Complex epigenetic regulation of alkaloid biosynthesis and host interaction by heterochromatin protein I in a fungal endophyte-plant symbiosis

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ABSTRACT

Epichloë festucae forms mutualistic symbiotic interactions with grasses of the *Lolium* and *Festuca* genera where it systemically colonises all aerial tissues of the host. Protection from insect and mammalian herbivory are the best-documented benefits of these associations. The two main classes of anti-mammalian alkaloids synthesised by *E. festucae* are the ergot alkaloids and indole diterpenes, of which ergovaline and lolitrems are the principal terminal products. Synthesis of both metabolites require multiple gene products encoded by clusters of 11 genes located at the subtelomeric regions of chromosomes one and three respectively. These loci are close to 'silent' in axenic culture but among the most highly expressed genes *in planta*. We show here that heterochromatin 1 protein (HepA) is an important component of the regulatory machinery that maintains these loci in a silent state in culture. Deletion of this gene led to derepression of *eas* and *ltm* gene expression under non-symbiotic culture conditions. Although there was no obvious culture phenotype RNAseq analysis revealed that around 1000 genes are differentially expressed (DE) in the Δ*hepA* mutant compared to wild type with just one third upregulated. Inoculation of the Δ*hepA* mutant into seedlings of *L. perenne* led to a severe host interaction phenotype characterised by a reduction in tiller length but an increase in tiller number. Hyphae within the leaves of these associations were much more abundant in the intercellular spaces of the leaves and colonised the vascular bundles. The lack of regulated hyphal growth within the leaves was accompanied by a dramatic change in the transcriptome with around 900 genes DE with two thirds of these upregulated. This major physiological change was accompanied by a decrease in *ltm* gene expression and loss of the ability to synthesize lolitrems highlighting the importance of restricted growth for synthesis of these metabolites. Our results show that HepA has an important role in controlling the chromatin state of these subtelomeric loci and their symbiosis specific regulation.

1. Introduction

Eukaryotic genomes are partitioned into discrete functional domains based on the degree of chromatin condensation; euchromatin is generally less condensed, more accessible to the transcriptional machinery and gene-rich; heterochromatin is highly condensed, much less accessible to the transcriptional machinery and relatively gene-poor (Grewal and Jia, 2007). Heterochromatin is mainly found at centromeres, sub-telomeres and rDNA clusters and has important roles in genome stability and integrity including chromosome segregation, telomere capping, and DNA repair (Allshire and Karpen, 2008; Ayoub et al., 2009; Fanti et al., 1998; Perrini et al., 2004). A key protein for formation of heterochromatin in eukaryotic organisms is heterochromatin protein 1 (Lachner et al., 2001; Maison and Almouzni, 2004). This protein is recruited to chromatin by recognition of di- and tri- methylated histone H3K9 via the N-terminal chromodomain of HP1. The C-terminal domain of this protein promotes dimerization and interaction with HP1-associated proteins such as the H3K9 methyltransferase resulting in methylation of adjacent nucleosomes and initiation of heterochromatin spreading leading to condensation and inactivation of the chromatin. Most of our knowledge about the modes of action of HP1 proteins has been acquired from studies with *Drosophila* HP1 (DmHP1a), *Schizosaccharomyces pombe* Swi6 and mammalian HP1α (Grewal and Elgin, 2002; Grewal and Jia, 2007; Lomberk et al., 2006).

Studies in *Neurospora crassa* have also provided key insights into heterochromatin formation and in particular the crucial link between H3K9 methylation and DNA methylation, a process critical for chromatin silencing (Tamaru and Selker, 2001). Initiation of heterochromatin formation is catalysed by the DCDC [defective in methylation (DIM)-5,-7,-9, Cullin 4, DNA damage-binding protein 1 (DDB1) complex] at highly degenerate DNA repeat sequences, many of which are products of repeat-induced point mutation (RIP), a genome defense system first unravelled in *N. crassa* (Lewis et al., 2010; Lewis et al., 2009; Tamaru and Selker, 2001). This complex trimethylates H3K9 (DIM-5) to create binding sites for multiple HP1 (Hpo)-containing complexes, which in turn direct methylation (DIM-2) of cytosine bases

in DNA and deacetylation of histones (Honda et al., 2012; Honda and Selker, 2008; Tamaru et al., 2003).

Heterochromatin also plays an important role in the regulation of secondary metabolite gene expression in filamentous fungi as the genes encoding the enzymes for these biosynthetic pathways are frequently organized as clusters in heterochromatic regions of the genome, such as sub-telomeric regions that frequently comprise mosaics of non-functional transposable elements (Brakhage, 2013; Palmer and Keller, 2010; Winter et al., 2018). Deletion of genes encoding heterochromatin protein-1 (HepA) or the histone H3 lysine 9 (H3K9) methyl transferase (ClrD) in *A. nidulans* and heterochromatin protein-1 (HEP1) in *Fusarium graminearum* results in enhanced expression of secondary metabolite genes (Reyes-Dominguez et al., 2012; Reyes-Dominguez et al., 2010). Deletion of *hepA* in *A. nidulans* led to increased expression of genes encoding enzymes for sterigmatocystin (STC), isopenicillin A and terraquinone B biosynthesis (Reyes-Dominguez et al., 2010). The onset of STC biosynthesis was accompanied by a decrease in HepA occupancy at *stc* gene promoters, however there was no change in HepA occupancy at promoters of neighbouring genes, suggesting the presence of chromatin boundary elements flanking the *STC* locus. Deletion of *clrD*, encoding the H3K9 methyltransferase, also led to increased expression of *STC* cluster genes and was accompanied by a reduction in the relative levels of H3K9 trimethylation. Interestingly, deletion of *Hep1* in *F. graminearum* led to increased expression of genes for aurofusarin biosynthesis but decreased expression of genes for trichothecene biosynthesis, suggesting that Hep1 can have both a repressive and activation role depending on the chromosome environment of the cluster (Reyes-Dominguez et al., 2012). Despite the differences in the direction of expression, H3K9me3 marks were depleted in the promoters of the genes in both clusters.

In contrast to the fungal systems described above many secondary metabolite biosynthesis clusters are silent when fungi are grown in axenic culture (Bok et al., 2009; Brakhage, 2013). While activation of these clusters can sometimes be achieved through the inactivation of genes that encode enzymes catalysing repressive marks such as H3K9me3 and H3K27me3 (Connolly et al., 2013), or by overexpression of cluster-specific transcription

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factors (Bergmann et al., 2007), it is becoming increasingly apparent that interaction of the fungus with another organism is crucial for the activation of many secondary metabolism pathways (Nützmann et al., 2011; Schroeckh et al., 2009). This is especially true for fungi that form symbiotic associations with plants, such as the associations between *Epichloë spp.* and temperate grasses where nearly all the secondary metabolite gene clusters identified to date are silent or very lowly expressed in axenic culture but highly expressed *in planta* (Fleetwood et al., 2007; Tanaka et al., 2005; Young et al., 2005; Zhang et al., 2009). The products of these biosynthetic clusters protect the host from biotic stresses such as insect and mammalian herbivory.

The two main classes of anti-mammalian alkaloids synthesized by *Epichloë* endophytes are the ergot alkaloids and indole diterpenes, of which ergovaline and lolitrem B are the principal final products for *Epichloë festucae* strain Fl1 (Schardl et al., 2013). The genes encoding the enzymes required for the synthesis of each of these metabolites are organised in clusters immediately adjacent to the telomeres of chromosome one (*EAS* locus) and chromosome three (*LTM* locus), respectively (Winter et al., 2018). Each locus contains 11 genes organised in three subclusters separated by large blocks of type I (retrotransposons) that have undergone RIP, and non-functional type II – principally miniature inverted transposable elements (MITEs) – transposons; genome features that favour formation of heterochromatin and gene silencing (Fleetwood et al., 2011; Schardl et al., 2013; Young et al., 2006). However, both the *eas* and *ltm* cluster genes are highly expressed *in planta* suggesting that these regions of the genome are significantly remodelled when *E. festucae* is in close association with its host. In support of this hypothesis we recently showed that the levels of H3K9me3 and H3K27me3 were significantly reduced at these loci in plant-infected tissue compared to axenic culture (Chujo and Scott, 2014). Furthermore, deletion of the H3K9 or H3K27 methyltransferases, encoded by *clrD* and *ezhB* respectively, lead to derepression of *eas* and *ltm* genes in axenic culture. However, the level of expression of the *eas* and *ltm* genes in these mutants *ex-planta* was still considerably less than observed *in planta* highlighting that there are

multiple regulatory layers controlling the chromatin remodelling and gene activation that leads to full derepression of these symbiosis-specific biosynthetic pathways.

The aim of this study was to determine the role of heterochromatin protein I in regulating the expression of genes in the *EAS* and *LTM* clusters using a combined genetic, biochemical and transcriptomics approach, with the goal of further understanding the mechanisms that underlie how high-level expression of these cluster genes occurs *in planta* in response to chromatin remodeling.

