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# Heterochromatin influences the secondary metabolite profile in the plant pathogen *Fusarium graminearum*

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ABSTRACT

Chromatin modifications and heterochromatic marks have been shown to be involved in the regulation of secondary metabolism gene clusters in the fungal model system *Aspergillus nidulans*. We examine here the role of HEP1, the heterochromatin protein homolog of *Fusarium graminearum*, for the production of secondary metabolites. Deletion of *Hep1* in a PH-1 background strongly influences expression of genes required for the production of aurofusarin and the main tricothecene metabolite DON. In the *Hep1* deletion strains AUR genes are highly up-regulated and aurofusarin production is greatly enhanced suggesting a repressive role for heterochromatin on gene expression of this cluster. Unexpectedly, gene expression and metabolites are lower for the trichothecene cluster suggesting a positive function of *Hep1* for DON biosynthesis. However, analysis of histone modifications in chromatin of AUR and DON gene promoters reveals that in both gene clusters the H3K9me3 heterochromatic mark is strongly reduced in the *Hep1* deletion strain. This, and the finding that a DON-cluster flanking gene is up-regulated, suggests that the DON biosynthetic cluster is repressed by HEP1 directly and indirectly. Results from this study point to a conserved mode of secondary metabolite (SM) biosynthesis regulation in fungi by chromatin modifications and the formation of facultative heterochromatin.



## 1. Introduction

*Fusarium graminearum* is a plant pathogenic fungus that causes *Fusarium* head blight (FHB) disease of small grain cereals and maize (Desjardins and Proctor, 2007; Leonard and Bushnell, 2003). *Fusarium* diseases can lead to contamination of grain with fungal metabolites reaching toxicologically relevant levels in food and feed. *F. graminearum* and other species of this genus are necrotrophic filamentous fungi with an unusual broad host range. Single isolates can infect wheat, barley, oat, maize, but also broad leafed plants. It has been hypothesized that the broad host range of *F. graminearum* and other species is caused by their ability to produce multiple secondary metabolites (SMs) which function as redundant suppressors of plant defence, targeting pathogen recognition, signal transduction and expression of defence related genes (Desjardins et al., 1993; McMullen et al., 1997; Paterson and Lima, 2010; Wu et al., 2010). Trichothecenes, zearalenone, fumonisins, moniliformin and fusaric acid are the major *Fusarium* mycotoxins (D'Mello and Macdonald, 1999). The trichothecene family toxin deoxynivalenol (DON) is one of the metabolites that act as a virulence factor required for spreading from the infection site. Consequently, the most prominent genetic locus co-segregating with *Fusarium* spreading resistance (quantitative trait locus, QTL) in wheat has been shown to co-localize with a QTL for increased ability to detoxify DON into nontoxic DON-3-*O*-glucoside (Lemmens et al., 2005).

The *F. graminearum* genome sequence revealed the existence of 15 putative polyketide synthase genes (PKSs), 20 non-ribosomal peptide synthases (NRPSs) and 17 putative terpenoid synthases genes (Cuomo et al., 2007). In *Fusarium* and other fungi, the genes

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coding for the individual steps in the biosynthesis of a given metabolite usually show physical linkage and appear as large, synchronously regulated gene clusters (Bohnert et al., 2010; Desjardins and Proctor, 2007; Keller and Hohn, 1997; Keller et al., 2005; Yu and Keller, 2005). Although hundreds of novel putative SM-biosynthesis genes have been discovered by fungal genome sequencing projects, for only a small fraction the corresponding secondary metabolites are known. Strategies such as overexpression of transcription factors residing within a putative SM cluster of unknown biosynthetic function (Bergmann et al., 2007) or the mimic of natural competition by co-cultivating fungal and bacterial strains (Schroeckh et al., 2009) have led to the discovery of novel fungal metabolites. One of the underlying molecular mechanisms that repress SM genes under standard laboratory conditions was recently identified in *Aspergillus nidulans*. It was shown that transcriptionally inactive sterigmatocystin (Reyes-Dominguez et al., 2010) and monodictyphenon clusters (Bok et al., 2009) are associated with chromatin that carries silencing histone methylation marks on H3 lysine 9 (H3K9me). Traditionally, this mark creates a binding site for Heterochromatin protein 1 (HP1) (Lachner et al., 2001), a non-histone chromatin protein that mediates the establishment and maintenance of heterochromatin (Maison and Almouzni, 2004). Heterochromatic structures are associated with gene silencing and low recombination rates and predominate at pericentromeres and telomeres. Heterochromatin contributes to important biological functions, such as chromosome segregation during cell division, telomere capping and response to DNA damage (Allshire and Karpen, 2008; Ayoub et al., 2009; Ball and Yokomori, 2009; Elgin and Grewal, 2003; Fanti et al., 1998; Perrini et al., 2004). HP1 is a highly conserved protein present in organisms from *S. pombe* (Swi6p) to *Homo sapiens* (HP1α, HP1β, and HP1γ) (Grewal and Elgin, 2002; Lomberk et al., 2006) and the absence of heterochromatin protein affects organisms differentially. A loss of HP1 leads to lethality in Drosophila (Lu et al., 2000) and correlates with metastasis in human breast cancer cells (Norwood et al., 2004). In *Neurospora crassa* the HP1 homolog *hpo* and the H3K9 methyltransferase Dim-5 have recently been found to be required for normal centromere formation (Smith et al., 2011). Despite their importance in heterochromatic gene silencing, HP1 family members have also been found in euchromatic, actively transcribed regions of the genome (Kwon and Workman, 2008; Shaffer et al., 1993). There they associate with a diverse set of regulators and also participate in transcriptional activation (Hediger and Gasser, 2006). Taken together, HP1 proteins are found at different locations that are related to the three established functions of HP1: heterochromatin formation, telomere capping and positive regulation of gene expression (Kwon and Workman, 2011).

