OE::*ggs2*^{loc}) or ectopically (Δ*Ffvel1*+OE::*ggs2*$^{ect\ and}$ Δ*Fflae1*+OE::*ggs2*^{ect}), respectively. The strains were grown for 3 days in 10 % ICI medium. 28S and 18S rRNA was visualized by EtBr staining as control.

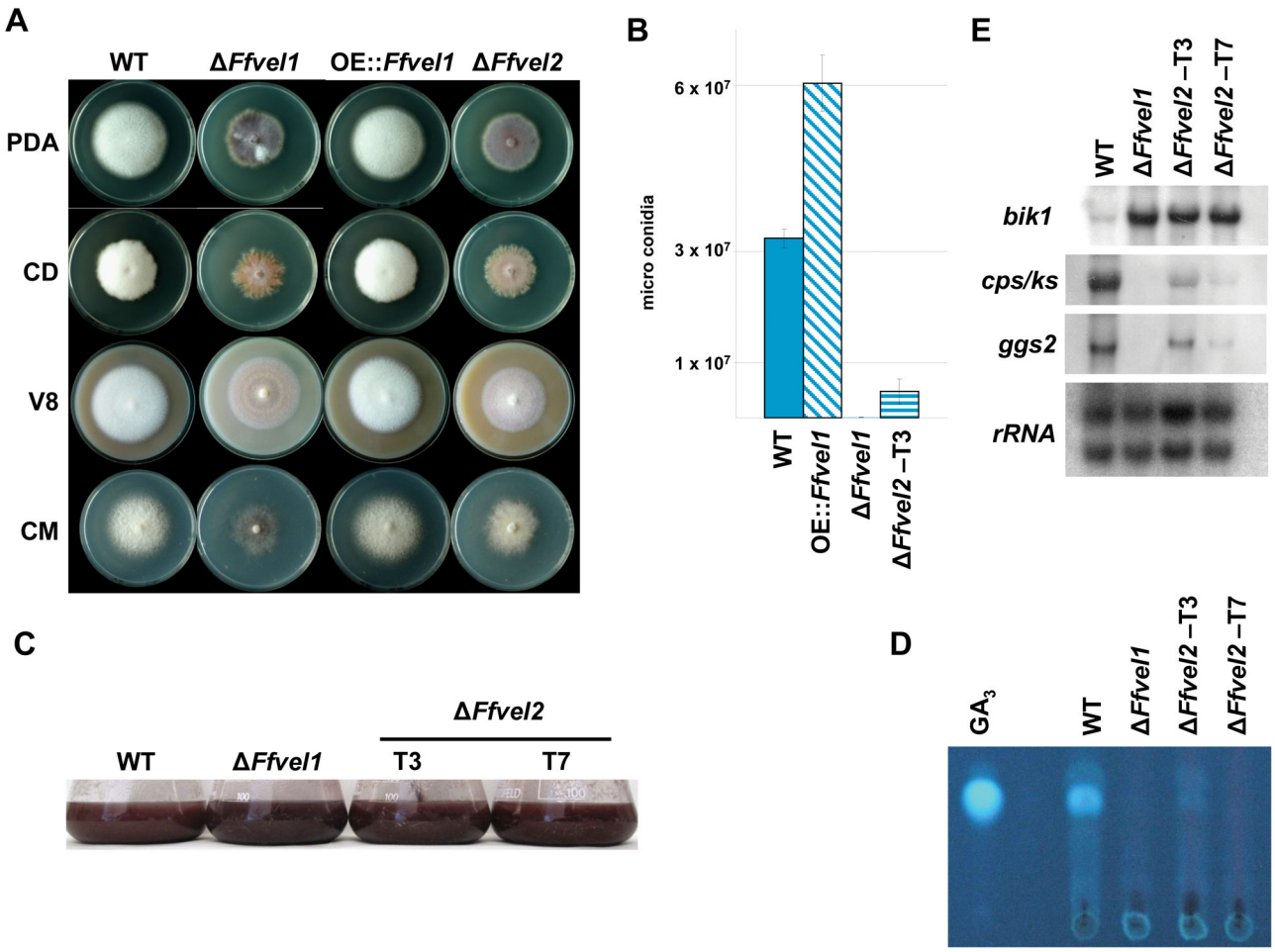

Figure 9. Phenotypical and secondary metabolite analysis in the *Ffvel2* knock-out strain compared to the *F. fujikuroi* wild type IMI58289

A Photographs of the *F. fujikuroi* wild type strain IMI58289, the *Ffvel1* and *Ffvel2* knockout strain and the overexpression strain OE::*Ffvel1* grown on different solidified media for 10 days under constant dark conditions. V8 = vegetable juice; CM = complete medium; PDA = Potatoe Dextrose Agar; CD = Czapek Dox (for details see *Experimental Procedures*).

B Spores produced by the *F. fujikuroi* wild type strain IMI58289, the *Ffvel1* and *Ffvel2* knock-out strains and the overexpression strain OE::*Ffvel1* grown for 10 days on V8 solidified media under constant light conditions. Experiment was carried out in triplicate; bars show standard deviations.

C Expression of selected gibberellin and bikaverin genes in the wild type and the *Ffvel1* and *Ffvel2* knock-out strains. The strains were grown for the indicated time in 10 % ICI medium. The northern blot was hybridized with the indicated probes. 28S and 18S rRNA was visualized by EtBr staining as control.

D Photographs of the *F. fujikuroi* wild type strain IMI58289, the *Ffvel1* and *Ffvel2* knock-out strains grown in 10 % ICI cultures for 3 days.

E Thin layer chromatogram of the *F. fujikuroi* wild type strain IMI58289 and the *Ffvel1* and *Ffvel2* knock-out strains grown in 10 % ICI cultures for the indicated time. GA_3 was used as standard (for details see *Experimental procedures*).

Table 1

Production of fumonisins and fusarin C by the *F. fujikuroi* wild-type strain IMI58289 and the Δ*Ffvel1* mutant

strain	fumonisins [ng/µl]		fusarin C [%]	
	FB_1	FB_2	− N[a]	+ N[b]
WT	0.17 (± 0.06)	1.19 (± 0.60)	1.50 (± 0.2)	100 (± 10.9)
Δ*Ffvel1*	n.d.	0.02 (± 0.01)	4.71(± 2.6)	43.96 (± 12.9)

[a] 10 % ICI;

[b] 100 % ICI;

n.d. = not detectable

Experiments were carried out in duplicate; values in brackets show standard deviations.

Table 2

Length of rice seedlings (*Oryza sativa*, L.) infected with agar plugs of different *F. fujikuroi* strains

strain	length [cm]
H_2O	21 (± 0.82)
WT	28.55 (± 0.67)
Δ*cps/ks*	16.9 (± 0.14)
Δ*Ffvel1*	17.48 (± 1.65)
Ffvel1^C	23.63 (± 1.11)
Δ*Ffvel2*	21.5 (± 0.99)
Δ*Fflae1*	19.4 (± 1.82)
GA_3	37.95 (± 1.91)

Experiments were carried out in triplicate; values in brackets show standard deviations.