The Global Regulatory Protein *VelA* Is Required for Symbiosis Between the Endophytic Fungus *Epichloë festucae* and *Lolium perenne*

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Epichloë species fungi form bioprotective endophytic symbioses with many cool-season grasses, including agriculturally important forage grasses. Despite its importance, relatively little is known about the molecular details of the interaction and the regulatory genes involved. The conserved velvet-domain protein *VelA* (or *VeA*) is a global regulator of a number of cellular and developmental functions in fungi. In this study, the *E. festucae velA* gene was functionally characterized in vitro and during interaction with perennial ryegrass. The *velA* gene is required in *E. festucae* for resistance to osmotic and cell wall-damaging stresses, repression of conidiation, and normal hyphal morphology during nutrient-limited in-vitro conditions. Expression of *velA* in *E. festucae* is light- and nitrogen-dependent and is tissue-specific in mature infected plants. In-plant studies showed that *velA* is required in *E. festucae* for a compatible interaction. Inoculating seedlings with mutant $\Delta velA$ induced callose deposition and H₂O₂ production, and a high level of seedling death was observed. In surviving plants infected with $\Delta velA$ mutant fungi, plants were stunted and we observed increased biomass and invasion of vascular bundles. Overall, this work characterizes a key fungal regulatory factor in this increasingly important model symbiotic association.

Fungi of the genus *Epichloë* form endophytic symbioses with cool-season grasses of the subfamily Pooideae, including agriculturally important forages, such as tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*), and are widely distributed in natural grasslands (Christensen et al. 2002; Leuchtmann et al. 1994; Schardl 2010). Although some sexual *Epichloë* strains can cause sterilization of host plants (choke disease), the symbiosis between *Epichloë* and host grasses is generally mutualistic and essential for grass forage production in some areas, including New Zealand and certain parts of the United States (Johnson et al. 2013a; Schardl 2001). Fungi receive all their nutrients from the

host plant and use the host seed as a means of dissemination, while protecting the plant from a range of biotic and abiotic stresses. Resistance to herbivory from insects is the best characterized of these and is mediated by production of four different classes of alkaloids: indole-diterpenes, ergot alkaloids, lolines, and peramine (Schardl 2001, 2010; Schardl et al. 2013a).

Epichloë hyphae do not colonize roots but are found in all tissues in above-ground parts of the plant from the meristem to the inflorescences, except for the vascular bundles (Christensen et al. 2002, 2008). Hyphal growth is restricted and dependent on host cell growth (Christensen et al. 2008). In the meristematic zone of a growing grass leaf, fungal hyphae grow by apical tip extension, but when they enter the leaf cell-elongating zone above the meristematic zone, they grow by intercalary growth, mirroring host development (Christensen et al. 2008). Hyphal growth ceases when the leaf stops growing, although hyphae remain metabolically active (Tan et al. 2001), which, based on transcriptomics analysis, Schmid et al. (2017) recently suggested may result from a balance of ongoing hyphal growth and death. These various growth types in planta result in a seldom-branched intercellular network of hyphae, parallel to the leaf axis, with hyphae tightly linked with the walls of neighboring plant cells.

E. festucae has been developed as a model system for mutualistic *Epichloë* interactions (Scott et al. 2012) and the genetics of alkaloid production has become well-understood (Schardl et al. 2013b). In addition, several mutants have been described that have an antagonistic plant-interaction phenotype when artificially infected into grass. Examination of these mutants implicate regulation of polar growth, hyphal fusion, and iron homeostasis and the ensuing effect on restriction of hyphal growth in planta as factors in maintaining mutualism (Charlton et al. 2012; Johnson et al. 2013b; Kayano et al. 2013; Takemoto et al. 2006, 2011; Tanaka et al. 2008, 2013). However, a hyperbranching adenyl cyclase deletion mutant did not significantly stunt infected plants despite an approximately twofold increased fungal biomass (Voisey et al. 2016), suggesting additional factors are involved. It has recently been suggested that altered chitin content in the fungal cell wall may contribute to the fungus avoiding a host defense response (Becker et al. 2015; Eaton et al. 2015), although the way in which *Epichloë* suppresses or avoids pathogen-associated molecular pattern-triggered and effector-triggered immunity to initiate and maintain a successful symbiotic interaction is still largely unknown.

The velvet gene, *veA* or *velA*, encodes an important conserved fungal regulator of a variety of growth and developmental characteristics (Bayram and Braus 2012). It is the best-characterized member of the velvet-domain family of genes that normally includes four members, *velA*, *velB*, *velC*, and *vosA*.

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The VelA protein shows light-dependent subcellular mobility in *Aspergillus nidulans* and, in the dark, interacts with the α -importin KapA to transfer into the nucleus (Bayram et al. 2008a; Stinnett et al. 2007). In the nucleus, velvet proteins form different complexes that lead to different regulatory effects, such as the VelA-VelB-LaeA trimeric complex termed the velvet complex, that is necessary for regulating fungal development and secondary metabolism in the dark (Bayram et al. 2008a, Sarikaya Bayram et al. 2010). The biochemical mechanism through which VelA exerts control over gene expression is not fully described, although, based on a crystal structure and yeast one-hybrid experiments with the VosA protein, it appears that the velvet domain mediates DNA binding (Ahmed et al. 2013).

The regulatory effect of VelA on sexual or asexual sporulation has been shown in a broad range of fungal species (Bayram and Braus 2012; Duran et al. 2007; Kato et al. 2003; Kim et al. 2002; Li et al. 2006), although sporulation can be either positively or negatively regulated, depending on the species. It has also been shown in several species that VelA is a global regulator of the expression of fungal secondary metabolite gene clusters (Choi and Goodwin 2011; Hoff et al. 2010; Kato et al. 2003; Lind et al. 2015; Mukherjee and Kenerley 2010). Additional phenotypes described for $\Delta velA$ mutants include hyphal hyperbranching, swelling, decreased cell-wall integrity (CWI), and resistance to various stresses (Choi and Goodwin 2011; Hoff et al. 2010; Karimi Aghchegh et al. 2014; Li et al. 2006).