*N. crassa*, a long standing model for chromatin research, has contributed considerably to the general concept of heterochromatin formation and its relation to DNA methylation. Extensive work established that in this system a multi-component methyltransferase complex containing the catalytic proteins Dim-5 (H3K9methylase) and Dim-2 (cytosine methylase) as well as adaptors and HP1 (*hpo* in *N. crassa*) are essential for chromosome function, heterochromatin formation and DNA methylation (Freitag et al., 2004; Honda and Selker, 2008; Lewis et al., 2010; Rountree and Selker, 2010; Smith et al., 2011; Tamaru et al., 2003).

Interestingly, deletion mutants of HP1 (*hepA*Δ) in *A. nidulans* did not show any cytological or morphological defect but a strong upregulation of several SM gene clusters. Indeed, our analysis revealed that heterochromatic structures were found on promoters within the repressed clusters in actively growing cultures (primary metabolism). When cultures entered the stage of secondary metabolism, these repressive marks were removed and replaced by activating marks. Strikingly, reorganization of histone post-translational modifications was restricted to promoters and coding regions of genes belonging to the sterigmatocystin (ST) cluster and did not occur in regions immediately outside of this SM cluster (Reyes-Dominguez et al., 2010). This type of regulation suggests that SM-regulators recognize border structures which are known to separate different chromatin domains (Cheung and Lau, 2005). Null mutants of the main component required for H3K9 methylation, the histone H3 methyltransferase DimE consequently also led to overexpression of several SM genes. Moreover, LaeA, a conserved general regulator of SM (Bok and Keller, 2004) and member of the *velvet* complex (Bayram et al., 2008), was found to be essential for removal of heterochromatic marks. Consequently, deletion of *dimE* or *hepA* fully (in solid cultures) or partially (in liquid cultures) bypassed the requirement for LaeA. Thus, LaeA might be acting at a higher-hierarchical level in SM regulation by preventing the establishment or mediating the removal of heterochromatic marks. Recently, an ortholog of LaeA was characterized in *Fusarium fujikuroi* (Wiemann et al., 2010) and we identified a putative homolog also in *F. graminearum* (FGSG_00657.3; unpublished observation).

In this study we show that the heterochromatin pathway also regulates secondary metabolism in *F. graminearum* and, using a *Hep1* deletion strain, we provide evidence that the balance between different secondary metabolites is changed in *Hep1* mutants.

## 2. Materials and methods

### 2.1. Strains and growth conditions

As reference wild type strain, PH-1 (FGSC 9075, NRRL 31084) was used throughout the study. For liquid cultures, 0.5 million of freshly prepared macroconidia were inoculated in 50 ml *Fusarium* minimal medium (FMM: 0.1% (w/v) $KH_2PO_4$, 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, 0.05% (w/v) KCl, 0.2% (w/v) $NaNO_3$ and 3% (w/v) Sucrose. This media was autoclaved and the trace elements were added (0.001% (w/v) Citric acid, 0.001% (w/v) $ZnSO_4{\cdot}6H_2O$, 0.0002% (w/v) $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 0.00005% (w/v) $CuSO_4{\cdot}5H_2O$, 0.00001% (w/v) $MnSO_4$, 0.00001% (w/v) $H_3BO_4$, 0.00001% (w/v) $Na_2MoO_4{\cdot}2H_2O$).

Strains were grown at 22 °C during 3 and 5 days in the dark without shaking. One millilitre of filtered media was reserved for chemical analyses of secondary metabolites. Roughly one quarter of the mycelial mass was collected by filtration and frozen in liquid nitrogen for RNA isolation and the rest was transferred to a 100 ml flask and treated with 1% formaldehyde (f.c.) for 15 min during shaking at 180 rpm and 37 °C. For solid cultures, 250 macroconidia were inoculated on the center of a Petri dish containing 25 ml of FMM and 2% agar. The strains were grown at 20 °C for 7 days. *F. graminearum* macroconidia were obtained by starting the culture with roughly 20 mg of fresh mycelium from a 72 h plate in a volume 100 ml mung bean media (10 g mung beans were thrown into approximately 500 mL of already boiling distillated water and cooked for 20 min. Afterwards the media was filtrated through a piece Miracloth (Calbiochem) to remove the bean debris. The solution was filled up to 1 l with distillated water and autoclaved). Cells were incubated by shaking at 18 °C and 100 rpm in a baffled 1 l flask during 1 week. The cultures were filtered and the macroconidia collected.

### 2.2. Nucleic acid analysis details for probes and restriction digests

For preparation of genomic DNA mycelia were collected from FMM plates, frozen in liquid nitrogen and ground to fine powder. Extraction of genomic DNA was essentially performed according to the method published for preparation of plant DNA (Ausubel et al., 1998) with the following modification: After the first chloroform extraction, DNA was directly precipitated with isopropanol, washed with 70% ethanol and dissolved in water containing