2. Materials and methods

2.1. Strains and growth conditions

Cultures of *Escherichia coli* were grown overnight in LB (Lysogeny Broth) broth or on LB agar supplemented when necessary with either ampicillin (100 µg/ml) or kanamycin (50 µg/ml) as previously described (Miller, 1972). Cultures of *E. festucae* were grown on 2.4% (w/v) potato dextrose (PD) agar (Sanderson and Srb, 1965), under conditions previously described (Moon et al., 2000; Moon et al., 1999). Liquid shake (200 rpm) cultures were grown for 4 d at 22°C in 100 ml conical flasks containing 50 ml 2.4% PD medium or MSM3 defined medium containing 3.67 mM KH_2PO_4, 3.44 mM K_2HPO_4, 15 mM sucrose, 5 mM glutamine, 2 mM $MgSO_4$, 0.6 µM thiamine and trace elements: 3.6 µM H_3BO_4, 1 µM $CuSO_4$, 0.7 µM KI, 0.8 µM FeNa-ethylenediaminetetraacetic acid, 1 µM $MnSO_4$, 0.5 µM $NaMoO_4$ and 0.4 µM $ZnSO_4$ (Blankenship et al., 2001). A description of all biological material is provided in Table S1.

2.2. Plant growth and endophyte inoculation conditions

Endophyte-free seedlings of perennial ryegrass (*Lolium perenne* cv. Samson) were inoculated with *E. festucae* by the method of (Latch and Christensen, 1985). Plants were grown in root trainers in an environmentally controlled growth room at 22°C with a photoperiod of 16 h of light (~100 $\mu E/m^2$ per sec) and 6 weeks after planting tested for the presence of endophyte by immunoblotting (Tanaka et al., 2005).

2.3. DNA isolation, PCR and sequencing

Fungal genomic DNA was extracted from freeze-dried mycelium as previously described (Byrd et al., 1990). For rapid extraction of genomic DNA on a small scale, mycelia were grown in PD broth for 4 d, transferred to lysis buffer (150 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1% SLS) and incubated at 70°C for 30 min. Genomic DNA was then isolated from the aqueous phase by sequential precipitation with 5 M potassium acetate, isopropanol and 70% ethanol and

resuspended in 20 μl H₂O. Plasmid DNA was isolated using a High Pure plasmid isolation kit (Roche) according to the manufacturer's instruction.

Standard PCR amplification was performed with *Taq* DNA polymerase (Roche). Where proofreading activity was required, the Expand High Fidelity™ PCR system (Roche), or *Pfx50*™ DNA Polymerase (Invitrogen) or Phusion High-Fidelity DNA Polymerase (Thermo Scientific) were used. The reaction mixture for *Taq* DNA polymerase or Expand High Fidelity™ PCR system (50 μl) contained 1× PCR Reaction buffer or 1× Expand High Fidelity Buffer, 0.3 μM forward and reverse primers, 200 μM dNTPs, 1 ng template DNA and 1.25 U of *Taq* polymerase or 2.6 U of Expand High Fidelity™ Enzyme Mix (Roche). The PCR parameters used were: one cycle at 94°C for 2 min; 30-40 cycles at 94°C for 15 sec, 50-65°C for 30 sec and 68-72°C for 1 min per kb followed by a final extension cycle of 7 min at 68-72°C. Reaction mixtures were stored at 4°C until analyzed. The reaction mixture for *Pfx50*™ DNA Polymerase (50 μl) contained 1× *Pfx50*™ PCR Mix, 0.3 μM forward and reverse primers, 300 μM dNTPs, 1 ng template DMA and 5 U of *Pfx50*™ DNA Polymerase. The PCR parameters used were: one cycle at 94°C for 2 min; 30-40 cycles at 94°C for 15 sec, 50-65°C for 30 sec and 68°C for 1 min per kb, followed by a final extension cycle of 5 min at 68°C. The reaction mixture for Phusion High-Fidelity DNA Polymerase (50 μl) contained 1× Phusion HF buffer, 0.5 μM forward and reverse primers, 200 μM dNTPs, 1 ng template DMA and 1 U of Phusion High-Fidelity DNA Polymerase. The PCR parameters used were: one cycle at 98°C for 30 sec; 30-40 cycles at 98°C for 10 sec and 72°C for 30 sec per kb, followed by a final extension cycle of 5 min at 72°C.

DNA fragments were sequenced using the dideoxynucleotide chain termination method with the Big-Dye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Applied BioSystems) and separated using an ABI3730 genetic analyzer (Applied BioSystems). Sequence data were assembled and analyzed using the MacVector sequence assembly software, version 12.0.5.

2.4. Preparation of deletion, complementation and HepA-EGFP constructs

Lists of all plasmids and the primer sequences used to prepare those constructs can be found in Tables S2 and S3.

To construct the *hepA* replacement construct (pTC10), the 1.9-kb 5' of *hepA* fragment and the 1.9-kb 3' of *hepA* fragment were prepared by PCR amplification from *E. festucae* genomic DNA, using the primer pairs TC31/TC32 and TC17/TC33, respectively. These fragments were then ligated into pCR™4Blunt-TOPO® (Invitrogen) to generate pTC8 and pTC9, respectively. After performing a sequence check, the 5' of *hepA* and the 3' of *hepA* DNA fragments were excised from pTC8 and pTC9 by *Eco*RI/*Kpn*I and *Xba*I/*Xho*I digestions, respectively, and were sequentially inserted between the corresponding sites of pSF15.15 (a pII99-based vector containing an *hph* (hygromycin resistance) cassette).

The 5.3-kb linear product of pTC10 (*hepA*) used for transformation was amplified with primer pair TC31/TC33 using the Expand High Fidelity PCR system (Roche) according to the manufacturer's instruction.

To construct the *hepA* complementation construct (pTC39), the 1.9- (5') and 2.0-kb (3') fragments of *hepA* were prepared by PCR amplification from *E. festucae* genomic DNA, using primer pairs TC58/TC59 and TC60/TC61, respectively. These fragments were then ligated into pCR™4Blunt-TOPO® (Invitrogen) to generate pTC23 and pTC24, respectively. After performing a sequence check, a *Not*I/*Sst*I fragment was excised from pTC23 and cloned into the same sites in pTC24 to generate pTC35. A *Sal*I/*Xho*I fragment containing the full-length *hepA* was excised from pTC35 and cloned into the corresponding sites in pSF17.1 to generate pTC39.

To construct the P*tefA*-*hepA*-*egfp* plasmid (pTC65), the 0.8-kb *PtefA* fragment and the 0.9-kb *hepA* cDNA fragment were prepared by PCR amplification from pPN114 and *E. festucae* cDNA, using primer pairs TC491/TC492 and TC493/TC494, respectively. These fragments were then ligated into pCR®-Blunt II-TOPO® (Invitrogen) to generate pTC61 and pTC62, respectively. After performing a sequence check, the *PtefA* fragment was excised from pTC61 by *Kpn*I/*Eco*RI digestion, and was sequentially inserted between the corresponding sites of pPN83 to generate pTC63. Then, the *hepA* cDNA fragment was excised from pTC62 by

*Eco*RI/*Nco*I digestion, and inserted between the corresponding sites of pTC63 to generate pTC64. Finally, the *PtefA-hepA* fragment was excised from pTC64 by *Kpn*I/*Not*I digestion, and inserted between the corresponding sites of pSF17.1 to generate pTC65.

2.5. E. festucae transformation

E. festucae protoplasts were prepared as previously described (Young et al., 2005). Protoplasts were transformed with 5 μg of linear PCR-amplified or circular plasmid DNA using the method previously described (Itoh et al., 1994). Transformants were selected on YPS media containing either hygromycin (150 μg/ml) or geneticin (200 μg/ml) and nuclear purified by three rounds of sub-culturing on PD medium containing the same antibiotic selection (Young et al., 2005).

2.6. DNA hybridization

E. festucae genomic digests separated by agarose gel electrophoresis were transferred to positively charged nylon membranes (Roche)(Southern, 1975) and fixed by UV light cross-linking in a Cex-800 UV light cross-linker (Ultra-Lum) at 254 nm for 2 min. DNA was labelled with digoxigenin-11-dUTP (DIG-11-dUTP) by primed synthesis using a DIG High Prime kit (Roche). Hybridizations were performed using the DIG High Prime DNA Labeling & Detection Starter Kit I (Roche) according to the manufacturer's instructions and visualized by nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) color detection.

2.7. RNA extraction, reverse transcription and quantitative RT-PCR

Total RNA was isolated from frozen mycelium or perennial ryegrass pseudostem tissue using either TRIzol® Reagent (Invitrogen) or RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. For normal RT-PCR, cDNA was synthesized by using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. For quantitative RT-PCR (qRT-PCR), total RNA was subjected to cDNA synthesis using the QuantiTect® Reverse Transcription Kit (Qiagen) according to the

manufacturer's instructions. qRT-PCR was performed using the SsoFast™ EvaGreen® Supermix (Bio-Rad) on a LightCycler® 480 System (Roche) with either three or four biological replicates and two technical replicates per reaction. Transcript levels were normalized to elongation factor 2 (EF-2) and 40S ribosomal protein S22 (S22). Sequences of PCR primers used for qRT-PCR analysis are provided in Table S4.

