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Dothistromin genes at multiple separate loci are regulated by AflR

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ABSTRACT

In fungi, genes involved in the production of secondary metabolites are generally clustered at one location. There are some exceptions, such as genes required for synthesis of dothistromin, a toxin that is a chemical analog of the aflatoxin precursor versicolorin A and made by the pine needle pathogen *Dothistroma septosporum*. The availability of the *D. septosporum* genome sequence enabled identification of putative dothistromin genes, including an ortholog of the aflatoxin regulatory gene *AflR*, and revealed that most of the genes are spread over six separate regions (loci) on chromosome 12 (1.3 Mb). Here we show that levels of expression of the widely dispersed genes in *D. septosporum* are not correlated with gene location with respect to their distance from a telomere, but that AflR regulates them. The production of dothistromin by *D. septosporum* in which the *AflR* gene was knocked out (*ΔDsAflR*) was drastically reduced, but still detectable. This is in contrast to orthologous *ΔAflR* mutants in *Aspergillus* species that lack any aflatoxin production. Expression patterns in *ΔDsAflR* mutants helped to predict the complete set of genes involved in dothistromin production. This included a short-chain aryl alcohol dehydrogenase (*NorB*), which is located on chromosome 11 rather than chromosome 12, but was 24-fold down regulated in *ΔDsAflR*. An orthologous set of dothistromin genes, organized in a similar fragmented cluster arrangement to that seen in *D. septosporum*, was found in the closely related tomato pathogen *Cladosporium fulvum* even though this species does not produce dothistromin. In *C. fulvum,* pseudogenization of key biosynthetic genes explains the lack of dothistromin production. The fragmented arrangement of dothistromin genes provides an example of coordinated control of a dispersed set of secondary metabolite genes; it also provides an example where loss of dothistromin production might have allowed adaptation to a new pathogenic lifestyle.

1. Introduction

Dothistroma septosporum is a globally important forest pathogen that causes red band needle blight disease of pine trees (Bradshaw, 2004). The disease symptoms are characterized by red bands in infected needles that contain dothistromin, a polyketide-derived toxin produced by the pathogen (Bassett et al., 1970). Dothistromin (DOTH) is produced in culture as well as in needle lesions, has a difuroanthraquinone structure and is structurally related to aflatoxin (AF), sterigmatocystin (ST) and versicolorin (VER) metabolites produced by some *Aspergillus* species (Gallagher and Hodges, 1972; Shaw et al., 1978).

Dothistromin is a broad-spectrum toxin that generates oxygen radicals by reductive oxygen activation (Youngman and Elstner, 1984) but its exact role in needle blight disease is unknown. It is not essential for pathogenicity as *D. septosporum* mutants lacking the ability to make DOTH are still able to infect pine (*Pinus radiata*) (Schwelm et al., 2009). Its toxicity to other fungi that inhabit pine needles led to the suggestion that it confers a competitive advantage for *D. septosporum* (Schwelm et al., 2009). However, in recent studies an effect on disease lesion formation and sporulation *in planta* has been shown, suggesting a role in virulence (Kabir and Bradshaw, unpublished results).

In fungi genes required for synthesis of secondary metabolites are generally clustered and co-regulated (Keller and Hohn, 1997; Martin and Liras, 1989). Precursors in DOTH biosynthesis are shared with those involved in aflatoxin biosynthesis but unlike the single cluster of genes involved in AF biosynthesis, in *D. septosporum* most DOTH genes are arranged at separate loci on a 1.3-Mb chromosome (Schwelm and Bradshaw, 2010; Zhang et al., 2007). Although 10 DOTH biosynthesis genes were found prior to the availability of the *D. septosporum* genome sequence (Bradshaw et al., 2002, 2006; Zhang et al., 2007) the remaining DOTH genes

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were difficult to locate since they are not contiguous. The *D. septosporum* genome sequence was assembled to chromosome level allowing identification of more biosynthesis genes at additional loci (de Wit et al., 2012). A similar fragmented arrangement of DOTH genes was also found in the genome of the closely related biotrophic tomato pathogen, *Cladosporium fulvum*, a species not known to produce DOTH (de Wit et al., 2012).

Clustering of secondary metabolite (SM) genes is thought to be important for co-regulation and for rapid production of SMs under specific environmental conditions when their presence is required (Chiou et al., 2002; Palmer and Keller, 2010). Studies of global regulation suggest that gene location is critical for regulation (Bok et al., 2009; Palmer and Keller, 2010; Perrin et al., 2007), leading us to question how the physically separated set of DOTH genes is (co)-regulated in *D. septosporum*. Studies with the first 10 DOTH genes to be identified showed that they are indeed co-regulated but also revealed an unusual pattern of expression. Dothistromin is produced mainly during the early exponential phase in culture, instead of during late exponential or stationary phases as is normally seen for SMs such as AF (Schwelm et al., 2008). Whether or not this type of regulation also occurs *in planta* is not known.

In *Aspergillus* spp., regulation of clustered AF and ST genes involves a pathway specific transcription factor AflR that belongs to the zinc binuclear domain (Zn_2Cys_6) class (Chang et al., 1995a; Payne et al., 1993). In *A. parasiticus* deletion of *aflR* (Δ*aflR*) abolished the expression of most AF pathway genes (Cary et al., 2000) and prevented production of AF. Overexpression of *aflR* in both *A. parasiticus* and *A. flavus* caused up regulation of AF gene transcription and AF accumulation (Chang et al., 1995b; Flaherty and Payne, 1997). Detailed analysis of gene transcription using microarrays identified 23 genes in *A. parasiticus* more highly expressed in the wild type than in the Δ*aflR* mutant. Eighteen of the genes differentially expressed on the microarray were AF biosynthetic genes with a putative consensus AflR binding site (5′-$TCGN_5CGR$-3′) in their promoters (Price et al., 2006) but more recent studies report that the promoters of almost all AF cluster genes contain AflR binding sites (Ehrlich, 2009; Ehrlich et al., 2008).