A role for VelA in fungus-host interaction has been shown in various fungi, including the commercial biocontrol agent *Trichoderma virens*, the most closely related fungus to *Epichloë* spp. that *velA* has, to date, been studied in, and plant and animal pathogens (Amai and Keller 2009; Duran et al. 2009; López-Berges et al. 2013; Mukherjee and Kenerley 2010; Myung et al. 2012; Wiemann et al. 2010). However, no differences in pathogenicity between $\Delta velA$ mutant and wild-type strains were observed in some other fungi (Chettri et al. 2012; Choi and Goodwin 2011; Dhingra et al. 2012; Kim et al. 2014).

Given the role of VelA in regulating hyphal growth and cell-wall biosynthesis in other fungi, we hypothesized that VelA may play an important role in regulating successful symbiosis between *E. festucae* and perennial ryegrass, in which these characteristics

are important features. In this study, the *E. festucae velA* homolog was identified, its transcription pattern in culture and in planta was described in detail, and its functional role was characterized in culture and in planta. We developed methods to detect plant responses in early stages of *E. festucae* infection, including cell death, H₂O₂ production, and callose and lignin deposition. We show that VelA in *E. festucae* is required for many of the processes described as being regulated by VelA in other species and that the mutant displays a severe incompatible interaction with perennial ryegrass, suggesting a key role in symbiosis for this dynamic fungal regulatory factor.

RESULTS

Identification of velvet family genes in *E. festucae*.

To identify the genes encoding velvet domain-containing proteins in *E. festucae*, the four *A. nidulans* velvet-domain protein sequences VelA, VelB, VelC, and VosA were used in a BLASTP search of the *E. festucae* F11 predicted protein database. As for most other filamentous ascomycetes, four proteins were identified. InterProScan analysis confirmed the presence of velvet domains in each of the four predicted protein sequences. A ClustalW alignment of these putative velvet domains and those of *A. nidulans* and *Magnaporthe oryzae* was performed and a phylogenetic tree was generated (Fig. 1A). This confirmed the presence of orthologs of VelA (EfM3.049680), VelB (EfM3.023360), VelC (EfM3.009960), and VosA (EfM3.010530) in *E. festucae*, which, in this fungus, we designate VelA, VelB, VelC, and VelD.

Examination of the sequence of each of the *E. festucae* velvet proteins revealed differences in sizes and predicted motifs, compared with the characterized *A. nidulans* proteins. The *E. festucae* VelA protein contains its velvet domain in a similar position to *A. nidulans* VelA but, rather than a nuclear export signal (NES) and nuclear localization signal (NLS) being encoded within the velvet domain as is the case in the *A. nidulans* protein, it has a predicted NLS at its C terminus (Fig. 1B) and no detectable NES. Furthermore, *A. nidulans* VelA has a proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) motif at its C terminus but no PEST motif was detected in *E. festucae* VelA. *E. festucae* VelB is around 100 amino acids (aa) longer than its *A. nidulans* ortholog and

Fig. 1. Characterization of velvet gene sequences in *Epichloë festucae*. **A**, An unrooted neighbor-joining tree generated from velvet domains of all four velvet proteins from *Aspergillus nidulans* (An), *Magnaporthe oryzae* (Mo), and *E. festucae* (Ef). **B**, Size and predicted domains and sequence motifs of velvet proteins in *E. festucae*. PEST = proline (P) glutamic acid (E) serine (S) and threonine (T)-rich sequence, TAD = transcription activation domain, NES = nuclear export signal, NLS = nuclear localization signal.

possesses a predicted NLS, which is absent in *A. nidulans* VelB. *E. festucae* VelC is predicted to be around 200 aa smaller than the *A. nidulans* ortholog, lacking the equivalent N terminal region of the protein. *E. festucae* VelD is approximately 100 aa smaller than its ortholog, VosA, in *A. nidulans* but shares an NLS around 250 aa from the N terminus.

To examine the indels in VelC and VelD further, multiple sequence alignments were performed. Alignments of predicted VelC sequences (Supplementary Fig. S1A) revealed that short N terminal sequences are found in VelC for all Clavicipitaceae family genera examined, while other Hypocreales species, such as *Fusarium graminearum*, share the extended N-terminal sequence with *Aspergillus* spp. and other fungi. The most parsimonious explanation is, thus, that a deletion occurred in the Clavicipitaceae common ancestor. No taxonomically congruous indels were observed among VelD sequences.

Upstream of the *velA* gene in the *E. festucae* genome a large intergenic distance was observed that, on further examination, contained nested retrotransposon relics. By checking the *velA* locus of different Sordariomycete species, it was shown that, instead of the retrotransposon nest, in *Neurospora crassa*, two genes are present, one of which is deleted in some Hypocreales species, such as *F. graminearum*, but neither gene is present in Clavicipitaceae genomes. Although an unusually large intergenic space is found upstream of *velA* in all *Epichloë* species, only three of 14 contained retrotransposons. In each case, the one or more retrotransposons were different, perhaps indicating that this locus is a hot spot for transposon insertion.

***velA* gene expression is influenced by light and nutrition and is tissue-specific in planta.**

The influence of light and nutrition (carbon and nitrogen catabolite source) on *velA* expression in *E. festucae* was tested by growing fungi in six different liquid media, with four combinations of carbon (glucose and sucrose) and nitrogen (nitrate and ammonium) sources, plus a rich medium (potato dextrose [PD]) and a starvation medium (just water) (Supplementary Table S2) in total darkness and full light conditions. In all media types, *velA* had higher expression in the light compared with the dark (Fig. 2), albeit this was only statistically significant in two

pair-wise comparisons. Also, in media containing NO₃ as the sole nitrogen source, *velA* had increased expression, but there was no consistent influence of carbon source on *velA* expression (Fig. 2). The highest expression was observed in starvation conditions (water) in light (Fig. 2).

To examine if *velA* expression in planta is tissue-dependent, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed on five different tissues of *E. festucae*-infected ryegrass, meristematic zone, extension zone, sheath, and the newest and oldest mature blades (Fig. 3A). Higher levels of *velA* expression were observed in tissues containing viable nongrowing hyphae (the blade and sheath), compared with tissues with actively growing hyphae (the meristem and extension zone), and older blades had higher expression compared with new blades (Fig. 3B).