For RNAseq analysis mycelia, grown for 10 days in MSM3 media, or pseudostem tissue harvested from ryegrass plants eight weeks post-inoculation, were snap frozen in liquid nitrogen. Total RNA was isolated from two biological replicates of each sample [0.5-0.6 g fresh weight mycelia or 3x 2 cm segments of pseudostem tissue harvested ~0.5 cm from the base of the tiller) using TRIzol reagent® (Invitrogen). Illumina TruSeq libraries were generated by New Zealand Genomics Limited and indexed. Libraries from the mycelial samples were run on an Illumina MiSeq using two 250-nucleotide paired-end runs whereas plant samples were run on three lanes of an Illumina HiSeq 2000 using a 100-nucleotide paired-end run. After sequencing, reads were de-multiplexed and sample assignments confirmed by mapping to the hygromycin resistance cassette (*hph*) present in the Δ*hepA* samples.

2.8. Microscopy

Cultures to be analyzed by light microscopy were inoculated onto a thin layer of PD agarose (2% w/v) layered on top of a base layer of PD agar (1.5% w/v) and grown for 5 d. Square blocks were cut from the agarose and placed in an imaging chamber (CoverWell, 20 mm diameter, 0.5 mm deep) (Molecular Probes) filled with 500 μl of distilled water and sealed with a 22 x 60 mm glass coverslip.

For fluorescence microscopy analysis, cultures were grown on a 5 mL layer of 1.5% (w/v) water agar cast over a sterile microscopy slide on a 20 mL 1.5% (w/v) water agar base. Hyphae were stained with 250 μg/ml Calcofluor White (Fluorescent Brighter 28; Sigma-Aldrich) in 50% glycerol (w/v) and examined under the Olympus IX71 (Olympus) inverted fluorescence microscope using the U-MWU2 Ultraviolet excitation cube (wideband).

For confocal microscopy analysis of HepA localisation, cultures were grown on a 5 mL layer of 1.5% (w/v) water agar cast over a sterile microscopy slide on a 20 mL 1.5% (w/v) water agar base. Nuclei were stained with 1 µg/mL DAPI (Sigma) in McIlvaine's buffer (pH 7)(McIlvaine, 1921) and the samples were observed using the Leica SP5 DM6000B confocal microscope (Leica Microsystems) (x40 oil immersion objective, numerical aperture [NA] = 1.3). DAPI was sequentially excited at 405 nm and emission was captured at 440-480 nm and GFP was sequentially excited at 488 nm and emission was captured at 515-545 nm.

Growth and morphology of *E. festucae* hyphae *in planta* was determined by staining leaves with aniline blue/Wheat Germ Agglutinin conjugated-AlexaFluor®488 (WGA-AF488; Molecular Probes/Invitrogen) as follows. Tillers of perennial ryegrass infected with *E. festucae* were soaked in 95% (V/V) ethanol overnight at 4°C, then treated with 10% potassium hydroxide for 3 h. The tissue was washed three times with PBS (pH 7.4) and incubated in staining solution (0.02% aniline blue, 10 ng/ml WGA-AF488, and 0.02% Tween 20 in PBS [pH 7.4]) for 30 min, followed by 10-min vacuum infiltration. Images of GFP fluorescence in *E. festucae* hyphae and *E. festucae* hypae and septa *in planta* were captured by confocal laserscanning microscopy using a Leica SP5 DM6000B (×40 oil immersion objective, NA = 1.3) (Leica Microsystems) with excitation wavelength of 405 nm and 488 nm, and acquisition windows of 449 nm to 555 nm and 498 nm to 558 nm for aniline blue and WGA-AF488, respectively.

2.9. Alkaloid analysis

Indole-diterpenes and ergot alkaloids were extracted from Epichloë-infected pseudostem tissue of *L. perenne* and analysed by LC-MS/MS as previously described (Rasmussen et al., 2012). Ergotamine was included as an internal standard for quantifying ergot alkaloids.

2.10. Bioinformatic analysis

E. festucae genes were identified by tBLASTn analysis of the *E. festucae* Fl1 (E894)

genome (http://csbio-l.csr.uky.edu/ef894-2011) with protein sequences obtained from either the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) or the Broad Institute (http://www.broad.mit.edu). Identity and similarity scores were calculated after ClustalW pairwise alignments of sequences (Thompson et al., 1994), using MacVector version 12.0.5. The *E. festucae* genome sequence data, as curated by C.L. Schardl at the University of Kentucky is available at http://csbio-l.csr.uky.edu/ef894-2011/.

Genome-wide gene expression of wild type and Δ*hepA Epichloë festucae* Fl1 was determined *in planta* and in culture using the general approach described previously (Ref ?). Read quality was checked using SolexaQA++ v. 3.1.4 (Cox et al., 2010). Using the dynamic trimming function of the same package, reads were trimmed such that all bases had a probability of error ≤ 0.05 and only reads ≥80 bases long were retained.

To validate samples, reads were first mapped to the unmodified *hepA* gene and the hygromycin gene in the deletion construct using the Burrows-Wheeler mapping algorithm implemented in Bowtie2 v.2.2.6 (Langmead et al., 2009). As expected, wild type samples showed matches to the *hepA* gene, but not the deletion construct; while Δ*hepA* mutant samples showed matches to the deletion construct, but not the *hepA* gene.

To determine genome-wide *E. festucae* gene expression, reads from each of two biological replicates were mapped separately for each condition to the set of EfM3 v.5 gene models (n = 8,324; http://www.endophyte.uky.edu) together with 6 additional secondary metabolite genes (*ltmE*, *ltmG*, *ltmJ*, *ltmK*, *ltmM* and *ltmS*). Reads that did not map uniquely to a single gene reference were excluded, and the number of reads that mapped to each gene was determined using the in-house software, mapcount (http://mpcox.github.io/mapcount/). Paired-end reads were treated as only a single 'hit'. Statistical significance, accounting for the variance between replicates, was calculated using Fisher's Exact Test (Fisher, 1922) as implemented in the R package (R development core team, 2013). DEGseq v. 1.24.0 A correction for multiple testing was applied using the False Discovery Rate (FDR) approach of (Storey and Tibshirani, 2003). The fold difference for each gene i was calculated from the raw read counts normalized by the total number of mapped reads as

$$FD_i = \frac{\max(condition1_i, condition2_i)}{\min(condition1_i, condition2_i)} \qquad \text{Equation 1}$$

The RNAseq data for *E. festucae* strains Fl1 and Δ*hepA* are available from the NCBI Sequence Read Archive (SRA) under Bioproject PRJNA447872. All statistical analyses were conducted by using GraphPad Prism6 (GraphPad Software).

3. Results

3.1. Identification and disruption of E. festucae heterochromatin protein-1

To identify an orthologue of the heterochromatin protein-1 gene in *E. festucae*, we performed a tBLASTn search of the *E. festucae* genome using as query the heterochromatin protein-1 amino acid sequence from *N. crassa* (HP1) (Freitag et al., 2004). This search identified a candidate homologue (gene model EfM3.043690), which we have called HepA (Bennett and Lasure, 1985; Reyes-Dominguez et al., 2010; Schardl and Scott, 2010). The *E. festucae* HepA protein shares 53.6%, 47.8% and 41.7% identity with heterochromatin protein-1 from *Fusarium graminearum*, *N. crassa* and *Magnaporthe oryzae*, respectively, and possesses both Chromo (a methyl-specific histone binding) and Chromo-Shadow (self-aggregation) domains, which are highly conserved features found across different fungal heterochromatin protein-1 (Fig. S1).

To investigate the biological role of HepA in *E. festucae*, a replacement construct, pTC10, was prepared and a PCR-amplified linear fragment of this plasmid introduced into the genome of *E. festucae* strain Fl1 by homologous recombination (Fig. S2A). PCR screening of 124 hygromycin-resistant (Hyg[R]) transformants identified three candidates – Δ*hepA*#109, #121 and #122 – that had PCR product patterns consistent with targeted replacement events. Southern

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analysis of genomic DNA digests from these transformants probed with a PCR fragment derived from pTC10 confirmed that all three candidates were single copy 'clean' replacement mutants at the *hepA* gene locus (Fig. S2B). Consistent with this analysis the *hepA* mRNA transcript found in the wild-type strain was not found in the *hepA* mutants but was restored upon introduction of pTC39, containing the full-length *hepA* gene under the control of its native promoter and terminator (Fig. S2C).

3.2. Culture phenotype of E. festucae ΔhepA

To investigate the role of HepA in *E. festucae* development, we compared the morphology and radial growth of Δ*hepA* mutants with the wild-type strain on PD agar medium. In contrast to Δ*clrD* (encoding the H3K9 methyl transferase) which has a severe reduction in radial growth (Chujo and Scott, 2014), the colony morphology of Δ*hepA* was similar to wild-type but with a slight reduction in radial growth (Fig. 1A). Introduction of a wild-type copy of *hepA* into Δ*hepA* rescued the wild-type growth phenotype, confirming complementation of this mutant (Fig. 1A). A closer examination of the edges of these colonies by light microscopy revealed there were no differences in the hyphal morphology of Δ*hepA* compared to wild-type and complemented strains. Given many previously isolated *E. festucae* symbiosis mutants are defective in cell-cell fusion (Becker et al., 2015; Kayano et al., 2013; Tanaka et al., 2013) we also examined this culture phenotype and found that Δ*hepA* was still able to form hyphal cell-cell fusions (Fig. 1B).