**Table 1**
Oligonucleotides used in this study.

| Name | Sequence 5′–3′ |
|---|---|
| Aur1pF | CATCCCATAACCTCGATCCAG |
| Aur1pR | CATGTGGATATGTAGAGAGTGG |
| Aur1_atg_F | ATGAGTTCCACAGACCCCC |
| Aur1_stop_R | ATGTTGATTTATGAGTTTGATTTCAC |
| Aur1_1043F | CTGAGTGATTTTTCAAAGAAGTTC |
| Pks12p_F | TCGTGGGGTATCTCTGAATTATGATTGTG |
| Pks12p_R | CATTGATGGGGTCATGTTGAATGAAC |
| Pks12_1100F | CGAGTCATCAAGCAAGAACTC |
| Pks12_R | GACATGTTGAAGAACCTAGCG |
| Tri5_864F | GAGTGTTTCATGCATGGCTACGTC |
| Tri5_1070_R | CTGAGCCTCCTTCACATCGTCC |
| Tri5p_F | CTTGCAGGGAATGAGAGAGCACG |
| Tri5p_R | GATGGCAAGGTTGTACTGGTAACAG |
| Tri6_253_F | TATCGAAAATTATATAACCACATC |
| Tri6_ORF_R | CCTTTGGTGCCGACTTCTTGCAGG |
| Tri6prom_F | AGAATACCTTTTAAACTGCCGTAGC |
| Tri6prom_R | TTCGAGGGTAGTCAAAATAGATGTTC |
| FGtubr | CTTCTTCCTCGTCAATTCCAG |
| FGtub_1200_f | CTATTCCCCCTCGTGGACTTAC |
| hpfusORFfw | TCAAAGGGGAAATACTGACGG |
| hpfusORFRv | TCGTAGAAACGAAGCATCTATG |
| Hep1_upstream_NotI_fwd | AATCAGCGGCCGCACCCAATTTATCACGGCATC |
| Hep1_upstream_SalI_rev | TGATTGTCGACTTTGAATACTTGGCGATATTGCG |
| Hep1_downstream_SpeI_fwd | AATCAACTAGTATCAACACTGAGGAAGCAGGC |
| Hep1_downstream_NotI_rev | TGATTGCGGCCGCCAAGGCTGGGAGTAAAGAGC |
| Hep1_upstream_locus_check_fwd | TGCTGACAGCCTTTGGTATG |
| Hep1_ORF_rev | GTGCGGCAGACATGAGCTA |
| Hep1_ko_cassette_rev | CAACGTGGACAGCTGGATAA |
| Hep1_downstream_locus_check_rev | ATGGCCAGTCACCGTGTTAT |
| FG_3531prom_F | TCCCAGCGGTATCACTCTA |
| FG_3531prom_R | TTTAACGAACGTGTTTATAACGAATGTATT |
| FG_3531_ORF_F | CAAGACCGAATGTGATTACAAGG |
| FG_3531_ORF_R | GGTCACCAGGAGTGTTCATGTCAG |
| FGSG_2319.3_ORF_fwd | GCAGCCGTCAATGAGCCAAA |
| FGSG_2319.3_ORF_fwd | TGCTGCAAGCTCAGGGCAAC |
| FGSG_2319.3_prom_fwd | CGGCAATTGGTCACGTCAGTCTT |
| FGSG_2319.3_prom_rev | TGCCATGGGACTGCGAGATAG |

50 μg/ml RNaseA. RNA was extracted from powdered frozen mycelia with TRIzol® (Invitrogen), following the instructions of the provider.

### 2.3. Plasmid constructions

Plasmids were constructed using standard PCR and cloning techniques (Sambrook and Russell, 2001). Sequences of the primers are given in Table 1. For *Hep-1* ORF deletion the following plasmids based on pBluescript II SK⁻ were used: pGW851 contains the N-terminal two thirds of the *hph* (hygromycin B phosphotransferase) coding region (777 bp; ATG-*Sac*II) fused to the *pki* promoter from *Trichoderma reesei* (Plasmid pGW852 contains the C-terminal 692 bp of *hph* (*Pst*I-stop) and the *T. reesei cbh1* terminator (Mach et al., 1994). (These plasmids contain a 443-bp overlap in the center of the *hph* coding region.)

The *Hep1* upstream fragment was amplified from PH-1 genomic DNA with primers *Hep1*_upstream_NotI_fwd and *Hep1*_upstream_SalI_rev and inserted in *Not*I–*Sal*I sites of plasmid pGW851 upstream of the pki promoter-hph fusion, resulting in plasmid pHep_fus_Up. The *Hep1* downstream fragment was amplified with primers *Hep1*_downstream_SpeI_fwd and *Hep1*_downstream_NotI_rev and inserted in *Spe*I–*Not*I sites of the pGW852 plasmid downstream of the hph-chb2 terminator fusion, the resulting plasmid was termed pHep_fus_Down. A linear fragment containing the *Hep1* upstream sequence and the N-terminal part of the hygromycin B phosphotransferase gene *hph* was cut out from plasmid pHep_fus_Up by *SacII*/*NotI* digestion. The *Hep1* downstream sequence was released from plasmid pHep_fus_Down together with the C-terminal part of the *hph* gene by *PstI*/*NotI* digestion. Strain PH-1 was co-transformed with both linear fragments that partly overlap within their *hph* sequences. Transformants were screened for hygromycin resistance and deletion of *Hep1* was identified by PCR. In the transformants carrying the replacement of the *Hep1* ORF by *hph*, a fragment of 764 bp was obtained using the oligonucleotides *Hep1*_upstream_locus_check_fwd/*Hep1*_ko_cassette_rev. Southern blotting was used to confirm the absence of Hep1 and DNA of two transformants showing a stable *hph* resistance after five consecutive selection rounds was digested with *Bam*HI. Blots were hybridized with a 700-bp radiolabeled probe amplified by PCR with the oligonucleotides Hpfus ORF_f and hpfusORFRv containing the *Hep1* ORF (data not shown).