We recently demonstrated an important role for the global SM regulator, VeA, in DOTH biosynthesis, suggesting common regulatory features with clustered genes in other fungal species (Chettri et al., 2012). Here we report characterization of the complete set of DOTH genes in *D. septosporum*, demonstrate that DsAflR is involved in regulating their expression and predict which gene products are required for the last steps of DOTH biosynthesis. We also show that in the closely related species *C. fulvum*, although CfAflR retains partial functionality, lack of DOTH production can be accounted for by pseudogenization of essential genes including *CfHexA* and *CfNor1* in this fungus.

2. Materials and methods

2.1. Strains and culture conditions

D. septosporum NZE10 (CBS128990) is a New Zealand wild-type forest isolate and the strain whose genome was sequenced by the Joint Genome Institute (JGI). Cultures were grown on Dothistroma Medium (DM), Dothistroma sporulation medium (DSM) (Bradshaw et al., 2000) or a minimal salts medium prepared with water in which pine needles had been soaked overnight (PMMG) as previously described (McDougal et al., 2011). Liquid cultures were grown for 7 days with agitation at 200 rpm, and then mycelia collected by filtration through Mira cloth.

The genome sequence of *C. fulvum* (race 0WU; CBS131901; isolated from a tomato in the Netherlands) was generated at Wageningen University (de Wit et al., 2012) and made publically available at the JGI portal.

2.2. Gene identification and sequence comparisons

The *DsAflR* gene was identified by blastp analysis of *D. septosporum* gene models available at the JGI genome site (http://genome.jgi-psf.org/Dotse1/Dotse1.home.html) using *A. parasiticus* (AAS66018) and *A. nidulans* (AAC49195) AflR as query sequences. The JGI DsAflR protein identification number (PID) is 75566. Alignments were performed using ClustalW (Larkin et al., 2007) and conserved regions (as summarized by Bhatnagar et al., 2003) were identified. Putative AflR transcription factor binding sites with the consensus sequence (TCG(N_5)CGR) (Ehrlich et al., 1999) that occurred within 1 kb of predicted translation start sites were identified, as well as variant sequences TCG(N_{10})TCG (Fernandes et al., 1998) and TCG(N_{11})CGR (Zhang et al., 2007), using Clone Manager Professional suite version 8 (Scientific & Educational Software, http://www.scied.com).

2.3. Gene knockout and complementation

Genomic DNA was extracted from freeze-dried mycelia of *D. septosporum* using the CTAB method described earlier (Moller et al., 1992). For the construction of a *D. septosporum DsAflR* knockout vector 1437 bp of coding region was replaced by an *A. niger glaA* promoter-hygromycin resistance gene cassette. The *DsAflR* replacement vector pR310 was constructed using a Multisite Gateway system (Invitrogen, Carlsbad, CA) as described previously for the *VeA* gene knockout (Chettri et al., 2012). Three entry clones were created by PCR amplification of a 5′ region of *DsAflR*, including nucleotides 692748–693934 of scaffold 12 (JGI genome coordinates), a selectable marker that confers hygromycin resistance (*hph*) and a 3′ region of *DsAflR*, including nucleotides 690012–691253. Primer sequences are shown in Supplementary Table S1. *D. septosporum* NZE10 was transformed with the three-fragment plasmid pR310 using methods described previously (Bradshaw et al., 2006) and transformants were single-spore purified.

Complementation of *D. septosporum AflR* replacement strain KO1 with *DsAflR* was achieved by co-transformation with a 1:1 ratio of *DsAflR* coding sequence flanked by 1 kb upstream and downstream sequence (i.e. nucleotides 690431 to 693567 of *D. septosporum* scaffold 12), and the vector pBCphleo (pR224) that confers phleomycin resistance. Transformants were selected on 10 μg/mL phleomycin and were single-spore purified. Confirmation of *DsAflR* gene replacement and complementation was determined by PCR and Southern hybridisation of *Bgl*I-digested DNA with a digoxigenin (DIG)-labeled probe encompassing *DsAflR* 3′flank and *hph* gene regions, following the protocol earlier described (Bradshaw et al., 2006) and as shown in Supplementary Fig. S1.

To determine if *CfAflR* is functionally orthologous to *DsAflR*, the *D. septosporum* Δ*DsAflR* mutant was transformed with a 2.9 kb genomic DNA fragment containing the *CfAflR* gene (nucleotides 85195–88132 of *C. fulvum* scaffold 130965), using methods outlined above. PCR primers used for amplification and for verification of constructs and transformants are shown in Supplementary Table S1.

2.4. Expression analysis and dothistromin assays

D. septosporum mutants and wild-type controls were grown in liquid DM or PMMG medium for 7 days at 22 °C with shaking (100–160 rpm). SMs were extracted from the media and the secreted DOTH assessed by TLC and quantified by HPLC using methods described previously for *D. septosporum* velvet gene mutants (Chettri et al., 2012). RNA was extracted from mycelia grown in

the same flasks and gene expression assessed by quantitative real-time (qRT)-PCR using methods published earlier (Chettri et al., 2012). Sequences of primers used for qRT-PCR are shown in Supplementary Table S1.

Data significance in comparisons, where required, was determined using a two-tailed *T*-test based on the null hypothesis of no significant difference. For qRT-PCR comparisons, REST 2009 (Relative Expression Software Tool) was used (Pfaffl et al., 2002). Pearsons correlation coefficients were calculated using http://www.stattools.net/.