3.3. Nuclear localization of E. festucae HepA is dependent on a functional histone H3 lysine 9 histone methyltransferase

To determine the localisation of the HepA protein in *E. festucae* cells we introduced a *hepA-egfp* fusion gene under the control of a *tefA* promoter into wild-type and Δ*clrD* strains and imaged these transformants by confocal laser scanning microscopy (CLSM). While the

HepA-eGFP fusion protein localized mainly to the nucleus in the wild-type strain, as confirmed by DAPI staining, it had a very diffuse localisation in the Δ*clrD* strain (Fig. 2). These results show that the localisation of *E. festucae* HepA to the nucleus is ClrD-dependent, a result similar to what was previously observed for the localisation of HP-1 in *N. crassa* and *L. maculans* (Freitag et al., 2004; Soyer et al., 2013).

3.4. E. festucae HepA is involved in LTM and EAS cluster gene silencing in axenic culture

We recently showed that ClrD is involved in the repression of the *ltm* and *eas* cluster genes in axenic culture (Chujo and Scott, 2014). We therefore tested whether disruption of *hepA* in *E. festucae* would also result in derepression of the *ltm* and *eas* cluster genes in axenic culture. The steady-state transcript levels of *ltm* and *eas* genes in the wild-type, Δ*hepA* and Δ*hepA/hepA* strains were analysed by RT-qPCR analysis using total RNA purified from culture grown mycelia of each of these strains. This analysis showed that seven (*ltmP*, *ltmQ*, *ltmF*, *ltmB*, *ltmG*, *ltmS* and *ltmM*) of the 11 *ltm* genes were derepressed in the Δ*hepA* strains relative to the wild-type, and that transcript levels were restored to wild-type levels in the Δ*hepA/hepA* complemented strains (Fig. 3A & B). However, three of the *ltm* genes remained transcriptionally silent in the Δ*hepA* strains (Fig. 3B). Similarly, some of the *eas* cluster genes including *lpsB*, *easE*, *easG*, *easA*, *dmaW* and *easC* were significantly derepressed in Δ*hepA* strains relative to wild-type (Fig. 3C & D), but others remained transcriptionally silent (Fig. 3D). These patterns of *ltm* and *eas* gene expression in the Δ*hepA* background are very similar to what we observed in the Δ*clrD* mutant background (Fig. S3).

We also analyzed transcript levels of some genes immediately adjacent to the *LTM* and *EAS* gene clusters including genes encoding a putative cytochrome P450 monooxygenase (EfM3.048280) and a cytochrome P450 demethylase (EfM3.048420), which are adjacent to the *ltm* cluster, and a chitinase (EfM3.051200) and cell wall glycoprotein (EfM3.054000), which are adjacent to the *eas* cluster. We found that the genes encoding the monooxygenase and the chitinase, which were recently shown to be on the boundaries of topologically associated

domains (TADs) for *LTM* and *EAS* (Winter et al., 2018), were derepressed in the Δ*hepA* background whereas the genes encoding the demethylase and glycoprotein remained transcriptionally silent (Fig. S4). These results suggest that HepA is one of several components important for the repression of *ltm* and *eas* cluster genes and other genes in the sub-telomeric regions (Schardl et al., 2013) of *E. festucae* when grown in axenic culture.

3.5. Transcriptome changes between ΔhepA and wild-type in axenic culture

To gain some insight into the effect of the Δ*hepA* mutation on gene expression in axenic culture high-throughput mRNA sequencing was performed on two biological replicates for each of Δ*hepA* and wild-type mycelia. 12.7% (1060/8330) of the genes in the *E. festucae* EfM3 gene model set (Schardl et al., 2013) were differentially expressed between the two samples, with 373 (35.2%) genes up-regulated and 687 genes (64.8%) down-regulated in the Δ*hepA* sample relative to the wild-type sample (File S1). These differentially expressed genes (DEG) were sorted into molecular function Gene Ontology (GO) categories based on their primary function (File S2; Fig. S5). While the largest group of DEG fell within the "Molecular Function Unknown" category, many others could be assigned putative functions. Strikingly, 34 genes encoding putative transcription factors were DE with the majority of these (27) being down regulated. Analysis of the changes in expression of the *ltm* and *eas* secondary metabolite cluster genes showed that five of the *ltm* and eight of the *eas* genes were up-regulated in Δ*hepA* compared to wild-type (File S3), a result independently confirmed by RT-qPCR (Fig. 3). Further analysis of candidate secondary metabolite genes identified eight encoding putative NRPSs, including *perA* (Tanaka et al., 2005), *sidN* (Johnson et al., 2013) and *lpsB* (Fleetwood et al., 2007), and nine putative cytochrome P450 monooxygenases, including *ltmP* and *ltmK* (Young et al., 2006), that were DE in Δ*hepA* compared to wild-type (File S3). The cytochrome P450 monooxygenases gene with the greatest fold increase (EfM3.014800) appears to part of a cluster of genes that includes a polyketide synthase (EfM3.014820) that was also upregulated.

3.6. Deletion of E. festucae hepA disrupts the symbiotic interaction with L. perenne

To determine the symbiotic interaction phenotype of the Δ*hepA* strains, seedlings of *L. perenne* were infected with two independent Δ*hepA* mutants and the wild-type strain. In contrast to plants infected with the wild-type strain, plants infected with the Δ*hepA* strains were stunted, with an increase in tiller number and a reduction of plant height (Fig. 4). Introduction of a wild-type copy of *hepA* into Δ*hepA*#109 rescued the wild-type symbiotic interaction phenotype (Fig. S6). To examine the cellular phenotype of the Δ*hepA* mutants *in planta*, we infiltrated sheath tissue with aniline blue/WGA-AF488, in order to stain the cell wall and chitin, respectively and analysed the samples by CLSM. Extensive hyphal colonisation was observed in plants infected with Δ*hepA* compared with wild-type. Hyphae in the leaf tissue of Δ*hepA*-infected plants were observed to form a complex tangle indicative of de-regulated growth (Fig. 5A). Multiple hyphae were found in the intercellular space where typically just one hypha was found in leaf tissue infected with the wild-type strain (Fig. 5B). In addition, multiple hyphae were observed within the vascular bundles of plants infected with Δ*hepA* whereas no hyphae were observed in this tissue for wild-type (Fig. 5C). These results demonstrate that HepA is essential for establishing and maintaining a mutualistic interaction between *E. festucae* and *L. perenne*.

3.7. Transcriptome changes in the hepA mutant association compared to wild-type

To identify fungal genes whose expression is altered between the Δ*hepA* antagonistic host association and the wild-type mutualistic association (Fig. 4), high-throughput mRNA sequencing was performed on two biological replicates for each of the two associations. 10.8% (899/8330) of the genes in the *E. festucae* EfM3 gene model set (Schardl et al., 2013) were differentially expressed between the two samples, with 571 (63.4%) genes up-regulated and

330 (36.6%) genes down-regulated in the Δ*hepA* sample relative to the wild-type sample (File S4). To gain insight into the nature of the gene expression changes, genes were sorted into molecular function Gene Ontology (GO) categories based on their primary function (File S5; Fig. S7). Although many of the differentially expressed genes (DEG) corresponded to those previously identified in three other symbiotic mutant associations (Eaton et al., 2015), around half (439/899) of the DEG were unique to the Δ*hepA* gene set (File S6; Fig. S8). A number of genes encoding products for primary metabolism, transport, host cell wall degradation, fungal cell wall structure and small secreted proteins were up-regulated in the Δ*hepA* sample, a result consistent with the proliferative growth of the Δ*hepA* mutant in host leaf tissue compared to the restrictive growth of the wild-type strain (Fig. 5). Among the genes that were down-regulated were a number encoding small secreted proteins and enzymes involved in secondary metabolism, including 10 of the 11 genes that comprise the *ltm* gene cluster (Fig. 6A; File S3). Nine of the 11 *eas* genes were up-regulated in the Δ*hepA* mutant compared to wild-type (Fig. 6C; File S3). Further analysis of candidate secondary metabolite genes identified seven putative NRPSs, including *perA* (Tanaka et al., 2005), *lpsA* (Panaccione et al., 2001) and *lpsB* (Fleetwood et al., 2007), and 16 putative cytochrome P450 monooxygenases, including *ltmP, ltmK* and *ltmJ* (Young et al., 2006), that were DE in Δ*hepA* compared to wild-type (Files S3 & S4), some upregulated and some downregulated.