Functional analysis of $\Delta velA$ mutant in vitro.

A targeted replacement of the *velA* gene with the hygromycin phosphotransferase (*hph*) gene, using a split-marker strategy, was used to generate a $\Delta velA$ mutant (Fig. 4A). Correct replacement was confirmed by PCR (Fig. 4B). Southern blot analysis of two mutants confirmed that both had single-copy replacement at the *velA* locus (Fig. 4C).

To perform complementation analysis, the *velA* gene was reintroduced back into mutant strain $\Delta velA\#3$ to generate $\Delta velA/velA$, confirmed via PCR and Southern blot (Fig. 4B and C). The transformant with *velA* expression levels most similar to that of wild-type strains, as measured by qRT-PCR, was chosen for further study (Supplementary Fig. S2).

Deletion of *velA* leads to stress susceptibility and increased conidiation.

Growth of wild type and $\Delta velA$ *E. festucae* was indistinguishable on PDA (PD agar). To test the influence of *velA* on osmotic stress, different strains were grown on 0 to 2 M sorbitol. After 52 days, wild-type and complemented colonies were substantially larger and had more aerial hyphae than $\Delta velA$ colonies, when grown on media containing 1.5 M sorbitol (Supplementary Fig. S3A).

CWI was tested by growing fungi on different concentrations of the cell wall-damaging agents Congo red (0 to 0.04%) and

Fig. 2. Effect of nutrition and light on *velA* expression. Expression of *velA* was measured by quantitative reverse transcription-polymerase chain reaction in *Epichloë festucae* growing for 24 h in six different liquid media in dark and light conditions. S = sucrose, G = glucose, NH₄ = (NH₄)₂SO₄, NO₃ = NaNO₃, PD = potato dextrose. Expression values were normalized to that of the gamma actin and 60S ribosomal protein L35 genes. Bars represent standard error of the mean calculated from three biological replicates. Results were analyzed using the multivariate analysis of variance Tukey's test, and statistically significant results ($P < 0.05$) are indicated with different letters. D = dark, L = light.

calcofluor white (0 to 0.03%). There was a slight difference in radial growth between wild-type and mutant colonies on 0.04% Congo red after 17 days and, after 52 days, the mutant had substantially less aerial growth. After 28 days on 0.02 and 0.03% calcofluor white, $\Delta velA$ had grown significantly more than the wild type ($P < 0.05$), but there was no significant difference between the wild type and complemented strains. Again, after 52 days, the mutant strain had substantially less aerial growth, compared with other strains at both concentrations, with significantly more radial growth.

Reduced growth under cell wall-stressing agents suggested a possible difference in mutant cell walls. This was examined further by testing the cell-wall sensitivity of different strains to lysing enzymes, by protoplast production assay. The number of protoplasts were counted after 2.5 and 5 h of digestion and a higher number of protoplasts were observed in $\Delta velA$ compared with wild type at both timepoints (Fig. 5A). There was no difference between wild-type and complemented strains, confirming that the observed phenotype was a result of *velA* deletion.

The number of conidia was increased more than three times in $\Delta velA$ relative to the wild-type strain while the number of conidia in the complemented strain was intermediate between wild type and mutant (Fig. 5B). Although full complementation was not observed, these results suggest that *velA* is a negative regulator of conidiation in *E. festucae*.

VelA is required for normal hyphal morphology in *E. festucae*.

The influence of VelA on fungal morphology in culture was tested by differential interference contrast (DIC) microscopy on

wild-type, $\Delta velA$, and $\Delta velA/velA$ strains of *E. festucae* growing on a thin layer (around 3 mm) of PDA and 3% water agar for 2 weeks. On PDA, no difference between wild-type and $\Delta velA$ strains was observed (Fig. 5C). In starvation conditions (3% water agar), an abnormal hyphal phenotype was observed in the $\Delta velA$ strain (Fig. 5C). In this condition, hyperbranching and convoluted hyphae were observed in the $\Delta velA$ strain compared with wild type. A wild-type hyphal phenotype was observed in $\Delta velA/velA$ strains, confirming that the abnormal phenotypes in $\Delta velA$ were due to *velA* deletion (Fig. 5C).

***velA* is required in *E. festucae* for establishing and maintaining a mutualistic interaction with the host plant.**

The role of VelA in the symbiotic interaction between *E. festucae* and *L. perenne* was investigated by inoculating *Epichloë*-free *L. perenne* seedlings with mycelia of wild type, $\Delta velA$, and $\Delta velA/velA$.

Plant defense responses are induced by $\Delta velA$ *E. festucae* upon inoculation and seedling infection is greatly reduced.

One month after inoculation, 74% of seedlings that were inoculated with the $\Delta velA$ strain had died, a significantly higher rate than those inoculated with wild-type or complemented strains (Fig. 6). A significantly lower infection rate (19% of total initially inoculated seedlings) was also observed among mature (>4 tillers) $\Delta velA$ -inoculated plants compared with the other strains (Fig. 6). No significant differences were detected between wild type and complemented strains (Fig. 6).

Fig. 3. Expression of *velA* in planta measured by quantitative reverse transcription-polymerase chain reaction. **A**, Ryegrass tissue types used during *velA* expression experiments. **B**, Relative expression levels of *velA* in different tissues of mature ryegrass. Expression values were normalized to that of the gamma actin and 60S ribosomal protein L35 genes. Bars represent standard error of the mean calculated from three biological replicates. Results were analyzed using the multivariate analysis of variance Tukey's test, and statistically significant results ($P < 0.05$) are indicated with different letters.

The plant response postinoculation was examined by measuring cell death (by trypan blue staining), H_2O_2 production, and callose and lignin deposition. There was no significant difference observed for cell death or lignin deposition between wild type and $\Delta velA$. Between wild type and the endophyte-free mock-inoculated control there was higher cell death in wild type but no difference in lignin deposition. Callose deposition staining showed higher levels of deposition in $\Delta velA$ -inoculated seedlings compared with wild type 4 days postinfection, and no callose was observed in either wild type or the endophyte-free mock-inoculated control (Fig. 7A and B).

