Regulation of dothistromin toxin biosynthesis by the pine needle pathogen *Dothistroma septosporum*.

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Abstract

Dothistromin is a virulence factor produced by the fungal pine needle pathogen *Dothistroma septosporum*. It is similar in structure to a precursor of aflatoxin and sterigmatocystin. Unlike most secondary metabolite genes in fungi, the genes for dothistromin biosynthesis are not clustered but spread over six loci on one chromosome. Another characteristic feature of dothistromin synthesis is that dothistromin is produced mainly during the early exponential growth phase in culture. These unusual features have been proposed to be adaptations for the biological role of dothistromin in the disease process. It was therefore of interest to determine whether the regulation of dothistromin production in *D. septosporum* differs from the regulation of aflatoxin and sterigmatocystin in *Aspergillus* spp. and to address the question of whether genes in a fragmented cluster can be co-regulated.

The availability of the *D. septosporum* genome facilitated identification of orthologs of the aflatoxin pathway regulatory genes *aflR*, *aflJ* and the global regulatory genes *veA* and *laeA*. These genes were functionally characterised by knockout and complementation assays and the effects of these mutations on the expression of dothistromin genes and the production of dothistromin were assessed.

Inactivation of the *DsAflR* gene (*ΔDsAflR*) resulted in a 10^4 fold reduction in dothistromin production, but some dothistromin was still made. This contrasted with *ΔAflR* mutants in *Aspergillus* species that produced no aflatoxin. Expression patterns in *ΔDsAflR* mutants helped to predict the complete set of genes involved in dothistromin biosynthesis.

AflJ was proposed to act as a transcriptional co-activator of AflR in *Aspergillus* spp. Disruption of *DsAflJ* resulted in a significant decrease in dothistromin production and dothistromin gene expression. Interestingly the expression of *DsAflR* was not

affected by deleting *DsAflJ,* while conversely *DsAflJ* transcript levels increased significantly in a *DsAflR* mutant compared to the wild type. Heterologous complementation with *A. parasiticus, A. nidulans* and *C. fulvum AflJ* failed to revert the dothistromin level to wild type suggesting species-specific function of AflJ.

VeA is an important regulator of secondary metabolism and development in fungi. Inactivation of the *D. septosporum* ortholog (*DsVeA*) resulted in reduced dothistromin production and showed the influence of DsVeA on the expression of other secondary metabolite backbone genes. Asexual sporulation was reduced but mutants were not compromised in pathogenicity. Overall, *D. septosporum* DsVeA showed functional conservation of the usual role in fungi.

LaeA is a global regulator of secondary metabolism and morphogenetic development, first identified in *Aspergillus nidulans*. Unexpectedly, DsLaeA exhibited an unusual repressive function on the dothistromin pathway and *DsLaeA* mutants exhibited an extended period of dothistromin production compare to WT *in vitro.* The mutation of *DsLaeA* showed varied responses in expression of other secondary metabolite genes and had differences in sporulation and hydrophobicity compared to the wild type.

Results from this study suggest that that some aspects of secondary metabolite gene regulation, such as coordinated control by the pathway specific regulator DsAflR, are conserved in the fragmented dothistromin gene cluster. However, DsAflJ appears to have a species specific role. The global regulators DsLaeA and VeA had conserved roles but most intriguing was that DsLaeA acted as a repressor of dothistromin biosynthesis, deviating from its usual function in other ascomycetes.

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The Author

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Chapter 1: Introduction

1.1 Dothistromin toxin

1.1.1 Dothistromin, a polyketide virulence factor with unusual genetic features.

Dothistromin is a toxic red coloured compound produced by *Dothistroma septosporum* (Bassett et al., 1970), the causal organism of dothistroma needle blight of pines. It is produced *in vitro* as well as *in planta* by the fungus (Bassett et al., 1970). One of the interesting aspects of dothistromin is its structural similarity to versicolorin B, a precursor of aflatoxin (AF) (Shaw et al., 1978; Bradshaw, 2004) (Figure 1.1).