We then checked whether the levels of indole-diterpene and ergot alkaloid metabolites found in these tissues correlated with these changes in gene expression. Metabolites were extracted from tissues infected with either Δ*hepA* or wild-type and examined by LC-MS/MS analysis. Consistent with the RNAseq (Figs 6A & C) and RT-qPCR analyses (Figs 6B & D) the relative levels of lolitrem B, the main lolitrem pathway product, and lolitriol, an abundant intermediate (Saikia et al., 2012), were not detectable in the Δ*hepA* mutant but present in wild-type (Fig. 6E). The absence of detectable levels of ergovaline in the extracts of the Δ*hepA* mutants is consistent with the decrease in expression of *lpsA*, which encodes an NRPS that catalyses the final biosynthetic step for ergovaline biosynthesis (Schardl et al., 2006)(Fig.

6E), even though most of the other *eas* cluster genes were up-regulated. Although the levels of the intermediate chanoclavine (Schardl et al., 2006) were less than those detected in wild-type the difference was not significant (Fig. 6E).

3.8. Core symbiosis genes differentially expressed in other symbiotic mutants are also differentially expressed in hepA mutant associations

Previously we compared fungal gene expression differences between a wild-type mutualistic association and three mutant (Δ*noxA*, Δ*proA* and Δ*sakA*) antagonistic associations and identified a core set of 182 DEG genes common to all three mutant associations with 143 up-regulated and 39 down-regulated (Eaton et al., 2015). By combining the Δ*hepA* DEG set (File S5) with the previous three data sets, using the same methods as previously used, we found that a set of 118 DEG (Fig. 7; File S7) were shared by all four symbiotic mutant associations, based on significant differences (corrected P<0.05) and a two-fold change cut-off. These core genes were mapped to the recently assembled telomere to telomere sequence of the Fl1 genome (Winter et al., 2018), but were not enriched on any particular chromosome or genome location such as proximity to AT-rich repeat sequences (Fig. S9). The 118 DEG encoded products associated with the biological processes highlighted in our previous study (Eaton et al., 2015), including primary metabolism, fungal cell wall structure and composition, host cell wall degradation, membrane transport, secondary metabolism and symbiont-host communication (Fig. S10). Given the commonality of these changes among very different symbiosis mutants most of the changes observed are likely to be a result of a 'breakdown' in the symbiosis rather than have a direct causal link to the type of mutation. Among the up-regulated genes were two plant hormone biosynthetic genes including IPT LOG (EfM3.064190) a bifunctional enzyme encoding an isopentenyl transferase (IPT) and a cytokinin specific phosphoribohydrolase (LOG: *lonely guy*)(Hinsch et al., 2015); and a FAD-dependent monooxygenase (EfM3.064200; YUCCA domain protein) responsible for the conversion of indole 3-pyruvate to auxin. These

two genes are part of a three gene cluster flanked by AT-rich isochores, but the third gene in this cluster (EfM3.063360), encoding a cytokinin-specific cytochrome P450 monooxygenase (Hinsch et al., 2015), is not significantly up-regulated in the Δ*hepA* data set. Of the 118 genes, 87 were up-regulated and 31 down-regulated. Of the 64 genes that 'dropped out' from the original 182 core gene set in this 4-way analysis, 63 were not significantly differentially expressed in Δ*hepA* whereas one (*thiA*/EfM3.032990 encoding a thiamine biosynthetic enzyme) had the opposite pattern of expression.

4. Discussion

Establishment of a mutualistic symbiotic interaction between *Epichloë festucae* and *Lolium perenne* results in a dramatic change in the transcriptome profile of both the host and mycosymbiont leading to major changes in host metabolism (Dupont et al., 2015). Among the many fungal genes that are preferentially expressed in the symbiotic physiological state are the *eas* and *ltm* genes, which encode enzyme products for the biosynthesis of ergot alkaloids and indole diterpenes (Fleetwood et al., 2007; Schardl et al., 2013; Young et al., 2006). These genes are organized in complex gene clusters immediately adjacent to the telomeres of the long arms of chromosomes I and III of *E. festucae* strain Fl1 (Winter et al., 2018). These genes are silent in culture but highly expressed *in planta*, with the *ltm* genes among the top 50 most highly expressed genes. We show here that chromatin remodeling of these subtelomeric loci is an important regulatory layer controlling expression of these genes and the biosynthesis of these secondary metabolites. Specifically, we demonstrate a key role for HP1 in controlling expression of these genes. We also show that this protein has a crucial role in establishing and maintaining a mutualistic symbiotic interaction. In the absence of a functional HepA *E. festucae* forms a highly antagonistic association with its host.

The derepression of *ltm* and *eas* gene expression observed in axenic cultures of *E. festucae* Δ*hepA* is consistent with our previous study showing that deletion of *clrD*, which encodes the H3K9 methyl transferase important for establishing the methylation marks to which HepA binds, also leads to derepression of these sub-telomeric secondary metabolite genes (Chujo and Scott, 2014). Like the Δ*clrD* mutant only a subset of the *ltm* (7/11) and *eas* (6/11) genes were significantly derepressed in axenic culture of the Δ*hepA* mutant compared to the wild type. In fact, most of the genes that remain silent in the Δ*hepA* mutant are the same genes that are silent in the Δ*clrD* mutant. In contrast, single gene deletions of *clrD* or *hepA* in *A. nidulans* and *F. graminearum* resulted in significant changes in expression of all the genes in the clusters studied and enhanced synthesis of the corresponding metabolites in axenic culture

(Reyes-Dominguez et al., 2012; Reyes-Dominguez et al., 2010). These and our previous expression analyses highlight the complexity of *ltm* and *eas* secondary metabolite gene regulation and the difficulty of mimicking in axenic culture through single gene deletions or through addition of plant metabolites (Chujo and Scott, 2014; May et al., 2008; Young et al., 2005), the conditions *in planta* that lead to full derepression of these genes. This lack of full derepression also made it difficult to identify and demarcate the boundaries of other potential secondary metabolite gene clusters likely to be regulated by H3K9me3 and associated binding of HepA (HP1).

Although just a subset of the *ltm* and *eas* cluster genes were differentially expressed in axenic cultures of the Δ*hepA* mutant, an analysis of the whole transcriptome highlights how the effects of this single gene deletion are much greater than an analysis of the *ltm* and *eas* genes alone would suggest, with approx. 13% of the genes showing a significant difference in expression between mutant and wild type. This is comparable to a similar analysis in *Leptosphaeria maculans* using an RNAi induced mutant of *HP1* where approx. 6% of the genes were significantly altered in their expression (Soyer et al., 2013). In *E. festucae* just one third of the genes differentially expressed were up-regulated, with the remaining two thirds being down-regulated, highlighting that HepA has both repressive and activating functions in regulating gene expression (Reyes-Dominguez et al., 2012; Shoji et al., 2014). However, it is unclear if such activating functions of HepA is direct, as it is also possible that HepA represses the expression of transcriptional repressors that in turn indirectly bring about the observed transcriptional changes.

Despite the major differences in gene expression between the *hepA* mutant and wild type in axenic culture there is little apparent impact on radial growth and hyphal morphology of the Δ*hepA* mutant, as has been observed for the corresponding mutants in *A. nidulans* (Reyes-Dominguez et al., 2010) and *F. graminearum* (Reyes-Dominguez et al., 2012). This is in contrast to some other fungal systems where HP1 deletions have been shown to strongly affect viability. Loss of Swi6 in *Schizosaccharomyces pombe* leads to loss of chromosome stability

(Allshire et al., 1995) and deletion of *hpo* in *N. crassa* gives rise to pronounced growth defects (Freitag et al., 2004). There was also no defect in the ability of Δ*hepA* hyphae to form tip to side hyphal fusions (Becker et al., 2015) that are important for the establishment of a hyphal network in axenic culture and cell-cell communication within that network (Leeder et al., 2013; Read et al., 2009; Simonin et al., 2010). Interestingly, deletion of *E. festucae clrD*, which encodes the H3K9 methyl transferase, results in a very severe axenic culture phenotype (Chujo and Scott, 2014).

In contrast to the very mild axenic culture phenotype, infection of *L. perenne* with the Δ*hepA* mutant led to a strong plant-interaction phenotype characterised by loss of apical dominance, with an increase in tiller number but a reduction in tiller length. This is a host interaction phenotype similar to that observed for the Δ*sakA* mutant (Eaton et al., 2010). These plants also had poorly developed root systems with fewer lateral roots. This loss of apical dominance and reduced lateral root growth is consistent with a reduction in auxin signalling and an increase in levels of cytokinins (Casimiro et al., 2001; McSteen, 2009). Interestingly, among the *E. festucae* genes that were upregulated in this mutant association, as well as in three others (Eaton et al., 2015), were two plant hormone biosynthetic genes; one (IPT-LOG) a bifunctional enzyme encoding an isopentenyl transferase (IPT) and a cytokinin specific phosphoribohydrolase (LOG: *lonely guy*) responsible for the synthesis of iP (isopentenyladenine) (Hinsch et al., 2015), and the other an FAD-dependent monooxygenase responsible for the conversion of indole 3-pyruvate to auxin. These two genes are part of a three gene cluster flanked by AT-rich isochores, but the third gene in this cluster encoding a cytokinin-specific cytochrome P450 monooxygenase that converts iP to tZ (*trans*-zeatin) (Hinsch et al., 2015), is not significantly up-regulated in the Δ*hepA*, or other symbiotic mutant, data sets. Inactivation of the gene for IPT-LOG (*de novo* pathway) together with that for tRNA-IPT (salvage pathway) in *Claviceps purpurea* led to a dramatic reduction in virulence on rye suggesting that fungal synthesis of cytokinins are important for host pathogenicity (Hinsch et al., 2016). The lack of expression of these genes in an *E. festucae* wild-type mutualistic

symbiotic interaction with *L. perenne* and their upregulation in the Δ*hepA* antagonistic interaction is consistent with the phenotypes observed for *C. purpurea*.