### 2.4. Transcriptional analysis by RT-qPCR

For reverse transcription quantitative real-time PCR (RT-qPCR), cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Biorad) following the instructions of the provider. The iQ™ SYBR® Green Supermix (Biorad) was used for amplification and detection of DNA in qPCR, using a 10-fold dilution of the cDNA of all analyzed transcripts. *Tri5* transcription was assessed using the primers Tri5_864F and Tri5_1070_R, *Tri6* with primers Tri6_253_F and Tri6_ORF_R, *Aur1* with Aur1_1043F and Aur1_stop_R, *Pks12* with Pks12_1100F and Pks12_R. Transcription of FGSG_2319.3, was analyzed using the primers FGSG_2319.3_ORF_fwd and FGSG_2319.3_ORF_rev. Transcription of FGSG_3531.3, was measured using primers FG_3531_ORF_F and FG_3531_ORF_R. All signals were normalized to the constitutively transcribed β-tubulin gene amplified with primers FGtub_1200_f and FGtubr. The BioRad (Hercules, CA) MyiQ cycler was used for amplification.

### 2.5. Secondary metabolite analysis

One milliliter of each culture filtrate was analyzed by LC–MS/MS as previously described (Vishwanath et al., 2009). Quantitative multi-target analysis for 186 metabolites was performed in the multiple reaction monitoring (MRM) mode with a QTrap 4000 LC–MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® $C_{18}$-column, 150 × 4.6 mm i.d., 5 μm particle size, equipped with a $C_{18}$ 4 × 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US). LC retention time and the intensity ratio of the two MRM transition that were monitored for each analyte agreed with the related values of an authentic standard within 0.1 min and 30% rel., respectively. All other details including analyte-dependent parameters can be found in Vishwanath et al. (2009).

### 2.6. ChIP coupled to quantitative PCR analysis

ChIP was carried out as described previously (Bernreiter et al., 2007) with the following modifications: Precipitation of the protein–antibody conjugate was performed by using Dynabeads® Protein A (Invitrogen). The washing step using the LiCl washing buffer was skipped and elution of the crosslinked chromatin from the Dynabeads® – antibody conjugate was performed in presence of SDS 0.1%/$NaHCO_3$ (10 mM). After the proteinase K treatment of the resuspended chromatin, the DNA was purified using the DNA extraction protocol as published. Antibodies used for ChIP were purchased from Abcam. Rabbit polyclonal antibody specific for histone H3 tri methyl K9 (ab 8898) and rabbit polyclonal antibody specific for the C-terminus of histone H3 (ab1791) were used. Two microliter of antibody were used per reaction of 200 μg total protein.

Amplification and detection of precipitated DNA in real-time qPCR was performed with iQ™ SYBR® Green Supermix (Biorad) following the instructions of the provider. The relative amounts of DNA were calculated by dividing the immunoprecipitated DNA by the input DNA. The resulting ratio was used to normalize the amount of DNA precipitated with histone H3-trimethyl K9 with total H3 by dividing through the values of histone H3 precipitation. At least two biological repeats and one technical (qPCR) repeat were carried out for each condition and strain and standard deviations were calculated based on these biological repeats. The promoter regions of the genes analyzed were amplified with the following primers:

Aur1pF and Aur1pR for *Aur1*, Pks12p_F and Pks12p_R for *Pks12*; Tri5p_F and Tri5p_R for *Tri5*; Tri6prom_F and Tri6prom_R for *Tri6*; FGSG_2319.3_prom_fwd and FGSG_2319.3_prom_rev for FGSG_2319.3; FGSG_03531.2_prom_fwd and FGSG_03531.2_prom_rev were used to amplify FGSG_03531.2.

### 2.7. Western blot

Mycelia (72 h of growth) were collected from PH-1 and *Hep1Δ* strains, washed with minimal media, dried and powdered under liquid nitrogen. Two hundred milligram were suspended in 2 ml cold buffer A (50 mM Tris, pH 7.5, 5 mM $MgOAc_2$, 20% glycerol, 5 mM EGTA, 3 mM $CaCl_2$ 100, 1 M Sorbitol, 7% Ficoll, 5 mM DTT, Protease inhibitors cocktail Sigma, 1:500). Once homogenized, 4 ml of buffer B (25 mM Tris, pH 7.5, 5 mM $MgOAc_2$, 10% glycerol, 5 mM EGTA, 5 mM DTT, Protease inhibitors cocktail Sigma, 1:500) were added. This suspension was centrifuged at 1500 g for 7 min at 4 °C. Whole cell extracts were mixed with an SDS sample buffer (10% glycerol, 100 mM DTT, 2% SDS, 0.1% bromophenol blue), incubated at 95 °C for 4 min and analyzed on a 15% SDS–PAGE gel. After blotting onto nitrocellulose membranes Westerns were carried out by incubation with polyclonal antibody against the C-tail of histone H3 (1:1000) or with polyclonal to histone H3 trimethyl K9 (1:1000).

## 3. Results and discussion

### 3.1. Identification and disruption of F. graminearum heterochromatin protein 1

The predicted sequence for the HP-1/HepA ortholog in *F. graminearum* was obtained by conducting a BLAST search (http://www.broadinstitute.org) using the *A. nidulans* HepA aminoacid sequence as a query. Only one sequence showing a value of $e^{-27}$ was retrieved. The putative protein FG08763.1, hereafter referred to as HEP1, contains a typical N-terminal chromodomain, a linker or hinge region and a C-terminal chromoshadow domain required for protein–protein interaction in HP1 proteins (Aasland and Stewart, 1995; Kwon and Workman, 2011; Lomberk et al., 2006). Notably, the first tyrosine in the chromodomain, which is conserved in other fungal HP1 proteins and is important for recognition of K9H3me3 by human HP1 (Nielsen et al., 2002), is substituted by a phenylalanine in all three sequenced *Fusarium* species, as shown in the alignment in Fig. 1. The significance of this conserved amino acid change for Hep1 function is not known at the moment.