3. Results and discussion

3.1. Organization and re-naming of the predicted set of D. septosporum dothistromin genes

The availability of the *D. septosporum* genome enabled rapid identification of a complete set of candidate DOTH biosynthetic and regulatory genes. Where justified, DOTH genes have been named according to their *Aspergillus parasiticus*/*A. flavus* orthologs in the AF biosynthetic pathway. After consultation with the AF research community it was decided to use the old-style descriptive gene names (e.g. *vbsA*, *verB*) instead of the newer *afl* letter codes (e.g. *aflK*, *aflL*) (Yu et al., 2004), and to keep original names for the regulatory genes *aflR* and *aflJ*. However, the Dothideomycete gene-naming convention of capitalization of the first letter of the gene name has been adopted for *D. septosporum* genes. Cross-references to *afl* (AF) and *stc* (ST) gene names are given in Table 1 and Fig. 1. In some cases this means a change of name for DOTH genes, such that *dotA* (Bradshaw et al., 2002) becomes *Ver1*, and genes previously called *moxA* and *cypA* (Bradshaw et al., 2006) become *MoxY* and *CypX* to highlight orthology with AF genes. When required to distinguish between orthologs from different species, the genes will be referred to with a prefix such as *DsAflR* for *D. septosporum AflR* and *CfAflR* for *C. fulvum AflR*.

Dothistromin genes are dispersed across chromosome 12 at six loci (de Wit et al., 2012) as shown in Fig. 1A. Thirteen of the enzyme-encoding genes are predicted to be required for biosynthetic steps up to versicolorin A (VA). The gene products and the chemistry involved in these steps have been well characterized (Ehrlich, 2009; Ehrlich et al., 2010; Wen et al., 2005; Yabe and Nakajima, 2004; Yu et al., 2004). For the remainder of this manuscript they will be referred to as 'core' genes. The proposed functions of these core genes and their roles in VA biosynthesis are shown in Fig. 1B and Table 1. Of these, gene replacement has confirmed the function of four including *PksA* (Bradshaw et al., 2006), *VbsA* (Zhang et al., 2007), *HexA* and *AdhA* (Supplementary Table S2). An additional gene, *Ver1* (originally called *dotA*) has also been functionally characterized and is thought to be required for a subsequent pathway step between VA and DOTH (Bradshaw et al., 2002). Putative orthologs of late pathway AF/ST genes occur at locus 5 (*OrdB*) and on chromosome 11 (*NorB*). No orthologs of the late pathway genes found in the *A. parasiticus* and *A. nidulans* AF and ST gene clusters that are required for conversion of VA to ST [*omtB* (*aflO*); *verA* (*aflN*)] or of ST to AF [*ordA* (*aflQ*); *omtA* (*aflP*)] were found in the *D. septosporum* genome by reciprocal BLAST analysis (Ohm et al., 2012).

Other genes that are not orthologous to AF/ST genes but for which a role in DOTH biosynthesis could be envisaged include *DotB*, a peroxidase-encoding gene adjacent to *Ver1* in locus 1

Table 1
Characteristics of dothistromin genes, grouped by *D. septosporum* mini-clusters.

New gene name	Dotse1 protein ID[b]	Amino acids	Introns	Gene copy number[c]	Wild-type expression in PMMG[d]	AF ortholog Ap	ST ortholog An	% ID[f] Ap	% ID An	Predicted function
Ver1[a,g]	192193	264	2	7	0.03	aflM	stcU	79.1	79.2	NAD(P) reductase
DotB	75412	415	0	11	0.09	–	(stcC)	–	24.0	Peroxidase
DotC	75413	581	3	15	0.22	(aflT[e])	–	31.2	–	MFS transporter
PksA[g]	192192	2400	2	2	0.13	aflC	stcA	55.1	58.2	Polyketide synthase
CypX[a]	139960	512	2	4	0.50	aflV	stcB	58.9	61.6	P450 monooxygenase
AvfA	75546	285	0	4	0.14	aflI	stcO	49.3	44.9	NAD(P) reductase
EpoA	57187	421	1	6	0.01	–	–	–	–	Epoxide hydrolase
MoxY[a]	75547	627	5	11	0.21	aflW	stcW	55.4	50.6	Flavin-binding monooxygenase
AflR[g]	75566	480	1	2	0.40	aflR	aflR	27.5	30.4	Regulatory protein
AflJ	57214	457	2	1	0.08	aflS	aflJ	37.1	40.8	Methyltransferase
Est1	75609	329	3	1	0.22	(aflJ)	(stcI)	29.6	27.8	Esterase (alpha/beta hydrolase)
OrdB	75648	268	0	1	0.11	aflX	stcQ	54.1	45.3	NAD(P) reductase
AvnA	57312	526	3	9	0.08	aflG	stcF	57.5	58.3	P450 monooxygenase
HexB	181128	1905	4	2	0.11	aflB	stcK	52.2	46.0	Fatty acid synthase
HexA[g]	66976	1693	2	2	0.28	aflA	stcJ	55.4	45.5	Fatty acid synthase
HypC	75655	186	1	1	0.37	aflZ	stcM	35.2	47.9	Anthrone oxidase
VbsA[g]	75656	648	1	4	0.45	aflK	stcN	72.3	73.1	VerB synthase (cyclase)
Nor1	75691	269	3	2	0.32	aflD	stcE	59.7	58.5	NAD(P) reductase
AdhA[g]	48495	307	2	2	0.06	aflH	stcG	58.1	60.6	Alcohol dehydrogenase
VerB	75692	521	2	7	0.42	aflL	stcL	67.1	67.6	Desaturase (P450 monooxygenase)
NorB	75044	392	0	4	0.18	aflF	stcV	60.7	43.6	Aryl alcohol dehydrogenase

Genes are listed by position (top to bottom) in loci 1–6 or on chromosome 11 (*NorB*). Details of additional and flanking genes are shown in Table S3.

[a] Old names are *dotA* (*Ver1*), *cypA* (*CypX*), *moxA* (*MoxY*).

[b] Protein identification (accession; PID) numbers refer to those at (http://genome.jgi.doe.gov/Dotse1/Dotse1.home.html). The PID numbers shown are the best gene models; old Dotse1 PID numbers in the gene catalog are *HexA* 75653, *PksA* 48345, *Ver1* 75411, *HypC* 66978.

[c] Copy numbers with *e*-value <1e−20 and >40% aa similarity.

[d] Mean expression determined by qRT-PCR and expressed relative to beta-tubulin gene expression (full data in Table S4).