Within the leaves of Δ*hepA* infected plants we observed an increase in hyphal number within the intercellular spaces and hyphal colonization of the vascular bundles, phenotypes not seen in wild type associations (Eaton et al., 2010; Tanaka et al., 2006). This proliferative growth phenotype has been previously observed in *L. perenne* associations containing symbiotic mutants defective in components of a cell-cell communication/cell fusion signaling network (Herzog et al., 2015; Steffens et al., 2016) including *noxA* and *noxR* which encode components of a NADPH oxidase complex (Takemoto et al., 2006; Tanaka et al., 2006); *symB* and *symC* which encode membrane-associated proteins (Green et al., 2017b); *mpkA, mkkA* and *so,* which encode components of the cell wall integrity MAP kinase pathway (Becker et al., 2015; Charlton et al., 2012); *mobC* which encodes a component of the striatin-interacting phosphatase and kinase (STRIPAK) complex (Green et al., 2017a), and *proA* encoding a transcription factor (Tanaka et al., 2013). A defining feature of the mutants in this cell-cell communication pathway is a defect in hyphal cell-cell fusion. However, unlike all these mutants, Δ*hepA* is able to undergo cell-cell fusion in culture and *in planta*, providing strong evidence that the proliferative growth response observed for the endophyte in these associations and the hypertillering of the host, are phenotypes not restricted to cell-cell fusion defects. Proliferative growth is a classic growth response of fungi to starvation, but how mutants still able to fuse and not undergo hyphal breakage trigger such a response is not clear. An alternative explanation is alkalinisation of the apoplastic fluid by both mutant types (Scott et al., 2018). Several fungal plant pathogens, including *F. oxysporum* are known to alkalinize the host apoplast thereby enhancing the virulence of the pathogen (Fernandes et al., 2017; Masachis et al., 2016) and expression of a dominant active allele of the *E. festucae pacC* (an alkaline mimic) results in the formation of aberrant convoluted hyphal structures *in planta* as well as hyphal breakage (Lukito et al., 2015). Further research, such as the use of a pH biosensor to monitor changes in the pH, is required to

better understand the physiological changes that are occurring when there is a 'breakdown' of a mutualistic interaction.

An analysis of the fungal transcriptome changes observed between ryegrass infected with the Δ*hepA* mutant versus wild type reflect the change in the interaction from mutualistic to a pathogenic-like interaction characterised by proliferative hyphal growth within the leaves of the host. Around two thirds of the genes were upregulated including genes encoding proteins for primary metabolism, transport, host cell wall degradation, fungal cell wall structure and small secreted proteins (Eaton et al., 2015). Surprisingly, most of the genes within the *EAS* cluster were up-regulated with the one exception of *lpsA*, which encodes an NRPS for catalysing the final step in the ergot alkaloid biosynthetic pathway in *E. festucae* strain Fl1 for the conversion of lysergic acid to ergovaline (Schardl et al., 2013). While the 11 genes within this cluster comprise a single topologically associated (TAD) domain, they are separated within this TAD into three sub-domains bounded by AT-rich blocks, with *lpsA* in the most telomere distal sub-domain (Winter et al., 2018). It will be interesting to see how the 3-D chromosome structure of the Δ*hepA* mutant *in planta* differs to that of wild type, once techniques are available to probe the chromosome structure of symbionts like *E. festucae in situ*. Among the genes that were down-regulated were a number encoding small secreted proteins (Hassing et al., 2018), and enzymes involved in secondary metabolism, including almost all of the genes that comprise the *LTM* gene cluster. The absence of detectable levels of lolitrem B, the main lolitrem pathway product, and lolitriol, an abundant intermediate (Saikia et al., 2012), are consistent with the general down regulation of this cluster. However, it is difficult to determine whether this transcriptional change is a direct effect of the Δ*hepA* mutation given the dramatic change in the whole plant and cellular phenotype of the symbiotic interaction.

The results of this study provide additional insights into how chromatin remodelling controls expression of symbiosis genes in the mutualistic symbiont *E. festucae*. In particular, this study has identified the importance of HepA in controlling the expression of the sub-telomeric *LTM* and *EAS* gene clusters. Identifying the specific plant stimuli that initiate the

signalling cascade leading to the chromatin remodelling necessary for high level expression of

these genes *in planta* will be an important area of future research pursuit.

Acknowledgements

Appendix A. Supplementary material

References

Allshire, R. C., Karpen, G. H., 2008. Epigenetic regulation of centromeric chromatin: old dogs, new tricks? Nat. Rev. Genet. 9**,** 923-937.

Allshire, R. C., Nimmo, E. R., Ekwall, K., et al., 1995. Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. Genes Dev. 9**,** 218-33.

Ayoub, N., Jeyasekharan, A. D., Venkitaraman, A. R., 2009. Mobilization and recruitment of HP1: a bimodal response to DNA breakage. Cell Cycle. 8**,** 2945-2950.

Becker, Y., Eaton, C. J., Brasell, E., et al., 2015. The fungal cell wall integrity MAPK cascade is crucial for hyphal network formation and maintenance of restrictive growth of *Epichloë festucae* in symbiosis with *Lolium perenne*. Mol. Plant-Microbe Interact. 28**,** 69-85.

Bennett, J. W., Lasure, L. L., Conventions for gene symbols. In: J. W. Bennett, L. L. Lasure, Eds.), Gene Manipulation in Fungi. Academic Press, London, 1985, pp. 537-544.

Bergmann, S., Schümann, J., Scherlach, K., et al., 2007. Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. Nature Chem. Biol. 3**,** 213-7.

Blankenship, J. D., Spiering, M. J., Wilkinson, H. H., et al., 2001. Production of loline alkaloids by the grass endophyte, *Neotyphodium uncinatum*, in defined media. Phytochemistry. 58**,** 395-401.

Bok, J. W., Chiang, Y. M., Szewczyk, E., et al., 2009. Chromatin-level regulation of biosynthetic gene clusters. Nature Chem. Biol. 5**,** 462-464.

Brakhage, A. A., 2013. Regulation of fungal secondary metabolism. Nat. Rev. Microbiol. 11**,** 21-32.

Byrd, A. D., Schardl, C. L., Songlin, P. J., et al., 1990. The β-tubulin gene of *Epichloë typhina* from perennial ryegrass (*Lolium perenne*). Curr. Genet. 18**,** 347-354.

Casimiro, I., Marchant, A., Bhalerao, R. P., et al., 2001. Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell. 13**,** 843-852.

Charlton, N. D., Shoji, J. Y., Ghimire, S. R., et al., 2012. Deletion of the fungal gene *soft* disrupts mutualistic symbiosis between the grass endophyte *Epichloë festucae* and the host plant. Eukaryot. Cell. 11**,** 1463-1471.

Chujo, T., Scott, B., 2014. Histone H3K9 and H3K27 methylation regulates fungal alkaloid biosynthesis in a fungal endophyte-plant symbiosis. Mol. Microbiol. 92**,** 413-434.

Connolly, L. R., Smith, K. M., Freitag, M., 2013. The *Fusarium graminearum* histone H3 K27 methyltransferase KMT6 regulates development and expression of secondary metabolite gene clusters. PLoS Genet. 9**,** e1003916.

Cox, M. P., Peterson, D. A., Biggs, P. J., 2010. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinf. 11**,** 485.

Dupont, P. Y., Eaton, C. J., Wargent, J. J., et al., 2015. Fungal endophyte infection of ryegrass reprograms host metabolism and alters development. New Phytol. 208**,** 1227-1240.

Eaton, C. J., Cox, M. P., Ambrose, B., et al., 2010. Disruption of signaling in a fungal-grass symbiosis leads to pathogenesis. Plant Physiol. 153**,** 1780-1794.

Eaton, C. J., Dupont, P.-Y., Solomon, P. S., et al., 2015. A core gene set describes the molecular basis of mutualism and antagonism in *Epichloë* species. Mol. Plant-Microbe Interact. 28**,** 218-231.

Fanti, L., Giovinazzo, G., Berloco, M., et al., 1998. The heterochromatin protein 1 prevents telomere fusions in *Drosophila*. Mol. Cell. 2**,** 527-538.

Fernandes, T. R., Segorbe, D., Prusky, D., et al., 2017. How alkalinization drives fungal pathogenicity. PLoS Pathog. 13**,** e1006621.

Fisher, R. A., 1922. On the interpretation of χ2 from contingency tables, and the calculation of P. J R Statist Soc 85**,** 87-94.