To study the function of the putative *F. graminearum* HEP1 protein, the gene was deleted in the PH-1 reference strain by replacement with a hygromycin resistance marker. Two independently selected transformants, *Hep1*ΔT-V7 and *Hep1*Δ-X1, carrying homologous gene replacements without additional integrations, (see Experimental procedures) were selected for further studies.

### 3.2. Absence of Hep1 does not show growth defects but causes an altered secondary metabolite profile

When compared to the PH-1 reference strain, the deletion of *Hep1* did not cause any noticeable difference in growth and biomass accumulation on solid or liquid media, respectively, and the production of macroconidia on mung bean broth was not decreased (data not shown).

However, a pronounced accumulation of a red pigment was observed in both tested deletion strains when they were grown on solid *Fusarium* minimal medium (FMM, Fig. 2A, shown for strain T-V7). This phenotype indicates that, similar to what we have found in *A. nidulans*, the deletion of the major component necessary for heterochromatin formation results in an altered secondary metabolite profile in *F. graminearum*.

In order to characterize the metabolites, both *Hep1* deleted strains were cultivated along with the HP-1 reference strain in FMM for 72 h and culture supernatants from two biologically independent experiments were analyzed by HPLC. The extraction and HPLC method applied here simultaneously targets 186 known fungal metabolites and the database comprises more than 20 *Fusarium sp.* metabolites (Vishwanath et al., 2009). Fig. 2B shows the results of quantitative HPLC which revealed that the mutant cultures overproduced aurofusarin in average between 3 and 4-fold but production of the main trichothecene type toxin deoxynivelenol (DON) was strongly reduced in the *Hep1* mutants.

### 3.3. The transcriptional activity of biosynthetic cluster genes mirror metabolite production levels

To further investigate the role of *Hep1* in regulation of aurofusarin and DON production we performed transcriptional analysis of

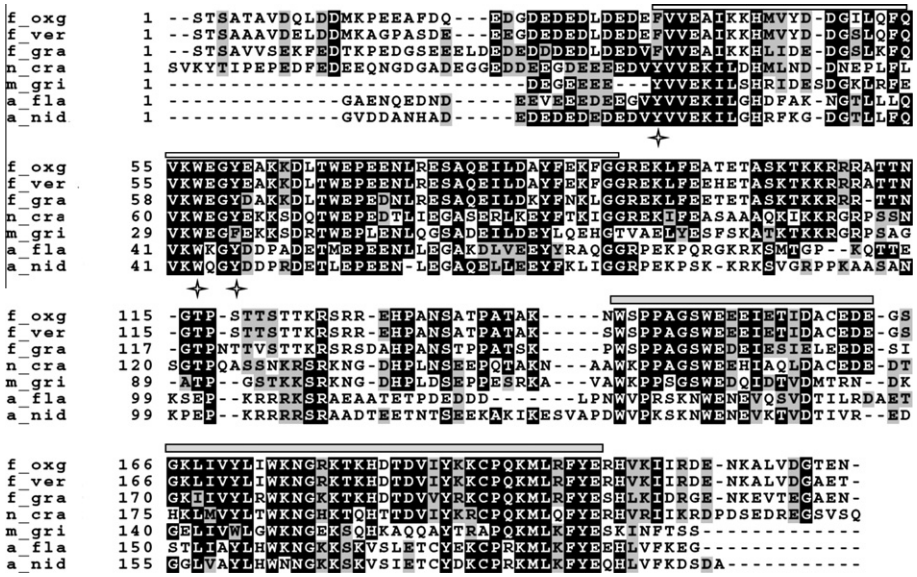


**Fig. 1.** Multiple sequence alignment of *F. graminearum* HEP1 with other putative or characterized fungal Heterochromatin protein 1 homologs. The alignment was performed using ClustlalW and similarities are visualized with Boxshade. f_oxg, *Fusarium oxysporum* FOXG_03019.2; f_ver, *Fusarium verticillioides* FVEG_01876.3; n_crassa, *Neurospora crassa* hpo (HP1); m_gri, *Magnaporthe grisea* AAR19295.1; a_fla, *Aspergillus flavus* AFL2G_02774; a_nid, *Aspergillus nidulans* HepA. The open horizontal bar is positioned above the predicted chromodomain and the filled horizontal bar is positioned above the predicted chromoshadow domain. Asterisks indicate residues that form a putative hydrophobic pocket presumably binding the N-methyl group in H3K9 (Nielsen et al., 2002).