Three times more H_2O_2 production was observed in mutant-inoculated seedlings compared with wild type-inoculated seedlings tested immediately after inoculation (Fig. 7C; Supplementary Fig. S4).

Vascular infection and intrahyphal hyphae in mature $\Delta velA$ *E. festucae*-infected plants.

Mature *L. perenne* plants infected with $\Delta velA$ *E. festucae* had reduced root length and foliar height compared with wild type-infected plants two months after inoculation (Fig. 8A and B). After a further month of growth, differences were even more pronounced in some of the mutant-infected plants, which were very stunted compared with wild type-infected plants (Fig. 8D). To analyze the influence of deleting *velA* on in-plant hyphal morphology, the top 4 cm of the youngest mature blades of plants infected with wild type, $\Delta velA$, and $\Delta velA/velA$ were examined using transmission electron microscopy (TEM), confocal, and light microscopy. TEM analysis of cross-sections showed a higher number of fungal hyphae in $\Delta velA$ mutant-infected plants compared with plants infected with wild-type and complemented strains

(Fig. 9A). In strong contrast with wild type, in $\Delta velA$ mutant-infected plants, the vascular bundles were highly colonized with hyphae that were vacuolated and irregular in size and shape. Some vascular-colonizing hyphae contained up to five intrahyphal hyphae (Fig. 9B). In nonvascular tissues in $\Delta velA$ mutant-infected plants, more than one hypha was frequently observed in a single intercellular space but, in wild-type and $\Delta velA/velA$ -infected plants, mostly there was only one hypha (Fig. 9C). Longitudinal sections of epidermal peels of blade samples were examined with aniline blue staining and a higher number of hyphae were observed in mutant-infected plants (Fig. 9D). In mutant-infected plants, hyphae were slightly convoluted compared with wild type-infected plants, but there was not any difference between wild type- and $\Delta velA/velA$ -infected plants (Fig. 9D). Chitin distribution was tested in in-plant hyphae by staining blade samples with Alexa Fluor 488-labeled wheat germ agglutinin (WGA), and no difference was observed in the chitin distribution in endophytic and epiphytic hyphae between different strains (Fig. 9D). The amount of epiphyllous hyphae on the exterior of the leaf was measured in stained tissues with AlexaFluor-WGA and similar amounts of epiphyllous hyphae were observed between different strains (Supplementary Fig. S5).

The relative number of fungal compartments in planta was estimated in blades, using qPCR, to determine relative levels of two single copy genes, *NRPS1* and *chitinase A* (Supplementary Fig. S6). This showed a twofold higher number of genomes in $\Delta velA$ -infected plants compared with the wild type. This analysis is only a proxy for biomass, but corresponds with microscopic observations. No significant difference in gene copy number was observed between complemented and wild

Fig. 4. Deletion of *Epichloë festucae velA* and confirmation by polymerase chain reaction (PCR) and Southern hybridization analysis. **A**, Physical map of wild-type genomic locus and $\Delta velA$ mutant locus, showing restriction sites for *HindIII* and *XbaI*, primers for deletion screening and replacement. **B**, PCR screening of deletion and complementation of different strains. WT = wild type. **C**, Southern blot results of *HindIII* (right picture) and *XbaI* (left picture) digestion of different strains by using probes shown in A.

type-infected plants confirming that the increased fungal material in $\Delta velA$ is due to *velA* deletion.

DISCUSSION

The velvet protein VelA is a key regulator of a broad range of fungal cellular and developmental functions, including sexual or asexual development, secondary metabolism, pathogenicity and fungal growth, and morphology (Bayram and Braus 2012; Calvo and Cary 2015; Calvo et al. 2016). Here, we characterized the role of VelA in the endophytic fungus *E. festucae* and its interaction with the host grass *L. perenne*. To our knowledge, this is the first report on the requirement for VelA in a symbiotic plant-microbe interaction.

VelA regulatory roles in fungal morphology, including hyper-branching and swelling, and sensitivity to different stresses has been shown in a range of different fungi (Choi et al. 2010; Jiang et al. 2011; Lan et al. 2014; López-Berges et al. 2013; Mukherjee and Kenerley 2010). These abnormal growth phenotypes are a likely result of regulatory effects of VelA on the expression of the genes that encode enzymes involved in the cell wall, such as chitinase (Hoff et al. 2010; Kamerewerd et al. 2011) and mannoprotein (Jeong et al. 2003). Although deleting *velA* in *E. festucae* resulted in little change to the hyphal morphology when growing in a rich medium, in starvation conditions, *velA* deletion led to abnormal morphologies of hyper-branching and convoluted hyphae. In addition, the $\Delta velA$ *E. festucae* was more sensitive to osmotic and cell wall-damaging stresses, along with a marked reduction in the time to degrade cell walls with lysing

Fig. 5. In vitro characteristics of different strains of *Epichloë festucae*. **A**, Sensitivity of different strains of *E. festucae* to lysing enzymes after incubating for 2.5 and 5 h. Bars represent standard error of the mean calculated from three biological replicates. Results were analyzed using the multivariate analysis of variance Tukey's test and statistically significant results ($P < 0.05$) are indicated with different letters. WT = wild type. **B**, Conidiation of different strains of *E. festucae*. Different strains were grown on 3% water agar for 15 days and conidiospores were counted from 20 individual fields (450 μ m) of view for each strain under a Leica DMR microscope in bright field. Bars represent standard error of the mean calculated from three biological replicates. Results were analyzed using the multivariate analysis of variance Tukey's test, and statistically significant results ($P < 0.05$) are indicated with different letters. **C**, Fungal morphology after growing for 2 weeks on 3% water agar were examined under differential interference contrast microscopy. Additional micrographs are presented in Supplementary Fig. S7.

enzymes that have β -glucanase, cellulase, protease, and chitinase activities. As for other fungi, VelA thus appears to be involved in regulating some aspect of cell-wall composition or repair.