[e] Brackets indicate orthology to *D. septosporum* gene is not supported by phylogenetic studies (Bradshaw et al., unpublished results).

[f] Percentage amino acid identities (% ID) to *A. parasiticus* (Ap) and *A. nidulans* (An) AF/ST genes determined by CLUSTALW whole sequence alignment.

[g] Function confirmed by gene knockout.

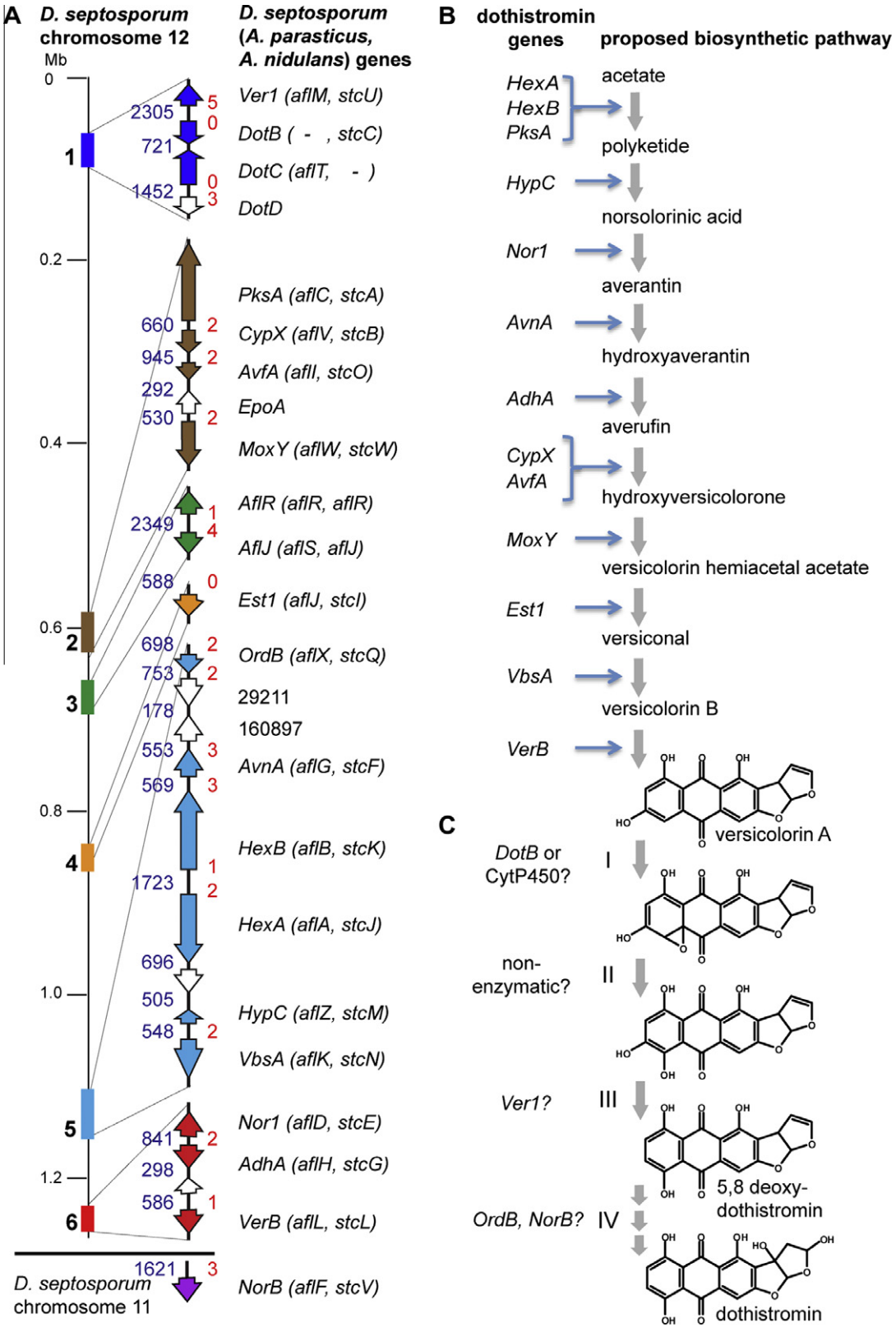

Fig. 1. The fragmented arrangement of dothistromin genes and proposed scheme for dothistromin biosynthesis. (A) The six loci (labeled 1–6) are shown along chromosome 12, with corresponding predicted dothistromin (DOTH) genes shown alongside as shaded arrows indicating the direction of transcription. Unshaded arrows represent genes not thought to have a role in DOTH biosynthesis but those discussed in the text are named. Sizes of intergenic regions (bp) are shown on the left. Numbers of putative AflR binding sites are shown on the right (see Table S6 for details). The *NorB* gene, shown at the bottom, is on chromosome 11 (152 kb from the telomere and 1621 bp from the nearest upstream gene). (B) The biosynthetic pathways of DOTH and aflatoxin have common steps as far as versicolorin A. *D. septosporum* DOTH genes are placed on the pathway based on orthology (or similarity in the case of *Est1*) to aflatoxin genes of known function. (C) A possible biosynthetic pathway from versicolorin A to DOTH is shown, based on that described by Henry and Townsend (2005), with predicted gene designations as discussed in the text.

(Bradshaw et al., 2002), *EpoA* an epoxide hydrolase gene located between *AvfA* and *MoxY* in locus 2, as well as two genes located between *OrdB* and *AvnA* in locus 5 that are predicted to encode a fungal transcription factor (JGI protein ID 29211) and NAD(P) reductase (JGI protein ID 160897) respectively. In addition to these is *Ds31* (PID 66854), a putative translation elongation factor gene with similarity to *stcT*, located seven genes downstream of *MoxY* (not shown in Fig. 1) (Zhang et al., 2007); the predicted protein contains a GST domain that could possibly be involved in epoxide detoxification (McDonald et al., 2005). Immediately flanking most of the loci are genes for which no putative function has been assigned as well as genes that would not be expected to have a role in DOTH biosynthesis, such as a glucose/mannose dehydrogenase gene (adjacent to *Ver1*) (Supplementary Table S3), highlighting the fragmented nature of this gene cluster. Homologs of the *A. parasiticus* AF cluster downstream genes *glcA, hxtA,* and *sugR* (Yu et al., 2000) were not located close to any of the DOTH cluster genes; the closest matches to these genes were found on chromosomes 1 and 2 in *D. septosporum*.