Fleetwood, D. J., Khan, A. K., Johnson, R. D., et al., 2011. Abundant degenerate miniature inverted-repeat transposable elements in genomes of epichloid fungal endophytes of grasses. Genome Biol. Evol. 3**,** 1253-1264.

Fleetwood, D. J., Scott, B., Lane, G. A., et al., 2007. A complex ergovaline gene cluster in epichloë endophytes of grasses. Appl. Environ. Microbiol. 73**,** 2571-2579.

Freitag, M., Hickey, P. C., Khlafallah, T. K., et al., 2004. HP1 is essential for DNA methylation in *Neurospora*. Mol. Cell. 13**,** 427-434.

Green, K. A., Becker, Y., Fitzsimons, H. L., et al., 2017a. An *Epichloë festucae* homolog of MOB3, a component of the STRIPAK complex, is required for establishment of a mutualistic symbiotic interaction with *Lolium perenne*. Mol. Plant Path. 17**,** 1480-1492.

Green, K. A., Becker, Y., Tanaka, A., et al., 2017b. SymB and SymC, two membrane associated proteins, are required for *Epichloë festucae* hyphal cell-cell fusion and maintenance of a mutualistic interaction with *Lolium perenne*. Mol. Microbiol. 103**,** 657-677.

Grewal, S. I., Elgin, S. C., 2002. Heterochromatin: new possibilities for the inheritance of structure. Curr. Opin. Genet. Dev. 12**,** 178-187.

Grewal, S. I., Jia, S., 2007. Heterochromatin revisited. Nat. Rev. Genet. 8**,** 35-46.

Hassing, B., Winter, D., Becker, Y., et al., 2018. Analysis of *Epichloë festucae* small secreted proteins in the interaction with *Lolium perenne*. PLoS One.

Herzog, S., Schumann, M. R., Fleissner, A., 2015. Cell fusion in *Neurospora crass*a. Curr. Opin. Microbiol. 28**,** 53-59.

Hinsch, J., Galuszka, P., Tudzynski, P., 2016. Functional characterization of the first filamentous fungal tRNA-isopentenyltransferase and its role in the virulence of *Claviceps purpurea*. New Phytol. 211**,** 980-992.

Hinsch, J., Vrabka, J., Oeser, B., et al., 2015. *De novo* biosynthesis of cytokinins in the biotrophic fungus *Claviceps purpurea*. Environ. Microbiol. 17**,** 2935-2951.

Honda, S., Lewis, Z. A., Shimada, K., et al., 2012. Heterochromatin protein 1 forms distinct complexes to direct histone deacetylation and DNA methylation. Nat. Struct. Mol. Biol. 19**,** 471-477, S1.

Honda, S., Selker, E. U., 2008. Direct interaction between DNA methyltransferase DIM-2 and HP1 is required for DNA methylation in *Neurospora crassa*. Mol. Cell. Biol. 28**,** 6044-6055.

Itoh, Y., Johnson, R., Scott, B., 1994. Integrative transformation of the mycotoxin-producing fungus, *Penicillium paxilli*. Curr. Genet. 25**,** 508-513.

Johnson, L. J., Koulman, A., Christensen, M., et al., 2013. An extracellular siderophore is required to maintain the mutualistic interaction of *Epichloë festucae* with *Lolium perenne*. PLoS Pathog. 9**,** e1003332.

Kayano, Y., Tanaka, A., Akano, F., et al., 2013. Differential roles of NADPH oxidases and associated regulators in polarized growth, conidiation and hyphal fusion in the symbiotic fungus *Epichloë festucae*. Fungal Genet. Biol. 56**,** 87-97.

Lachner, M., O'Carroll, D., Rea, S., et al., 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature. 410**,** 116-120.

Langmead, B., Trapnell, C., Pop, M., et al., 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10**,** R25.

Latch, G. C. M., Christensen, M. J., 1985. Artificial infection of grasses with endophytes. Ann. Appl. Biol. 107**,** 17-24.

Leeder, A. C., Jonkers, W., Li, J., et al., 2013. Early colony establishment in *Neurospora crassa* requires a MAP kinase regulatory network. Genetics. 195**,** 883-898.

Lewis, Z. A., Adhvaryu, K. K., Honda, S., et al., 2010. DNA methylation and normal chromosome behavior in Neurospora depend on five components of a histone methyltransferase complex, DCDC. PLoS Genet. 6**,** e1001196.

Lewis, Z. A., Honda, S., Khlafallah, T. K., et al., 2009. Relics of repeat-induced point mutation direct heterochromatin formation in *Neurospora crassa*. Genome Res. 19**,** 427-437.

Lomberk, G., Wallrath, L., Urrutia, R., 2006. The Heterochromatin Protein 1 family. Genome Biol. 7**,** 228.

Lukito, Y., Chujo, T., Scott, B., 2015. Molecular and cellular analysis of the pH response transcription factor PacC in the fungal symbiont *Epichloë festucae*. Fungal Genet. Biol. 85**,** 25-37.

Maison, C., Almouzni, G., 2004. HP1 and the dynamics of heterochromatin maintenance. Nat. Rev. Mol. Cell Biol. 5**,** 296-304.

Masachis, S., Segorbe, D., Turrà, D., et al., 2016. A fungal pathogen secretes plant alkalinizing peptides to increase infection. Nat. Microbiol. 1**,** 16043.

May, K. J., Bryant, M. K., Zhang, X., et al., 2008. Patterns of expression of a lolitrem biosynthetic gene in the *Epichloë festucae*-perennial ryegrass symbiosis. Mol. Plant-Microbe Interact. 21**,** 188-197.

McIlvaine, T. C., 1921. A buffer solution for colorimetric comparison. J. Biol. Chem. 49**,** 183-186.

McSteen, P., 2009. Hormonal regulation of branching in grasses. Plant Physiol. 149**,** 46-55.

Miller, J. H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, New York.

Moon, C. D., Scott, B., Schardl, C. L., et al., 2000. The evolutionary origins of *Epichloë* endophytes from annual ryegrasses. Mycologia. 92**,** 1103-1118.

Moon, C. D., Tapper, B. A., Scott, B., 1999. Identification of *Epichloë* endophytes *in planta* by a microsatellite-based PCR fingerprinting assay with automated analysis. Appl. Environ. Microbiol. 65**,** 1268-1279.

Nützmann, H. W., Reyes-Dominguez, Y., Scherlach, K., et al., 2011. Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. Proc. Natl. Acad. Sci. USA. 108**,** 14282-14287.

Palmer, J. M., Keller, N. P., 2010. Secondary metabolism in fungi: does chromosomal location matter? Curr. Opin. Microbiol. 13**,** 431-436.

Panaccione, D. G., Johnson, R. D., Wang, J., et al., 2001. Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. Proc. Natl. Acad. Sci. USA. 98**,** 12820-12825.

Perrini, B., Piacentini, L., Fanti, L., et al., 2004. HP1 controls telomere capping, telomere elongation, and telomere silencing by two different mechanisms in *Drosophila*. Mol. Cell. 15**,** 467-476.

R development core team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rasmussen, S., Lane, G. A., Mace, W., et al., 2012. The use of genomics and metabolomics methods to quantify fungal endosymbionts and alkaloids in grasses. Methods Mol. Biol. 860, 213-226.

Read, N. D., Lichius, A., Shoji, J. Y., et al., 2009. Self-signalling and self-fusion in filamentous fungi. Curr. Opin. Microbiol. 12, 608-615.

Reyes-Dominguez, Y., Boedi, S., Sulyok, M., et al., 2012. Heterochromatin influences the secondary metabolite profile in the plant pathogen *Fusarium graminearum*. Fungal Genet. Biol. 49, 39-47.

Reyes-Dominguez, Y., Bok, J. W., Berger, H., et al., 2010. Heterochromatic marks are associated with the repression of secondary metabolism clusters in *Aspergillus nidulans*. Mol. Microbiol. 76, 1376-1386.

Saikia, S., Takemoto, D., Tapper, B. A., et al., 2012. Functional analysis of an indole-diterpene gene cluster for lolitrem B biosynthesis in the grass endosymbiont *Epichloë festucae*. FEBS Lett. 586, 2563-2569.

Sanderson, K. E., Srb, A. M., 1965. Heterokaryosis and parasexuality in the fungus *Ascochta imperfecta*. Amer. J. Bot. 52, 72-81.

Schardl, C. L., Panaccione, D. G., Tudzynski, P., Ergot alkaloids-biology and molecular biology. In: G. A. Cordell, (Ed.), Alkaloids: Chemistry and Biology. Academic Press, San Diego, CA 2006, pp. 425-443.

Schardl, C. L., Scott, B., Recommendations for gene nomenclature for Epichloë species and related Clavicipitaceae. In: C. A. Young, G. E. Aiken, R. L. McCulley, et al., Eds.), Epichloae, endophytes of cool season grasses: implications, utilization and biology. Proceedings of the 7th International Symposium on Fungal Endophytes of Grasses. The Samuel Roberts Noble Foundation, Lexington, 2010, pp. 84-87.