In analysis of conidiation, sporulation was significantly increased in the $\Delta velA$ strain compared with wild type. Although this phenotype was not fully complemented, the phenotype was significantly ameliorated in the mutant strain with reintroduced *velA*, and, consistent with the role of VelA in other species, VelA does appear to regulate conidiation in *E. festucae*. Conidiation repression was also reported from deleting other

genes in *E. festucae*, including the gene encoding CWI mitogen-activated protein (MAP) kinase kinase *mkkA*, the downstream MAP kinase gene *mpk*, NADPH oxidase genes *noxA* and *noxB*, and *proA*, which encodes a C6-Zn transcription factor that is essential for sexual fruiting body maturation in *Sordaria macrospora* (Becker et al. 2015; Kayano et al. 2013; Tanaka et al. 2013). Each of these genes have been suggested to function together as a network in regulating conidiation. Whether VelA is a downstream regulator within this network or independently exerts control over conidiation cannot yet be determined.

Fig. 6. Role of *Epichloë festucae velA* on host infection and survival. Perennial ryegrass seedlings were inoculated with different strains of *E. festucae* and, after 1 month, the percentages of **A**, dead and **B**, infected plants per total inoculated seedlings were estimated. Bars represent standard error of the mean, calculated from three independent inoculation experiments. Results were analyzed using the multivariate analysis of variance Tukey's test, and statistically significant results ($P < 0.05$) are indicated with different letters. WT = wild type, E- = endophyte free mock-inoculated control.

Fig. 7. Callose deposition in meristematic region of inoculated seedlings with wild type and $\Delta velA$ mutant strains of *Epichloë festucae* 4 days postinoculation, stained with aniline blue solution. **A**, Sample pictures of stained seedlings with different fungal strains. **B**, Percentage of seedlings with callose deposition in each treatment. E- = endophyte free mock-inoculated control. **C**, Percentage of seedlings with H_2O_2 staining by 3,3'-diaminobenzidine in seedlings inoculated with different strains.

The drivers of the transcription of *velA* itself are dynamic and differ among those fungi studied. Inducers include exposure to light (Bayram et al. 2008b), nitrogen source (López-Berges et al. 2014; Wiemann et al. 2010) or carbon source (Karimi Aghcheh et al. 2014), as well as during fungal plant disease progression (Li et al. 2015; Schumacher et al. 2012). Our results show that *velA* transcription level in *E. festucae* is nitrogen but probably not carbon source dependent and appears to be controlled via nitrogen catabolite repression, based on higher expression in nitrate than ammonia. Expression is up-regulated by exposure to light, which is also the case for other Sordariomycete species examined, *N. crassa* and *Trichoderma reesei* (Bayram et al. 2008b; Karimi Aghcheh et al. 2014), but not *A. nidulans*

(Eurotiomycetes) or *Botrytis cinerea* (Leotiomyces) (Bayram et al. 2008a; Schumacher et al. 2015), which are constitutively expressed in light and dark. Whether VelA in *Epichloë* spp. and other Sordariomycetes is also nuclear-localized in response to light conditions, as is the case for *A. nidulans*, will be an interesting future study, noting that the presence and position of predicted NLS and other sequence motifs in VelA are highly variable between different fungi, with the putative NLS in the predicted *E. festucae* VelA sequence in a different sequence context compared with *A. nidulans*, and there is no obvious NES.

E. festucae has three distinct growth phases synchronized with host plant development: tip growth in the meristematic zone, intercalary growth in the plant cell expansion zone, and a

Fig. 8. Plant morphology measurements after inoculation with different strains of *Epichloë festucae*. **A**, Root size, **B**, shoot size, **C**, number of tillers 2 months after inoculation. Bars represent standard error of the mean calculated from three plants. Results were analyzed using the multivariate analysis of variance Tukey's test, and statistically significant results ($P < 0.05$) are indicated with different letters. **D**, Morphology of infected plants with different strains of *E. festucae* 3 months after inoculation. E- = endophyte free mock-inoculated control.

Fig. 9. Hyphal morphology of ryegrass blades infected with different strains of *Epichloë festucae*. **A**, Cross sections stained with toluidine blue visualized with light microscopy showing higher distribution of $\Delta velA$ hyphae. **B**, Transmission electron microscopy (TEM) micrographs show vascular bundles of the blades infected with different strains. A circle indicates intrahyphal hyphae. **C**, TEM micrographs of fungal hyphae in the mesophyll area of infected blades. **D**, Longitude hyphal growth and chitin distribution in endophytic hyphae stained with aniline blue in mixture with Alexa Fluor 488-labeled wheat germ agglutinin and visualized using confocal microscope. Hyphae appear bright blue and chitin appears bright green.

phase in nongrowing plant tissues in which mycelia maintain metabolic activity but with no or very little increase in apparent biomass. We observed a tissue-specific expression pattern for *velA*, in which *velA* transcripts were much more abundant in blades than sheath and significantly higher in sheath than meristem and extension zone tissues. Greater expression in sheath and blades compared with actively growing tissues perhaps suggests specific expression in hyphae not actively extending, although transcript levels were very much higher in blades than sheath, suggesting other factors to be more important in regulating tissue-specific expression of *velA* than the different types of hyphal growth in these tissues. Blades, which are exposed to more light, show higher expression than the other tissues, and also, the highest expression was observed in the oldest blade, which will have a different nutritional profile from the presumably more nutrient-rich young blade. In combination, plant and culture expression data are consistent with light and nutrition being dominant regulators of *velA* expression, although additional regulatory features in the complex plant environment cannot be ruled out.