3.2. D. septosporum AflR regulates dothistromin biosynthesis

Because of the lack of tight clustering of functional genes, and the expectation that not all DOTH genes will be orthologs of AF/ST genes, we sought evidence for co-regulation of all predicted DOTH genes by the putative pathway regulator AflR. An alignment of the predicted DsAflR amino acid sequence with those from *A. parasiticus* and *A. nidulans* suggested conservation of the Zn_2Cys_6 zinc binuclear cluster domain but the presence of a glutamine-rich region in DsAflR (Fig. 2).

To test the hypothesis that DsAflR regulates the expression of DOTH biosynthetic genes, three independent knockout mutants were made in *D. septosporum* and one of them (KO1) was complemented with *DsAflR* by co-transformation. Southern hybridization showed that, whilst complemented strain CO1 had one copy of *DsAflR*, strain CO2 contained more than one copy (Supplementary Fig. S1). The ability of *ΔDsAflR* knockout mutants to produce DOTH was tested in two different culture media: a rich complex medium (DM) and a minimal salts medium containing pine needle extract (PMMG). At least a 10^4-fold and a 10^5-fold reduction in DOTH levels was shown in *ΔDsAflR* mutants compared to the wild type in these respective media (Table 2), but low levels of DOTH production (above background levels) remained (see also *Supplementary* Fig. S2). The complemented mutant with additional copies of *DsAflR* produced higher levels of DOTH than the wild type (Table 2), as would be expected for an AflR overexpression mutant (Flaherty and Payne, 1997).

In *A. flavus, A. parasiticus* and *A. nidulans,* disruption of *AflR* led to mutants completely lacking the ability to produce either AF or ST (Fernandes et al., 1998; Payne et al., 1993; Price et al., 2006). This suggests that, although DsAflR is needed for high levels of expression, other regulatory proteins may be able to provide low-level activation of DOTH genes. The putative transcription factor (PID 29211) that is encoded at locus 5 has only 22–23% identify with the AflR proteins. It is ~150 aa longer but shows some conservation of N- and C-terminal zinc cluster and binding specificity domains with DsAflR. Whether this protein or another transcription factor has a role in DOTH regulation, and therefore can account for the residual expression levels in the *ΔDsAflR* mutant, is not known. An alternative explanation for the low level of DOTH production in the absence of DsAflR might relate to the dispersed distribution of DOTH genes compromising similar levels of epigenetic control as the tightly clustered AF genes, thereby enabling their activation by basal transcription factors.

3.3. DsAflR is the key regulator of dothistromin biosynthetic genes

To test the prediction that DOTH genes would be down regulated in *ΔDsAflR* mutants, qRT-PCR assays were carried out to compare gene expression with that in wild-type and *DsAflR*-complemented *D. septosporum* strains. The 13 core genes were all significantly down regulated in a *ΔDsAflR* mutant in PMMG medium, showing from 1.6-fold (*PksA*) to 90.9-fold (*AvnA*) down-regulation compared to the wild type (Fig. 3). Down-regulation was also shown for the functionally confirmed DOTH *Ver1* gene (11.9-fold) as well as for other AF/ST orthologs with unassigned roles