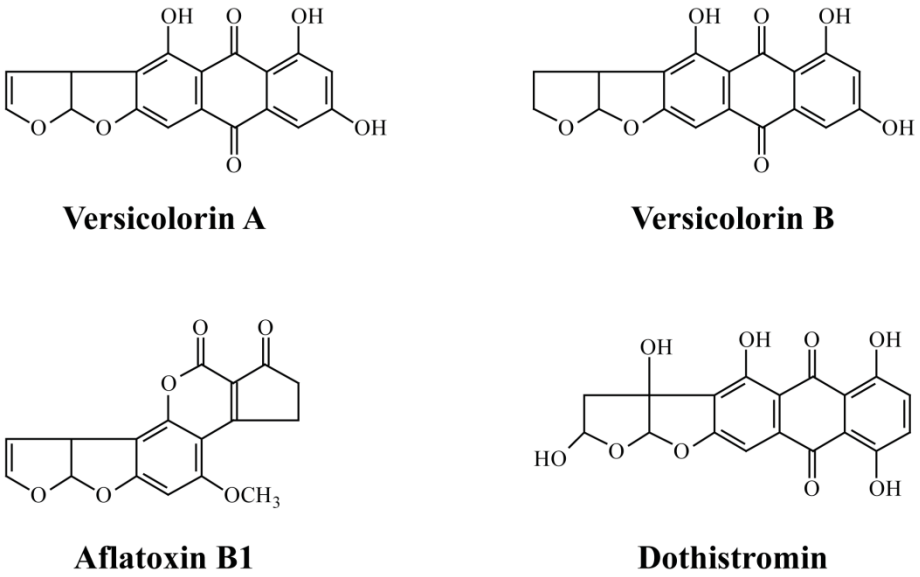


Figure 1.1: Chemical structures of aflatoxin B1, versicolorin B, dothistromin and versicolorin A

Dothistromin is a non host-selective toxin and was initially suspected to play a role in dothistroma needle blight as symptoms could be reproduced by injection of dothistromin into the needle (Shain and Franich, 1981). Subsequently Schwelm et al

(2009) demonstrated that a dothistromin-deficient *D. septosporum* strain was still able to infect radiata pine and proposed that dothistromin confers a competitive advantage for survival in the niche. However, further analysis of these mutants showed decreased mesophyll colonization, smaller lesions and fewer spores thus showing the role of dothistromin as a virulence factor (Kabir et al., 2014).

Based on the structural similarity between dothistromin and an intermediate of aflatoxin (AF) and sterigmatocystin (ST) it was speculated that *D. septosporum* might contain orthologs of genes involved in AF/ST biosynthesis in *Aspergillus* spp. AF and ST are well characterized fungal secondary metabolites due to their importance as a natural carcinogens (Yu and Ehrlich, 2011). In *Aspergillus parasiticus* AF biosynthesis involves 27 enzymatic steps and over 27 structural and two regulatory genes, *aflR* and *aflJ* (Ehrlich, 2009). Secondary metabolite biosynthesis genes in filamentous fungi are generally present in clusters and located in telomere-proximal regions of fungal genomes (Georgianna et al., 2009; Keller and Hohn, 1997). In *Aspergillus flavus* and *A. parasiticus* the aflatoxin pathway genes are clustered within a 75 kb region of the chromosome approximately 80 kb away from the telomere and have conserved synteny (Townsend, 1997; Chang et al., 2005; Yu et al., 2004a). In *A. nidulans* the ST cluster contains 25 genes and spans over a 60 kb region without maintaining synteny with the AF cluster (McDonald et al., 2005).

Dothistromin biosynthetic genes orthologous to AF/ST genes were originally identified from *D. septosporum* by hybridisation of gene libraries with AF/ST gene probes (Bradshaw et al., 2002, 2006; Zhang et al., 2007). Although AF/ST and dothistromin biosynthesis pathways are predicted to bear a considerable degree of similarity (Shaw et al., 1978), there are fundamental differences in the genetics and regulation of dothistromin biosynthesis. Unlike AF/ST genes, dothistromin genes are

not tightly clustered, but arranged in several loci on a 1.3 Mb chromosome. Before the *D. septosporum* genome sequence was available considerable effort was made in vain to find an entire dothistromin gene cluster (Bradshaw et al., 2006; Schwelm and Bradshaw, 2010). Prior to the release of the *D. septosporum* genome sequence by JGI in 2010 (http: //genome.jgi.doe. gov/Dotse1/Dotse1.home.html), only ten putative dothistromin biosynthesis genes had been identified. The 30.2 Mb genome was sequenced at 34 fold coverage and assembled to chromosome level (De Wit et al., 2012). The availability of genome at the start of this project greatly accelerated the work by helping to find the remaining dothistromin biosynthetic and regulatory gene candidates and revealed the extent to which these genes were widely dispersed in one chromosome (De Wit et al., 2012).