Analysis of the plant interaction revealed that *VelA* is required for successful benign symbiotic infection. By deleting *velA* in *E. festucae*, its ability to infect perennial ryegrass seedlings was significantly reduced and around 70% of inoculated seedlings died. We hypothesized that plant defense responses to the mutant fungus played a role in the seedling death and reduced infection. Previous analyses of plant responses to *Epichloë* spp. have focused on mature infected plants and, in general, support a slight response to wild-type *Epichloë* spp. (Ambrose et al. 2015; Eaton et al. 2010; Johnson et al. 2003; Zhang et al. 2011). However, the lack of consensus on the host response to *Epichloë* spp. in normal plant infection is highlighted by three recent transcriptomics studies, each of which focused on a benign, symptomless association. In an *E. coenophiala* interaction with tall fescue, the apparent plant transcriptional response was very slight (Dinkins et al. 2017). A ‘moderate’ effect, including on plant defense and hormone biosynthetic genes, was observed across different tissues in an *E. festucae* var. *lolii* interaction with perennial ryegrass (Schmid et al. 2017). In contrast, in the non-native *E. festucae* F11 interaction with perennial ryegrass (the same system used in this study), reprogramming of more than one-third of host transcriptional output was reported (Dupont et al. 2015). As the events that lead to plant death and lack of infection for the $\Delta velA$ *E. festucae* clearly occur at or soon after the inoculation of seedlings, in this study, we developed methods to directly measure plant responses, including cell death, H_2O_2 production, and callose and lignin deposition in seedlings, during the initial infection process. No obvious difference in cell-death responses in the inoculated meristematic zone between mutant and wild type–infected seedlings were observed that could explain the substantial seedling death. However, there was more cell death in wild type and mutant than mock-inoculated control seedlings, suggesting that there is some *Epichloë* recognition by the plant, consistent with studies referenced above. No impact on lignin deposition was observed from *Epichloë* inoculation, but there was a significant amount of callose deposition in response to mutant but not wild-type inoculation. The mutant also induced significant reactive oxygen species (ROS) production after inoculation. Overall, wild-type *E. festucae* induces a small response from the plant but *velA* mutant fungi induce a significant plant response, consistent with the remarkably high amount of seedling death and low infection rates with the mutant.

In the small number of surviving infected ryegrass plants with $\Delta velA$ mutant *E. festucae*, strong incompatible phenotypes and abnormal in-plant hyphal growth were observed. Mutant-infected plants were stunted and their root size was reduced, although the degree of stunting was variable, suggesting a large host-genotype effect, suppressor mutations, or both. Microscopic

and molecular analysis of the blades showed that mutant hyphae are highly vacuolated and increased by around two times compared with a wild type–infected plant, although we note that any in-plant biomass measurements are only estimates and the qPCR method we used estimates compartment number, which may vary in stunted plants compared with host cell number. TEM analysis showed that mutant hyphae heavily colonized the host’s vascular bundles that are hyphae-free in wild type–infected plants. Similar interaction phenotypes were shown previously for some other *E. festucae* mutants involved in fungal ROS production and its regulation or iron homeostasis (Becker et al. 2015; Eaton et al. 2010; Johnson et al. 2013b; Kayano et al. 2013; Takemoto et al. 2006; Tanaka et al. 2006, 2008, 2013). Intrahyphal hyphae were also observed in some of these associations (Becker et al. 2015; Green et al. 2017). As for condensation, discussed above, whether *VelA* functions downstream of or in a complex with any of the proteins encoded by these other genes remains to be determined.

While the interpretation for incompatible plant interaction phenotypes for most of the mutants listed above is essentially loss of restricted hyphal growth, significantly increased fungal biomass, at least away from the shoot apex, does not always lead to severe antagonistic plant-interaction phenotypes (Voisey et al. 2016) and, in the $\Delta velA$ interaction at least, induction of plant defense responses appears to play a role in the lack of compatibility. How *E. festucae* represses plant responses in compatible interactions is not well-known, but recent studies suggest that *E. festucae* may evade chitin-induced responses by covering or modifying its cell-wall chitin (Becker et al. 2015). Although no difference in the cell-wall chitin of in-plant hyphae was observed between $\Delta velA$ *E. festucae* and wild type, based on culture results discussed above and consistent with described roles in other fungi, it seems *VelA* in *E. festucae* does regulate some aspect of the cell-wall constitution. Additionally, *VelA* may regulate some factor that sequesters the cell wall from plant receptors, such as the collagen-like Mcl1 in *Metarhizium* (Wang and St Leger 2006) or the Ecp6 LysM-containing effector in *Cladosporium* spp. (Bolton et al. 2008). Based on the widespread role of effector proteins in other plant-microbe interactions, including mutualisms (Kloppholz et al. 2011; Plett et al. 2011, 2014a and b), it seems likely that other types of effector proteins are also produced by *E. festucae*. Future planned transcriptomics experiments will more fully explain the mechanisms regulated by *VelA* that are required for successful benign interaction.

In conclusion, we have demonstrated that *VelA* in *E. festucae* F11 is a regulator of fungal biology and development and of establishing and maintaining the mutualistic interaction of the fungus with *L. perenne*, at least partially by avoiding plant defense responses. Elucidating the biochemistry of the *Epichloë* cell wall and effector production regulated by *VelA* in planta and their role in the interaction will be areas of considerable future interest.

MATERIALS AND METHODS

Strains and growth conditions.

Epichloë festucae strains (Supplementary Table S1) were grown on 2.4% PD (Difco Laboratories) plus 1.5% agar (Difco Laboratories) and were maintained at 22°C. For liquid cultures, 2 cm² of actively growing mycelium on PDA were added to 2-ml sterile tubes with 750 μ l sterile distilled (d)H₂O and two sterile ceramic beads. The mycelium was then fragmented, using a Precellys 24 tissue disruptor machine (Bertin Technologies) for four cycles of 45 s at 4,200 rpm. This homogenate was inoculated into sterile 250-ml flasks containing 100 ml of PD broth and was incubated at 22°C for 7 days, with shaking. Plasmids were cloned and maintained in two *Escherichia coli* strains, DH5 α and Top 10 (Life Technologies). Both strains

were grown in liquid Luria-Bertani (LB) medium or on LB agar plates, supplemented with either kanamycin (50 µg/ml) or ampicillin (100 µg/ml) when appropriate.

To detect the influence of light and nutrition on gene expression, mycelium from four 100-ml cultures were harvested by filtering through two pieces of sterile 3M Whatman paper in a sterilized Büchner funnel and were washed with 200 ml of sterilized water. The mycelium was scraped from the Whatman paper using a sterile spatula and was divided into six different media in 250-ml sterilized flasks. Three replicates for each medium were incubated, either in the dark or in the light, at 22°C for 24 h. The mycelium was again harvested, using a Büchner funnel, and was washed with sterile dH₂O and was then frozen in liquid nitrogen for RNA extraction.