Schardl, C. L., Young, C. A., Hesse, U., et al., 2013. Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci. PLoS Genetics. 9**,** e1003323.

Schroeckh, V., Scherlach, K., Nützmann, H. W., et al., 2009. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA. 106**,** 14558-14563.

Scott, B., Green, K., Berry, D., 2018. The fine balance between mutualism and antagonism in the *Epichloë festucae*-grass symbiotic interaction. Curr Opin Plant Biol. 44**,** 32-38.

Shoji, K., Hara, K., Kawamoto, M., et al., 2014. Silkworm HP1a transcriptionally enhances highly expressed euchromatic genes via association with their transcription start sites. Nucleic Acids Res. 42**,** 11462-11471.

Simonin, A., Palma-Guerrero, J., Fricker, M., et al., 2010. Physiological significance of network organization in fungi. Eukaryot. Cell. 11**,** 1345-1352.

Southern, E. M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98**,** 503-517.

Soyer, J. L., El Ghalid, M., Glaser, N., et al., 2013. Epigenetic control of effector gene expression in the plant pathogenic fungus *Leptosphaeria maculans*. PLoS Genet. 10**,** e1004227.

Steffens, E. K., Becker, K., Krevet, S., et al., 2016. Transcription factor PRO1 targets genes encoding conserved components of fungal developmental signaling pathways. Mol. Microbiol. 102**,** 792-809.

Storey, J. D., Tibshirani, R., 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. USA. 100**,** 9440-9445.

Takemoto, D., Tanaka, A., Scott, B., 2006. A p67(Phox)-like regulator is recruited to control hyphal branching in a fungal-grass mutualistic symbiosis. Plant Cell. 18**,** 2807-2821.

Tamaru, H., Selker, E. U., 2001. A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. Nature. 414**,** 277-283.

Tamaru, H., Zhang, X., McMillen, D., et al., 2003. Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa*. Nat Genet. 34**,** 75-79.

Tanaka, A., Cartwright, G. M., Saikia, S., et al., 2013. ProA, a transcriptional regulator of fungal fruiting body development, regulates leaf hyphal network development in the *Epichloë festucae-Lolium perenne* symbiosis. Mol. Microbiol. 90**,** 551-568.

Tanaka, A., Christensen, M. J., Takemoto, D., et al., 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic association. Plant Cell. 18**,** 1052-1066.

Tanaka, A., Tapper, B. A., Popay, A., et al., 2005. A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol. Microbiol. 57**,** 1036-1050.

Thompson, J. D., Higgins, D. G., Gibson, T. J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22**,** 4673-4680.

Winter, D. J., Ganley, A. R. D., Young, C. A., et al., 2018. Repeat elements organise 3D genome structure and mediate transcription in the filamentous fungus *Epichloë festucae*. PLoS Genet. 14**,** e1007467.

Young, C. A., Bryant, M. K., Christensen, M. J., et al., 2005. Molecular cloning and genetic analysis of a symbiosis-expressed gene cluster for lolitrem biosynthesis from a mutualistic endophyte of perennial ryegrass. Mol. Gen. Genomics. 274**,** 13-29.

Young, C. A., Felitti, S., Shields, K., et al., 2006. A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. Fungal Genet Biol. 43**,** 679-693.

Zhang, D. X., Stromberg, A. J., Spiering, M. J., et al., 2009. Coregulated expression of loline alkaloid-biosynthesis genes in *Neotyphodium uncinatum* cultures. Fungal Genet. Biol. 46**,** 517-530.

Figure legends

Fig. 1. Axenic culture phenotype of *E. festucae* Δ*hepA* and Δ*hepA/hepA* mutant strains. (A) Colony morphology of wild-type Fl1 (WT), Δ*hepA* and Δ*hepA/ΔhepA* strains grown on PD agar for 10 days. (B) Hyphal fusion of wild-type Fl1 (WT), Δ*hepA* and Δ*hepA/ΔhepA* strains grown on water agar for 9 days, stained with Calcofluor White. Images were taken with the fluorescence microscope. Bars = 20 µm. Hyphal fusions are indicated by white circles.

Fig. 2. *E. festucae* HepA localizes to nuclei in a ClrD-dependent manner. *E. festucae* wild-type and Δ*clrD* #125 (PN2864) mutant strains expressing the HepA-GFP fusion protein were examined using confocal laser scanning and DIC microscopy. DAPI was used to stain DNA. Bar = 20 µm.

Fig. 3. Derepression of *ltm* and *eas* genes in cultured Δ*hepA* mutant strains. (A) Physical map of the lolitrem B (*LTM*) biosynthetic gene cluster locus. Gray arrows indicate selection of genes for which fold changes in steady-state transcript levels are shown in this figure. (B) Expression levels of *ltm* genes in cultured wild-type (WT), Δ*hepA* #109 (PN2778), Δ*hepA* #121 (PN2779), Δ*hepA/hepA* #14 (PN2926) and Δ*hepA/hepA* #19 (PN2928) mutant strains. Values for each mRNA expression level were normalized to that of 40S ribosomal protein S22. Bars represent log2 value of mean ± SEM (n=3) relative to that of cultured WT. Letters show significant differences where *P* is at least <0.05 as determined by one-way ANOVA followed by Tukey's multiple comparisons test. Transcript expression levels were measured by qRT-PCR using primers listed in Table S4. (C) Physical map of the ergovaline (*EAS*) biosynthetic gene cluster locus. Gray arrows indicate selection of genes for which fold changes in steady-state transcript levels are shown in this figure. (D) Expression levels of *eas* genes in cultured wild-type (WT), Δ*hepA* #109 (PN2778), Δ*hepA* #121 (PN2779), Δ*hepA/hepA* #14 (PN2926) and Δ*hepA/hepA* #19 (PN2928) mutant strains. Values for each mRNA expression level were normalized to that of 40S ribosomal protein S22. Bars represent log2 value of mean ± SEM (n=3) relative to that

of cultured WT. Letters show significant differences where *P* is at least <0.05 as determined by one-way ANOVA followed by Tukey's multiple comparisons test. Transcript expression levels were measured by qRT-PCR using primers listed in Table S4.

Fig. 4. Symbiotic phenotype of *E. festucae* Δ*hepA* mutant strains. (A) Phenotype of perennial ryegrass plants 12 weeks after inoculation with *E. festucae* wild-type Fl1 (WT), Δ*hepA* #109 (PN2778) and Δ*hepA* #121 (PN2779) mutant strains. (B) Tiller number of perennial ryegrass plants infected with *E. festucae* wild-type Fl1 (WT), Δ*hepA* #109 (PN2778) and Δ*hepA* #121 (PN2779) mutant strains. The box plot represents the tiller number of ryegrass plants 12 weeks after inoculation with *E. festucae* WT and Δ*hepA* mutant strains ($n = 67$, 47 and 43 for wild-type, Δ*hepA* #109 and Δ*hepA* #121 strains, respectively); ***P <0.001 and ****P <0.0001, determined by one-way ANOVA followed by Dunnett's multiple comparisons test. C. Height of perennial ryegrass plants infected with *E. festucae* wild-type Fl1 (WT), Δ*hepA* #109 (PN2778) and Δ*hepA* #121 (PN2779) mutant strains. The box plot represents the height of ryegrass plants 12 weeks after inoculation with *E. festucae* WT and Δ*hepA* mutant strains ($n = 67$, 47 and 43 for wild-type, Δ*hepA* #109 and Δ*hepA* #121 strains, respectively); ****P <0.0001, determined by one-way ANOVA followed by Dunnett's multiple comparisons test.

Fig. 5. *In planta* cellular phenotype of *E. festucae* Δ*hepA* mutant strains. (A) Confocal depth series images of longitudinal sections through 12 week old *L. perenne* pseudostem tissue inoculated with wild-type Fl1 (WT), Δ*hepA* #109 (PN2778) and Δ*hepA* #121 (PN2779) mutant strains, showing hyphae stained with aniline blue/WGA-AF488. Images were generated by maximum intensity projection of 10 x 1 μm confocal z-stacks. Bar = 50 μm. (B) Transmission electron micrographs of cross-sections of wild-type Fl1 (WT), Δ*hepA* #109 (PN2778) and Δ*hepA* #121 (PN2779) mutant hyphae in the intercellular space of perennial ryegrass. pc, plant cell. Bar = 1 μm. (C) Transmission electron micrographs of vascular tissue

of wild-type Fl1 (WT), Δ*hepA* #109 (PN2778) and Δ*hepA* #121 (PN2779) mutant-infected perennial ryegrass plant. Hyphae of endophyte are indicated by arrowheads. Bar = 5 μm.

Fig. 6. Deletion of *hepA* leads to altered *ltm* and *eas* gene expression and reduction of secondary metabolite biosynthesis *in planta*. (A) RPMK of all ltm genes (B) RPMK of all eas genes (C) Alkaloid levels

Fig. 7. Distribution of differentially expressed *Epichloë festucae* genes among the four symbiotic mutants. Venn diagram showing the number of differentially expressed genes shared by all four symbiotic mutants. Differentially expressed genes are defined as those that are statistically significant and greater than two-fold difference in expression relative to the wild-type association.