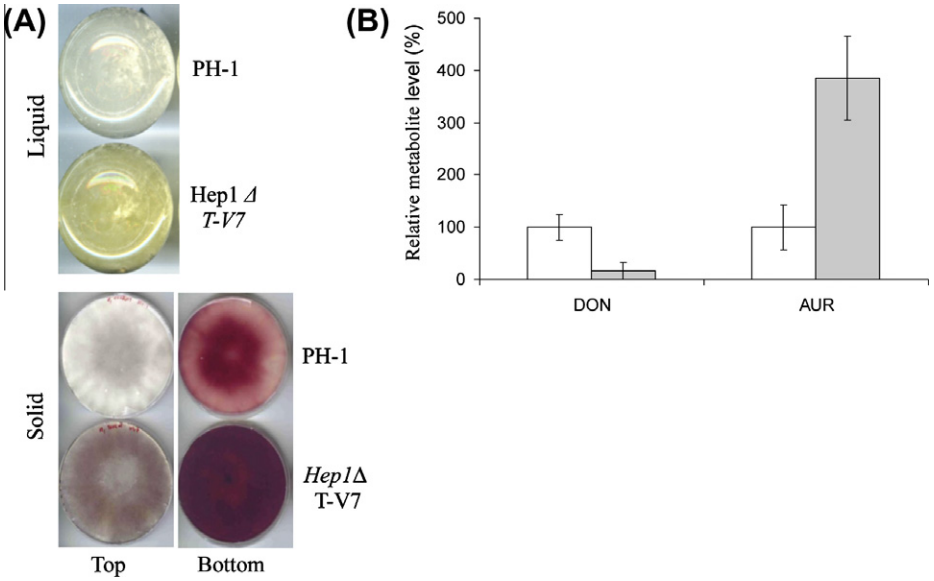


**Fig. 2.** Deletion of *Hep1* causes overproduction of aurofusarin and reduction in DON levels. (A) The red pigment aurofusarin strongly accumulates in the *Hep1* deletion mutants (shown here for the transformant T-V7) in liquid FMM media after 72 h (upper panel) and on solid FMM after 5 days (lower panel) of incubation. Plates are shown from the bottom and from the top. (B) Quantification of aurofusarin and DON metabolite levels in FMM culture supernatants of PH-1 (open bars) and two different *Hep1*Δ deletion mutants (filled bars). Metabolite levels for PH-1 have been arbitrarily set to 100%. Error bars indicate the standard deviation of two biological and two technical repetitions in two different *Hep1*Δ strains (T-V7 and T-X1).

indicator genes involved in the production of these metabolites. As both deletion strains behaved similarly in metabolite production, all further downstream analysis was carried out with two *Hep1*Δ-TV7 strain.

The genes responsible for aurofusarin biosynthesis are arranged in a cluster (schematically drawn in Fig. 3A) that spans around 30 kb (Malz et al., 2005). We analyzed the transcriptional activity of *Aur1*/*GIP2*, encoding a positively acting transcription factor required for the full expression of the cluster and *PKS12*, a polyketide synthase gene required for aurofusarin biosynthesis (Frandsen et al., 2006; Gaffoor et al., 2005; Kim et al., 2005). To test if alterations in the transcriptional profile are specific for the cluster we included FGSG_2319.3 in our analysis. This putative open reading frame (ORF) lies downstream of *Aur1* and is transcribed in the opposite direction (Fig. 3A). A strong increase in transcription (threefold to eightfold) of the cluster genes *Aur1*, *PKS12*, and of the neighboring ORF FGSG_2319.3 was observed in the *Hep1*Δ strain (Fig. 3B). Higher transcript levels of the genes belonging to the AUR cluster agree with the increased metabolite levels found in the mutants strains (Fig. 2) and indicate a conserved function

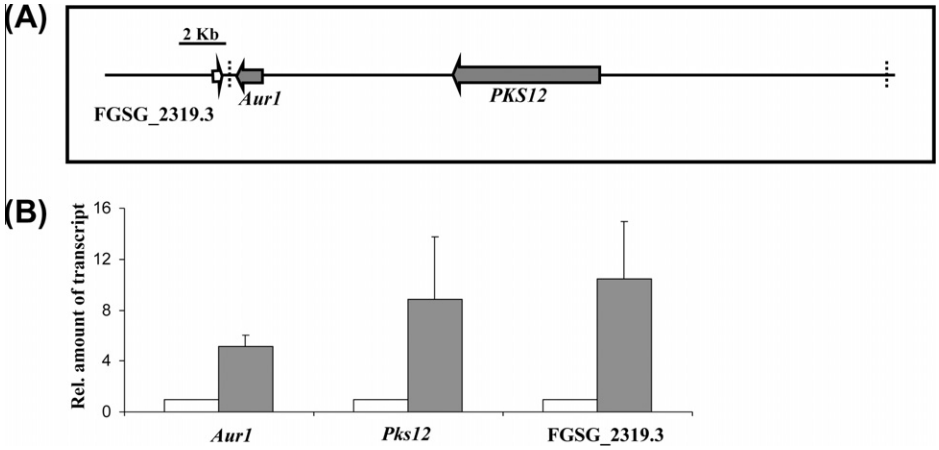


**Fig. 3.** The aurofusarin biosynthetic cluster is upregulated in the *Hep1* deletion mutant. (A) Schematic representation of the AUR gene cluster. Only the relative position of genes analyzed in this study is indicated. Dotted vertical lines indicate the proposed 3′ and 5′ limits of the cluster. FGSG_2319.3 is the first predicted gene that is not proposed to form part of the cluster and is therefore drawn here as the first gene positioned immediately outside the AUR cluster. B. RT-qPCR transcriptional analysis of the AUR-cluster regulator *Aur1*, the biosynthetic gene *Pks12* and the predicted non-cluster gene FGSG_2319.3 in the wild type strain PH-1 (open bars) and in the *Hep1*Δ strain (filled bars). Relative amount of the specific transcripts were obtained by normalization against the amount of beta-tubulin transcript. Average values and standard deviations are derived from two biological and two technical repetitions.