Stress-sensitivity tests were performed by growing mycelia on PDA plates supplemented with 1.5 M sorbitol (Sigma-Aldrich), 1.5 M NaCl (Sigma-Aldrich), 0.04% Congo red (Sigma-Aldrich), 0.03% calcofluor white (Sigma-Aldrich), and 60 µM menadione (Sigma-Aldrich).

Hyphal morphology was examined by growing fungi on a thin layer (around 3 mm) of PDA or 3% water agar for 2 weeks at 22°C and was observed with DIC microscopy using a Zeiss Axioplan microscope.

Plant inoculation growth conditions and infection determination.

Endophyte-free 7- to 10-day-old seedlings of perennial ryegrass (*L. perenne* 'Nui') were inoculated as described by Latches and Christensen (1985). Seedlings were transferred, after 2 weeks to soil in root trainers (194 × 125 × 110 mm; Flight Plastic Packaging) in the greenhouse at 22°C with 16 h of 650 W/m² light and 8 h of dark. Infected plants were detected by immunoblotting (Gwinn et al. 1991; Simpson et al. 2012) and light microscopy after plants had at least four tillers.

To measure fungal gene expression during seedling infection, seedlings were inoculated with different fungal strains and were grown under 24-h light or 24-h dark. After freezing seedlings, samples from 4 cm upwards and 0.5 cm downward from the meristem were collected for RNA extraction.

Examination of conidiation in vitro.

Slides coated with 3% water agar were inoculated with approximately 1 mm² of fungi in triplicate and were incubated at 22°C for 15 days in total darkness, in which condition conidiation is induced in *E. festucae* (Tanaka et al. 2013). The conidiospores were counted from 20 individual fields (450 µm) of view for each strain.

Protoplast production assay.

Fungi of different strains were cultured in triplicate in 250-ml flasks containing 100 ml of 60 mM glucose and 30 mM NaNO₃ with 9.38 ml of M-100 salt solution (Mycological Society of America. Mycology Guidebook Committee 1974) for 7 days, at 22°C, in 24-h dark, at 180 rpm. Mycelia were collected by centrifugation for 10 min at 4,000 rpm (using a Heraeus Megafuge 8R) and were washed with water once, and, after drying on filter paper, around 50 mg (±5 mg) of mycelium was resuspended in 6 ml of OM buffer (1.2 M MgSO₄, 10 mM Na₂HPO₄, pH 5.8) containing 15 mg of lysing enzymes per milliliter from *Trichoderma harzianum* (Sigma). Resuspended mycelium in lysing solution was incubated at 120 rpm and 30°C in six-well cell culture plates, and reactions were stopped after 2.5 and 5 h of incubation (Mey et al. 2002). The number of produced protoplasts was measured by averaging five counts of 1-mm² squares of a Neubauer hemocytometer.

DNA extraction and PCR.

Fungal genomic DNA (gDNA) was extracted from frozen mycelium as previously described (Al-Samarrai and Schmid

2000). DNA extraction of fungal transformants was done using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research). Plasmid DNA was extracted and purified using a DNA-spin plasmid DNA purification kit (iNtRON Biotechnology).

Taq DNA polymerase (New England BioLabs) was used for standard PCR and iProof high-fidelity DNA polymerase (Bio-Rad) was used when proofreading activity was required. Cycling conditions were designed based on the manufacturer's instructions. A list of primers used is presented in Supplementary Table S3.

RNA extraction and quantitative real-time RT-PCR analysis.

Total RNA was extracted using a Direct-zol RNA MiniPrep kit (Zymo Research). Complementary DNA (cDNA) was synthesized by using an iScript cDNA synthesis kit (Bio-Rad) from 2 µg of RNA. Real time qPCR was performed using SsoAdvanced universal SYBR green supermix with 1 µl of cDNA in a CFX96 real-time PCR detection system (both from Bio-Rad), using primers that amplified target genes (Supplementary Table S4). RNA samples alone (no reverse transcription) and water-only (no template) controls were used to detect gDNA and experimental contamination. To choose the most efficient primer pairs, first by using a gradient temperature PCR, the annealing temperature with the lowest cycle threshold (Ct) was chosen, using the cDNA sample to be used for expression analysis as template. Then, primer efficiency tests of each pair were performed by generating standard curves of five tenfold dilutions of cDNA sample. Primer pairs with 95 to 105% amplification efficiency were chosen. Transcript levels were calculated using comparative ΔCt normalized to gamma actin and 60S ribosomal protein L35 using the geNorm algorithm automated in CFX manager software (Bio-Rad).

Using qPCR to estimate relative fungal biomass in planta.

Quantitative real time PCR was performed for two single-copy genes, *chitinase A* and *NRPS1* (*nonribosomal peptide synthetase 1*), on 5 ng of gDNA isolated from the blades of wild type- and mutant *velA E. festucae*-infected plants with a CFX96 real-time PCR detection system (Bio-Rad), as described by Rasmussen et al. (2007). Fungal biomass was estimated from the number of copies of *chitinase A* or *NRPS1* per total infected plant gDNA.

Detection of plant responses.

For plant cell death, callose deposition, and lignin production tests, endophyte-free etiolated seedlings of *L. perenne* were inoculated as described above.

Plant cell death was detected based on the method of Koch and Shlusarenko (1990). At different timepoints after inoculation, seedlings were stained by boiling for 1 min in lactophenol-trypan blue (a mixture of 10 ml of lactic acid [98%], 10 ml of glycerol, 10 ml of phenol, as well as 10 mg of trypan blue dissolved in 10 ml of distilled water). After staining, seedlings were decolorized for 30 min in chloral hydrate (2.5 g of chloral hydrate dissolved in 1 ml of distilled water). Stained seedlings were mounted in chloral hydrate and observed under a Nikon Ti-E research inverted microscope (Nikon DsRi1 camera) in bright field.