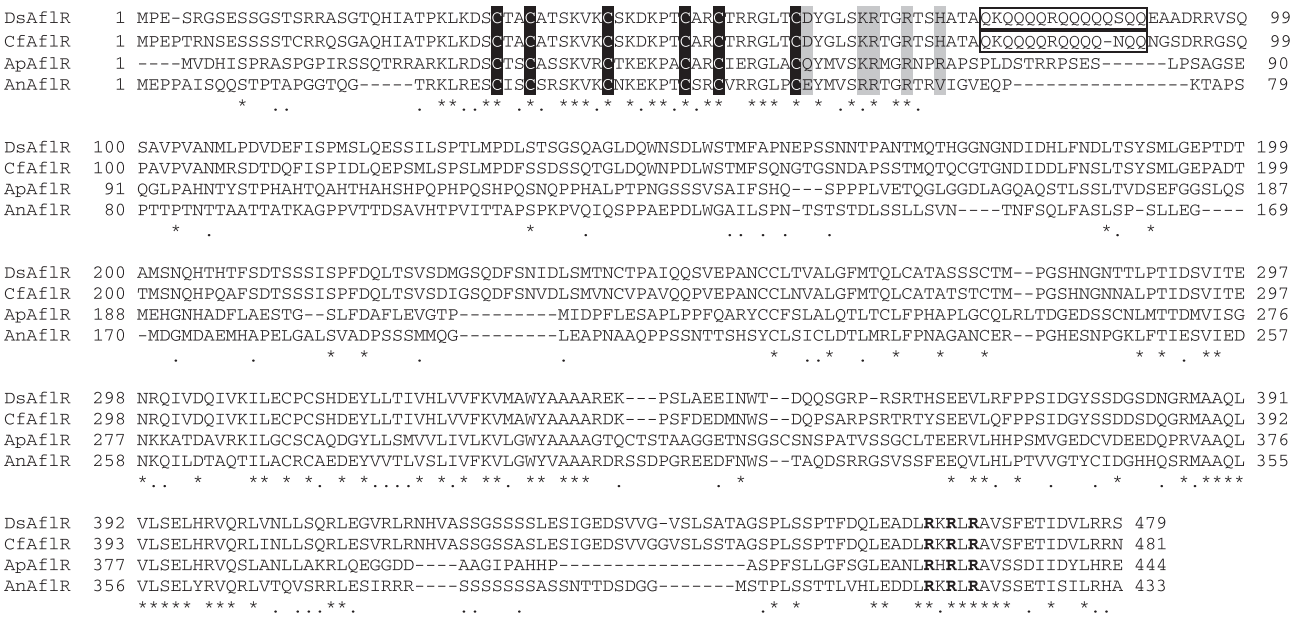

Fig. 2. Multiple alignment of predicted AflR protein sequences. AflR sequences are from *Dothistroma septosporum* (Ds), *Cladosporium fulvum* (Cf), *Aspergillus parasiticus* (Ap) and *Aspergillus nidulans* (An). A conserved zinc binuclear domain (Zn_2Cys_6) is highlighted in black. Amino acids highlighted with gray are thought to determine DNA-binding specificity in *Aspergillus* spp. and are similar in all four fungi. C-terminal arginine residues (bold) implicated in AflJ binding in *A. parasiticus* (Chang, 2003) are also conserved. *D. septosporum* and *C. fulvum* proteins have a glutamine-rich motif (boxed) which is not found in the *Aspergillus* proteins.

Table 2
Dothistromin production in *Dothistroma septosporum AflR* mutants.

Strain	Dothistromin production (pg/mg DW)	
	DM medium	PMMG medium
WT	45,091 ± 10,807	8192 ± 1437
KO1	0.15 ± 0.030*	0.16 ± 0.100*
KO2	0.12 ± 0.012*	0.36 ± 0.156*
CO1	55,947 ± 7676	6136 ± 517
CO2	150,205 ± 12,404*	16,217 ± 1737*

Dothistromin secreted into growth media by wild type (WT), *DsAflR* knockout mutants (KO1, KO2) or mutant KO1 complemented with *DsAflR* (CO1, CO2). Values are means ± SD ($n = 3$).
* Significantly different from wild type ($P < 0.05$). Assay resolution limit is <0.1 pg/mg.

in DOTH biosynthesis (*OrdB*, 8.1-fold; *NorB*, 23.8-fold). In addition, genes predicted to encode a peroxidase (*DotB*) and NADP reductase (160897) showed 4.4-fold and 2.7-fold down regulation in the *ΔDsAflR* mutant, respectively. Genes encoding the transcription factor PID 29211 (locus 5) and an epoxide hydrolase (*EpoA*, locus 2) did not show significant down-regulation.

In *A. parasiticus* all AF orthologs of DOTH genes, except *aflI* (*avfA*) and *aflF* (*norB*), were down regulated in a Δ*aflR* knockout strain compared to the wild type (Georgianna and Payne, 2009; Payne et al., 1993; Price et al., 2006). It is notable that in *D. septosporum DsNorB* showed strong down-regulation despite being located on a separate chromosome to the majority of DOTH genes. Also of interest is that in *D. septosporum DsAflJ* is 8-fold *up* regulated in the Δ*DsAflR* mutant. To determine whether this was due to a mutation in the *DsAflJ* regulatory region introduced by the *DsAflR* knockout procedure, the shared *DsAflR-AflJ* intergenic region was sequenced, but no mutations were found. Whether or not DsAflJ acts as a repressor of DOTH biosynthesis requires further investigation. In *A. parasiticus*, ApAflJ is thought to be a co-regulator of aflatoxin biosynthesis and binds to ApAflR. Three C-terminal arginine residues of ApAflR are implicated in this binding (Chang, 2003); these amino acids are conserved in DsAflR. However, *DsAflJ* did not complement an *A. parasiticus* mutant lacking *aflJ* (Ehrlich, unpublished results), suggesting AflJ may not be functionally conserved between these species.

In *D. septosporum*, the multi-copy complemented strain CO2 displayed 2-fold overexpression of *DsAflR* and correspondingly higher levels of expression for 9 out of the 13 'core' pathway genes, but 2.4-fold down-regulation of *DsAflJ* (Supplementary Table S4). Expression of DOTH genes by cultures grown in rich DM medium showed a less consistent trend compared to those in PMMG pine extract medium but most DOTH genes were down-regulated in a Δ*DsAflR* mutant (Supplementary Table S4). Overall the patterns of DOTH gene expression in the Δ*DsAflR* mutant are consistent with DsAflR being the pathway-specific Zn_2Cys_6 transcriptional regulator of DOTH biosynthesis and with DsAflJ having some, possibly repressive, role in DOTH biosynthesis.

SM gene clusters are usually located within the sub-telomeric region of chromosomes. It has been suggested that such location may affect epigenetic control of transcription of these clustered genes (Palmer and Keller, 2010). Dothistromin genes are unique in being co-regulated even though the genes are separated into six loci that are dispersed over one chromosome (Fig. 1). Analysis of gene expression levels in wild-type *D. septosporum* showed no correlation with the chromosomal positions of genes (in terms of distance from a telomere) in either pine extract (PMMG) or rich (DM) media (Supplementary Table S5). With regard to the effect of DsAflR on gene expression, genes showing the strongest down-regulation in the Δ*DsAflR* mutant compared to the wild type (>30-fold) are located within 140 kb of a telomere (*DotC*, *AvnA* and *AdhA*/*VerB* at loci 1, 5 and 6, respectively), whilst genes located in the more central loci 2, 3 and 4 showed a maximum of 11-fold down-regulation. However, overall there was not a significant correlation between chromosomal position of genes and the effect of deleting *DsAflR* on expression of those genes ($r = 0.24$, $P = 0.34$) (Supplementary Table S5). Overall these results suggest that the position of a DOTH gene in the chromosome, with respect to distance from the telomeres, is not a strong determinant of gene expression level.

Consistent with a role for DsAflR in regulation of DOTH biosynthesis, most core DOTH genes in *D. septosporum* contain between one and five putative upstream AflR binding sites (TCG(N_5)CGR) (Fig. 1A; Supplementary Table S6). The number of predicted AflR sites does not appear to be important; *VerB* has only one such site but was 83-fold down-regulated in the Δ*DsAflR* mutant, whilst *Ver1* has five AflR sites but was only 12-fold down-regulated. In studies with *Aspergillus spp.* it was shown that although some AF/ST genes possess more than one AflR binding site, in many cases the one closest to the translation start site is sufficient for gene activation (reviewed in Bhatnagar et al., 2003).