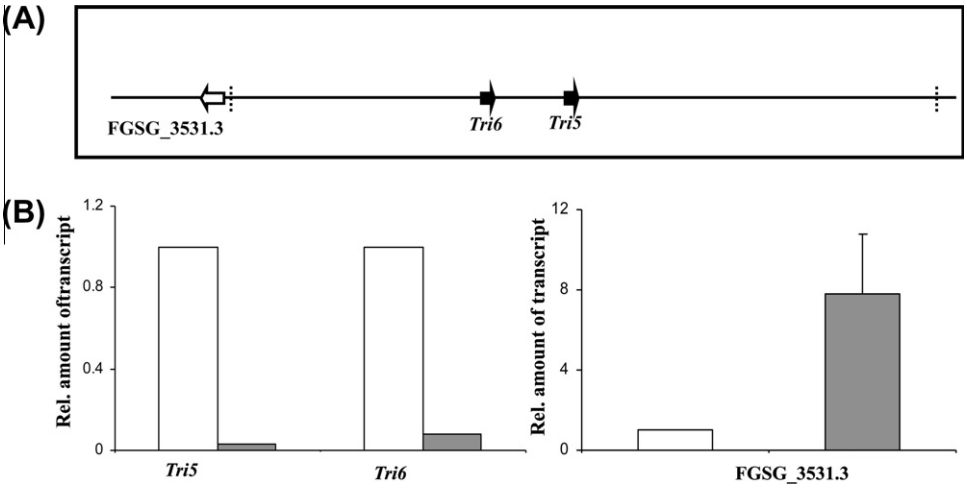


**Fig. 4.** The cluster for DON biosynthesis is repressed in the *Hep1* deletion mutant. (A) Schematic representation of the DON gene cluster. Only the relative position of genes analyzed in this study is indicated. Dotted vertical lines represent the proposed 3′ and 5′ limits of the cluster. FGSG_3531.3 is the first predicted non-cluster gene. (B) RT-qPCR transcriptional analysis of the DON-cluster regulator *Tri6*, the biosynthetic gene *Tri5* and the predicted non-cluster gene FGSG_3531.3 in the wild type strain PH-1 (open bars) and in the *Hep1*Δ strain (filled bars). Relative amounts of the specific transcripts were calculated by normalization against the amount of beta-tubulin transcript. Expression of the non-cluster gene is shown in a separate graph due to the roughly 10-fold stronger overall values obtained in qPCR. Average values and standard deviations are derived from two biological and two technical repetitions.

of *Hep1* in repressing secondary metabolite gene clusters. Surprisingly, the transcript of FGSG_2319.3, a gene encoding a hypothetical protein was also up-regulated. This is in contrast to the situation in *A. nidulans* where the expression of genes flanking the sterigmatocystin biosynthetic cluster is not changed in the HepA deletion strain (Reyes-Dominguez et al., 2010). Our results on AUR cluster gene expression thus may indicate that FGSG_2319.3 forms part of the AUR cluster or that *Hep1* control reaches beyond the limits of this cluster. To distinguish between these two possibilities, the function of FGSG_2319.3 in aurofusarin biosynthesis will need to be studied by gene disruption and metabolite profiling.

In *F. graminearum*, the genes involved in trichothecene biosynthesis have been shown to reside at more than one locus in the genome (Jurgenson et al., 2002; Kimura et al., 2007; Meek et al., 2003), although most *Tri* genes have been found within the 25 kb *Tri5*-cluster (Kimura et al., 2003). Within this cluster (schematically drawn in Fig. 4A) we analyzed transcription of *Tri6*, one of the positive pathway-specific regulators (Hohn et al., 1999; Proctor et al., 1995b), and *Tri5*, a gene that encodes a trichodiene synthase (Proctor et al., 1995a). FGSG_3531.3 was analyzed as control gene presumably not forming part of the cluster.

As shown in Fig. 4B the absence of *Hep1* leads to strongly decreased transcription of *Tri5* and *Tri6* whereas transcription of neighboring FGSG_3531.3 is increased in the mutant strain. Transcriptional down-regulation of the two genes inside the *Tri5*-cluster is in agreement with reduced DON levels in the Hep1 mutants (Fig. 2) but this effect contrasts the classical role of HP1 in formation of transcriptionally inactive constitutive or facultative heterochromatin. However, HP1 homologs do not always follow code (Cryderman et al., 2005; Li et al., 2002). For example HP1 interacts with transcriptional regulators, proteins involved in DNA repair and with the heterogeneous nuclear ribonucleoproteins (hnRNPs) that are known to be involved in RNA processing. Genome-wide

high-resolution mapping studies in different organisms showed elevated dosage of HP1 proteins at euchromatic sites containing developmentally regulated genes and in some localization studies the presence of HP1 was shown to be RNA polymerase-II dependent (Kwon and Workman, 2008, 2011; Piacentini et al., 2009). Thus, it is not entirely surprising that also in *F. graminearum*, HEP1 mediates both repressing and activating functions, depending on the localization of the investigated genes. It could also be that HEP1 is involved in the repression of a factor negatively influencing the transcription of DON biosynthetic genes and consequently lack of HEP1 function could lead to up-regulation of the DON repressor. This type of negatively acting genes have been found in a recent *F. graminearum* microarray experiment (Gardiner et al., 2009). As these genes presumably encode enzymes and not transcriptional regulators, they have been proposed to function in the production of a metabolite that negatively regulates DON biosynthetic genes at the transcriptional level.

It is noteworthy that – in contrast to genes inside the cluster – the DON-cluster flanking gene FGSG_3531.3 is up-regulated. This finding suggests that the mechanism responsible for *Tri5* and *Tri6* down-regulation is DON cluster specific and does not affect the entire genomic region. It is thus likely that Hep1 indeed negatively regulates a larger chromosomal region encompassing also the DON cluster, and thus performs the canonical role of heterochromatin formation. At the moment it is not possible to exactly identify the chromosomal localization of AUR and TRI clusters because the *F. graminearum* physical map is still in progress (http://mips.helmholtz-muenchen.de/genre/proj/FGDB (Guldener et al., 2006). According to preliminary bioinformatic results, however, it is unlikely that these two clusters are positioned in classical heterochromatic regions such as subtelomeres or pericentromeres (Ulrich Guldener, personal communication).