Callose deposition was detected based on the method of Knox (1979), by staining seedlings 3 days postinoculation for 30 min in aniline blue solution (0.1% water-soluble aniline blue in 0.1 M tripotassium orthophosphate [K₃PO₄]) that had been decolorized in the dark for 24 h. Stained seedlings were rinsed and mounted in water and callose deposition was observed under a Leica DMR microscope (Leica DC500 camera) using an I3 filter cube (excitation 450 to 490 nm, emission >515 nm), using *Impatiens walleriana* pollen tubes as a positive control.

Lignin production was tested using a modified method from Tanaka et al. (2014). Seedlings were stained 2.5 days post-inoculation with 1% safranin O (C.I. 50240; BDH) in water for 10 min in the dark, and lignin was observed under a Leica DMR microscope (Leica DC500 camera) in bright field.

Hydrogen peroxide (H₂O₂) production was detected by uptake of 3,3'-diaminobenzidine (DAB) (Thordal-Christensen et al. 1997). Seedlings were inoculated by placing (without any incision) 1- to 2-mm² of fungal culture on agar blocks 1 cm above the meristem. Immediately after inoculation, seedlings were stained with freshly prepared 1 mg of DAB-HCl (pH 3.8) per milliliter for 4 h in the dark. Stained seedlings were observed using a Leica DMR microscope (Leica DC500 camera) in bright field.

Preparation of deletion and complementation constructs.

E. festucae F11 protoplasts were prepared as described by Young et al. (2005) and were transformed with 2 to 5 µg of either circular plasmid DNA or plasmids digested with restriction enzymes or PCR products based on Fleetwood et al. (2007). Transformants were selected on PDA medium supplemented with hygromycin at 200 µg/ml or geneticin at 300 µg/ml, and were nuclear-purified through three rounds of subculture from the outer edge of the colonies on the same medium.

A split-marker strategy was used to generate the *ΔvelA* mutant as described by Rahnama et al. (2017). To generate knock-out vectors pMR5 and pMR6, 3' (2,364 bp) and 5' (1,863 bp) flanking regions (Fig. 4A) of the *velA* gene were amplified, using primers MR5, MR6, MR7, and MR8, with the appropriate attB sites. The PCR products were cloned into vectors pSM1 (GenBank accession KX904528) and pSM2 (GenBank accession KX904529) using Gateway BP Clonase II enzyme and were transformed into Invitrogen One Shot TOP10 cells, according to manufacturer's instructions, and were selected on 50 µg of kanamycin per milliliter.

The *ΔvelA* complementation strain *ΔvelA/velA* was constructed by cotransformation of *E. festucae* *ΔvelA* protoplasts with 5 µg of pII99 plasmid (Namiki et al. 2001) and a 2× molar equivalent of high fidelity PCR product of the *velA* gene containing 1,894-bp 5' and 878-bp 3' flanks amplified from *E. festucae* F11 gDNA with primers MR21 and MR22.

Southern blot analysis.

E. festucae gDNA (3 µg) was digested with *Bam*HI restriction enzymes at 37°C and was separated by agarose gel electrophoresis. DNA was transferred to a positively charged nylon membrane (Hybond-N+; Amersham) by capillary transfer (Southern 1975). DNA was fixed on the membrane by UV cross linking for 1 min using Gel Doc EZ System (Bio-Rad). The membrane was probed and detected using DNA labeling and Detection Starter Kit II (Roche, Switzerland). The membrane was visualized by exposure to a Fuji LAS-4000 Imaging System (Fujitsu Life Sciences) for 10 min.

Microscopy.

TEM analyses were done on 2-cm samples from the top of the youngest mature leaf blades in order to examine standardized tissue types and developmental stage. Samples were fixed in 0.05 M sodium cacodylate (pH 6.8) containing 2.5% glutaraldehyde for 2 h at room temperature. Samples were washed three times in 0.05 M sodium cacodylate (pH 6.8) buffer and were postfixed in 1% osmium tetroxide in 0.025 M buffer for around 1 h at room temperature. Samples were dehydrated through an ethanol series (30, 50, 70, 90, and 100% [twice for 100%]) and were transferred to neat epoxy resin for 32 h with rotation and were then embedded in flat molds and were polymerized for 48 h at 60°C. Samples were cross-sectioned on a

Leica EM UC6 ultramicrotome equipped with a Diatome diamond knife, and then, sections were collected on 200-mesh copper grids and were stained with 2% aqueous uranyl acetate and Reynolds' lead citrate. Images were taken using a Philips CM12 TEM operating at 120 kV, equipped with a Gatan Bioscan 1-megapixel digital camera.

Chitin distribution analyses were done on 4-cm samples from the top of the youngest mature leaf blades. Blade samples were stained using WGA-AF488 Alexafluor (Molecular Probes) solution, were prepared based on the method of Dupont et al. (2015), and were observed using an Andor Revolution spinning disc confocal microscope equipped with an Andor Ixon 885 camera.

Bioinformatics analyses.

Predicted velvet domain-containing protein homologs were identified in *E. festucae* by BLASTP analysis of *E. festucae* F11 (E894) predicted proteins. After aligning the sequences using ClustalW and editing them by removing gaps, a tree was generated using the Neighbor-joining method. Manual sequence analysis and annotation, ClustalW pairwise alignment, phylogenetic tree construction, and InterProScan analyses were performed using Geneious software version 8.1.6. Motif prediction in protein sequences was performed online as follows: NLS sequences were predicted using cNLS Mapper, NES sequences were predicted using the NetNES 1.1 server, and PEST regions were predicted using ePESTfind.

Statistical analysis.

All statistical analyses were performed with SPSS software (IBM Corp.). Statistical significance was determined using independent sample *t* tests or Tukey's multivariate analysis of variance test for multivariate comparisons.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

cNLS Mapper:

http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi
 ePESTfind: <http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind>
Epichloë festucae F11 assembly 2011-03 website:
<http://csbio-l.csr.uky.edu/ef894-2011>
 NetNES 1.1 server: <http://www.cbs.dtu.dk/services/NetNES>