The only core DOTH gene without a putative AflR-binding sequence TCG(N_5)CGR is *DsEst1*. This gene is predicted to fulfil the role of *estA* in AF/ST biosynthesis, although it is not considered an ortholog based on its low (<30% aa) identity to *A. parasiticus* and *A. nidulans estA* (Table 1) and its placement in a separate clade

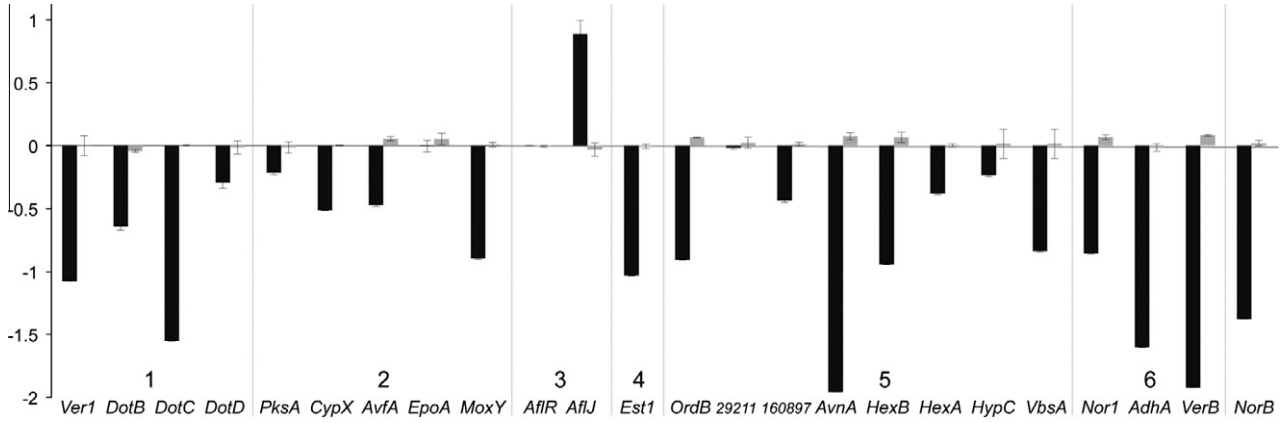

Fig. 3. Expression of dothistromin genes in *D. septosporum AflR* mutants. Gene expression was evaluated by quantitative real-time PCR and is shown as $\log_{10} x$-fold differences in expression (mean ± SEM) in Δ*DsAflR* mutant KO1 (black bars) and the *DsAflR* complementation mutant CO1 (gray bars) relative to expression in the wild type. The genes are grouped according to locus (1–6) on chromosome 12, or on chromosome 11 for NorB.

by phylogenetic analysis (Bradshaw et al., unpublished results). Despite 11-fold down-regulation in the Δ*DsAflR* mutant, *DsEst1* has neither the canonical AflR-binding site nor the variant TCG(N_{10})TCG site that was speculated to be a target for AflR in *A. nidulans* (Fernandes et al., 1998). However, it does contain another variant (TCG(N_{11})CGR) that was previously noted upstream of many DOTH genes in *D. septosporum* (Bradshaw et al., 2002; Zhang et al., 2007); whether this site is functional is not known. Intriguingly, in *A. parasiticus*, the activity of the *estA*-encoded esterase was shown to be complemented by another unknown cytosolic esterase enzyme which is not encoded in the AF cluster (Chang et al., 2004; Yabe and Nakajima, 2004); it would be interesting to determine whether the unknown esterase is orthologous to DsEst1.

3.4. A proposed biosynthetic pathway for dothistromin

Based on the production of 5,8-dihydroxyanthraquinone intermediates by DOTH-producing fungi (Danks and Hodges, 1974), a biosynthetic scheme was proposed for synthesis of DOTH (Henry and Townsend, 2005) that involves epoxidation of VA. Using this scheme, we suggest roles for DsAflR-regulated genes in the final steps of DOTH biosynthesis in *D. septosporum* (Fig. 1C). In step I, it is possible that DotB, a predicted chloroperoxidase with weak (24%) amino acid identity to StcC (McDonald et al., 2005) performs epoxidation of the A-ring of VA. Alternatively, a cytochrome P450 (CytP450), such as CypX or AvnA (CytP450 enzymes encoded by the DOTH cluster and thought to function in the earlier part of the pathway) could catalyze this step. There is a precedent for enzymes catalyzing more than one step in AF biosynthesis; this has been shown for the CytP450 OrdA, the esterase EstA and the cyclase VbsA (Chang et al., 2004; Sakuno et al., 2005; Yabe and Nakajima, 2004). It is also possible that another, uncharacterized, cytochrome P450 enzyme encoded in the *D. septosporum* genome is responsible for this step.

Opening of the epoxide in Step II could potentially be achieved by the epoxide hydrolase EpoA, encoded by a gene divergently transcribed from *MoxY*. However, *EpoA* does not appear to be regulated by DsAflR, *D. septosporum EpoA* knockout mutants produced a compound indistinguishable from DOTH using an ELISA assay (Jin, 2005) and a syntenically positioned *EpoA* gene in the DOTH-producing fungus *Passalora arachidicola* is a pseudogene (Zhang et al., 2010). Together these results suggest EpoA is not required for DOTH biosynthesis, but other epoxide hydrolases may have the ability to complement this hydrolysis. Alternatively, opening of the epoxide ring could be achieved non-enzymatically as suggested for ST and related compounds (Henry and Townsend, 2005). Indeed hydroxylation of anthraquinones can be achieved directly by a single monooxygenase as shown for aurofusarin (Frandsen et al., 2006).