### 3.4. Despite their differential expression, aurofusarin and DON biosynthesis clusters carry reduced heterochromatic marks in the Hep1 mutant

In order to investigate if the differences of transcriptional activity in the mutant strain are correlated with changes in heterochromatic marks, we performed chromatin immunoprecipitation (ChIP) experiments on the promoters of genes belonging to the Aurofusarin and *Tri-5* gene clusters. The antibodies used for ChIP were previously used to detect the C-terminus of histone H3 (H3-Cterm) as well as di- and trimethylated lysine 9 of histone H3 (H3K9me) in *A. nidulans*. We first tested the reactivity of these antibodies in *F. graminearum* whole cell extracts by western blot. In these experiments a single band was detected with both antibodies and no differences were observed in the global levels of H3K9me between the wild type control and the *Hep1* deleted strain (Supplementary Fig. S1). This indicates that deletion of *Hep1* does not lead to a reduction of H3K9 methylation on a genome-wide scale. When we tested the presence of H3K9me at the SM gene promoters we found that the level of K9H3me3 is drastically decreased in the *Hep1*Δ strain in regulatory and catalytic genes of the aurofusarin cluster. The flanking FGSG_2319.3 gene is also affected in the same way by the deletion of *Hep1* (Fig. 5A). The same reduction of the heterochromatic mark was detected for the Tri 5 cluster, the promoter regions of *Tri5*, *Tri6* and for the flanking putative gene FGSG_3531.2 (Fig. 5B).

These ChIP results are similar to the ones obtained in *A. nidulans*, where loss of HepA leads to a strong reduction of H3K9 methylation at specific loci. This phenomenon has first been described in fission yeast, where the HP1 homologue SWI6 interacts with the H3K9methyltransferase Clr4 and both proteins are required for propagation and perpetuation of K9H3me3. Consequently, loss of SWI6 results in a lower H3K9 methylation

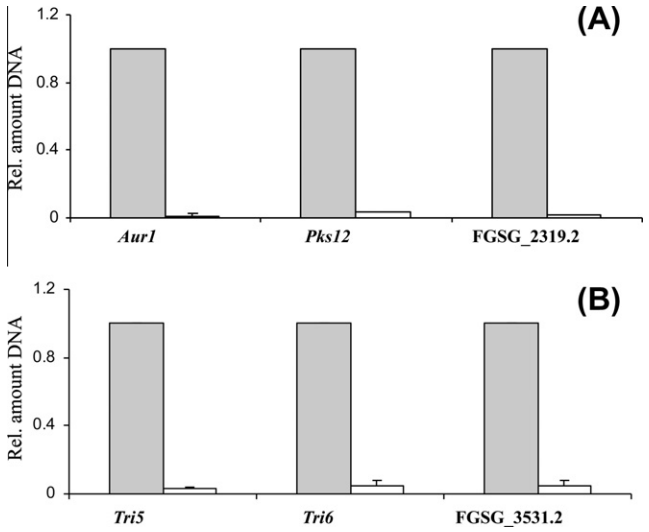


**Fig. 5.** Heterochromatic marks are lost in the *Hep1*Δ strain. Chromatin immunoprecipitation (ChIP) analysis of the heterochromatic mark K9H3me3 analyzed in the promoter region of two AUR cluster (panel A) and two DON cluster (panel B) genes. Promoters of the predicted genes FGSG_2319.3 and FGSG_3531.3 flanking the AUR and DON clusters, respectively, were used as non-cluster controls. Relative amounts of DNA precipitated with the H3K9me3-specific antibody were calculated relative to the amount of DNA precipitated with the antibody recognizing the histone H3 C-terminus. Wild type H3K9 trimethylation levels (filled bars) were arbitrarily set to 1 and mutant levels (open bars) are shown relative to the wild type. Average values and standard deviations are derived from two biological and two technical repetitions.

levels (Richards and Elgin, 2002). A similar situation has been described in mouse (Aagaard et al., 1999), Drosphila (Schotta et al., 2002), and *A. nidulans* (Reyes-Dominguez et al., 2010). Our actual data indicate that also in *F. graminearum*, HEP1 is responsible for the recruitment of the not yet characterized *F. graminearum* H3K9 methyltransferase.

## 4. Conclusions

Lack of heterochromatin protein is lethal in *Drosophila* or shows severe phenotypes such as loss of chromosome stability in *S. pombe* (Allshire, 1995; Ekwall et al., 1996) or the loss of DNA methylation accompanied by severe growth defects in *N. crassa* (Freitag et al., 2004; Rountree and Selker, 2010). Here we identified a second fungal species – next to *A. nidulans* – in which loss of HP1 does not cause obvious growth or developmental defects but influences the secondary metabolite profile. This finding strengthens the hypothesis that facultative heterochromatin mediated by heterochromatin protein 1 and histone H3K9 methylation influences the expression of mycotoxin gene clusters. In contrast to *A. nidulans*, where SM genes are generally up-regulated in HepA mutants, Hep1 deletion in *F. graminearum* can lead to both, elevated and reduced transcription. If Hep1 is involved in the repression of a negative factor of DON gene expression or if Hep1 functions as DON activator awaits clarification. As the secondary metabolite profile influences pathogenicity in *F. graminearum Hep1* and H3K9me may be involved in regulation of virulence in this fungus. Infection assays with susceptible wheat lines using PH-1 wild type and Hep1 mutants are in progress.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.11.002.

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