Step III involves deoxygenation of ring A to yield the 5,8-dihydroxyanthraquinone. This step is most likely catalyzed by the NADPH dehydrogenase encoded by *Ver1* (*dotA*) as suggested previously (Henry and Townsend, 2005). This gene was functionally analyzed and shown to be required for DOTH biosynthesis, with gene knockout mutants accumulating a yellow pigment consistent with a stable VA-type intermediate (Bradshaw et al., 2002).

The last stages of DOTH biosynthesis (Step IV) are proposed to involve hydroxylation of the bisfuran. We suggest that enzymes encoded by *OrdB* and *NorB*, both regulated by DsAflR, might have oxidative roles here. In *A. parasiticus*, OrdB is an NAD(P)-dependent oxidoreductase that functions in conversion of VA to ST (Ehrlich and Yu, 2010) and in *A. nidulans* an orthologous gene is possibly required for biosynthesis of monodictyphenone from emodin (Chiang et al., 2010). *NorB* encodes an aryl alcohol dehydrogenase that functions in late steps of AF biosynthesis and is postulated to function mainly in oxidation of alcohol groups (Ehrlich et al., 2008). Similar functions to these are predicted for OrdB and NorB in DOTH biosynthesis, although in *D. septosporum NorB* is located on a separate chromosome than all the other proposed DOTH genes. The *D. septosporum* genome does contain other genes similar to *NorB* (with >40% aa similarity and *e*-value <1e−20; Table 1), but those copies are located on chromosomes 1 and 2. An alternative possibility for Step IV is that CytP450 enzymes such as AvnA and CypX might perform these steps in addition to previously proposed steps.

Disruption of *D. septosporum DotB, OrdB* and *NorB* is in progress to test the hypotheses outlined above. Defining the last stages of the DOTH biosynthetic pathway is likely to be challenging due to multiple copies, and therefore possible functional redundancy, of the genes involved (Table 1). Accordingly, *ordB* and *norB* mutants of *A. flavus* have leaky phenotypes (Ehrlich, 2009). It is possible that other genes not mentioned here are involved in DOTH biosynthesis; discussion of some other cluster-associated genes and their possible roles is provided in Supplementary Text 1.

3.5. Cladosporium fulvum AflR partially complements DsAflR but some pathway genes are non-functional

C. fulvum contains a complete set of DOTH genes but does not produce DOTH (de Wit et al., 2012). Thus we investigated regulatory and structural genes in this species. The complete *C. fulvum CfAflR* gene was transformed into *D. septosporum* Δ*DsAflR* mutant KO1. In two independent transformants, *CfAflR* gene expression levels were comparable to those of *DsAflR* in wild-type *D. septosporum* ($P = 0.29$ and 0.14 for the respective transformants), suggesting that the *CfAflR* promoter is fully functional. Dothistromin was produced in these complemented transformants at levels ~100-fold higher than those in the Δ*DsAflR* mutant. However these DOTH levels were less than 0.1% of *D. septosporum* wild type levels (mean ± SD of 33.8 ± 1.8 and 25.2 ± 4.0 pg DOTH/mg DW for the two *CfAflR* complemented transformants respectively), suggesting a deficiency in CfAflR protein function. The predicted CfAflR protein contains the same conserved domains as DsAflR (Fig. 2) but whether other sequence differences between these two proteins affect CfAflR function is not yet known.

The suggested deficiency in CfAflR function lead us to question whether the DOTH biosynthetic genes are functional in *C. fulvum*. Manual annotation and, where necessary, cDNA sequencing, was carried out to ascertain structures of all the predicted DOTH genes of *C. fulvum*, and showed predicted amino acid identities ranging from 74% (AvfA) to 98% (Ver1) compared to *D. septosporum* gene products (Supplementary Table S7). Closer inspection showed that *CfHexA* contains a premature stop codon and two frameshift mutations, whilst *CfNor1* has a 7 nt insertion causing immediate nonsense (Supplementary Fig. S3). Both HexA and Nor1 are required for early steps of DOTH biosynthesis (Fig. 1), hence these mutations block the dothistromin biosynthetic pathway. Pseudogenization and down-regulation of genes associated with necrotrophy, such as toxin biosynthetic genes and cell wall degrading enzymes, are postulated to be adaptive changes related to the biotrophic lifestyle of *C. fulvum* (de Wit et al., 2012). The remarkable conservation of some of the predicted proteins and partial complementation of the *ΔDsAflR* mutant by *CfAflR* suggest only recent loss of the capacity to produce dothistromin in *C. fulvum*.

4. Conclusions

Dothistromin biosynthetic genes in *D. septosporum* are co-regulated by an AflR-like transcription factor even though they are arranged in multiple separated loci. This feature enabled prediction of genes involved in a proposed biosynthetic pathway for DOTH.

The fragmented arrangement contradicts the more usual finding that fungal SM genes are clustered at a single locus (Khaldi et al., 2010) and our results show that positioning of such clusters at subtelomeric regions of the chromosome does not necessarily affect co-regulation as had been previously suggested (Palmer and Keller, 2010; Yu and Keller, 2005). Preliminary phylogenetic analysis suggests that the ancestral gene cluster for dothistromin biosynthesis more closely resembled the unified clusters found for ST and AF biosynthesis, but while individual portions in the cluster became separated in the Dothideomycete lineage, they retained their ability to be co-regulated by a single transcription factor. Questions have arisen as to whether or not the original cluster was introduced by horizontal gene transfer (Slot and Rokas, 2011).

Our results also show that AflJ is regulated differently in *D. septosporum* compared to in *Aspergillus* spp. and suggest that DsAflJ may have a role in biosynthesis separate from that of an AflR co-activator. Furthermore, although a fragmented arrangement of DOTH genes and a partially functional AflR ortholog is present in the tomato pathogen *C. fulvum*, the biotrophic lifestyle of this fungus may have made production of DOTH unnecessary for its adaptation. Dissimilarities between the organization of DOTH and AF genes are of value for better understanding the evolution and regulation of aflatoxin-like gene clusters in fungi.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2012.11.006.

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