Another distinct feature of dothistromin biosynthesis is that it is produced mainly during early exponential phase in culture, instead of during late exponential and stationary phases as is normally seen for secondary metabolites such as AF/ST (Schwelm et al., 2008; Cleveland & Bhatnagar, 1990; Payne & Brown, 1998). Due to the dispersed arrangement of dothistromin genes and unusual timing of biosynthesis, it was interesting to see if regulatory mechanisms known to control AF/ST biosynthesis in *Aspergillus* spp. are conserved in *D. septosporum.*

The role of regulatory proteins AflR and AflJ in AF/ST biosynthesis is well established in *Aspergillus* spp. (Ehrlich and Montalbano, 1995; Ehrlich et al., 1999) AflR binding sites were predicted upstream of dothistromin genes (Bradshaw et al., 2002, 2006), leading to the question of whether an AflR-like protein is involved in the regulation of dothistromin. In addition to this pathway specific regulator, the global regulatory VeA/LaeA complex and chromatin modification is known to regulate subtelomerically-located AF/ST biosynthesis gene clusters (Bok and Keller, 2004;

Keller et al., 2005; Bayram et al., 2010; Bayram and Braus, 2012; Amare and Keller, 2014). The dispersed nature of the dothistromin genes led to questions of whether they are also regulated by global regulators and chromatin modification.

The next sections of this chapter will provide an overview of topics relevant to this project including Dothistroma needle blight (DNB) disease and the causal organism, secondary metabolism in fungi with emphasis on polyketides, and secondary metabolite regulation. Further information about the regulatory proteins AflR, AflJ, VeA and LaeA is in chapters 3-6 in which the corresponding genes have been studied. Note that gene naming has been done according to the usual convention for the fungal species and the class to which it belongs, for example *aflR* for *Aspergillus nidulans*, a Eurotiomycete, and *AflR* for *Dothistroma septosporum,* a Dothideomycete.

1.2. Dothistroma needle blight

Dothistroma needle blight, which is also known as red band disease, is one of the devastating diseases of *Pinus* species. Historically it was a major problem in the southern part of the world including Brazil, Kenya, Zimbabwe, New Zealand, and South Africa where the host and pathogen were both introduced, causing extensive damage (Ivory 1967, Gibson 1972, Bradshaw, 2004). Now dothistroma needle blight is considered one of the most important diseases of pines globally (Barnes et al., 2004) and global climate change has been suggested as a contributing factor to this disease severity (Wood et al., 2005).

1.2.1 Causal organism and the disease.

Two species are known as causal organisms of DNB: *D. septosporum* (Dorog.) Morelet and *Dothistroma pini* Hulbary (Barnes et al., 2004) that belong to the class

Dothideomycetes and order capnodiales. The first DNA-based evidence for these *Dothistroma species* in different countries was provided by Barnes et al (2004). The study revealed that *D. septosporum* was distributed worldwide while *D. pini* was confined only to the North Central U.S.A. though it was later reported from the Ukraine (Groenewald et al., 2007) and France (Fabre et al., 2012). The teleomorphic form of *D. septosporum* was first described as *Scirrhia pini* (Funk and Parker, 1966) but later renamed as *Mycosphaerella pini* E. Rostrup and Monk. The teleomorph has been reported in Canada, parts of the USA, Germany, Yugoslavia, Poland, and Portugal (Reviewed by Bradshaw, 2004). In parts of the Southern Hemisphere where *D. septosporum* is exotic, only the anamorph has been reported (Barnes et al., 2004; Bradshaw, 2004; Evans, 1984). There has been no report of a sexual stage for *D. pini*.

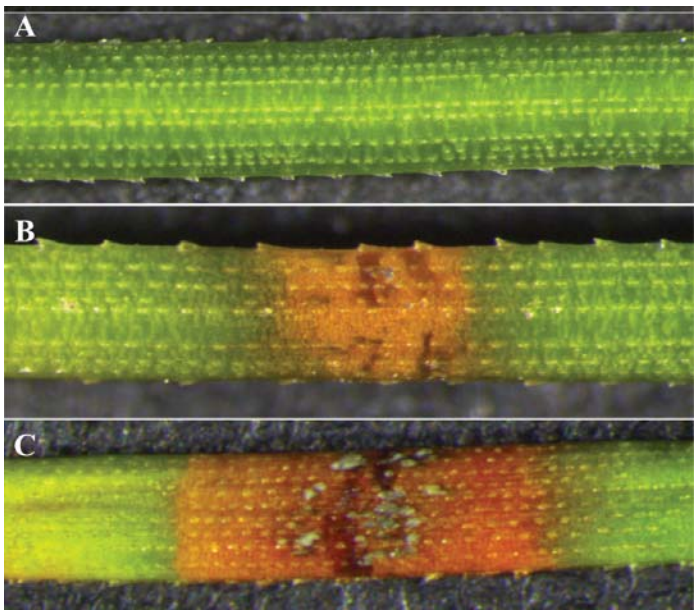


Figure 1.2: Dothistroma needle blight symptoms (Courtesy Shahjahan Kabir)

A) Uninfected needle B) Infected needle showing characteristic red band due to mycotoxin dothistromin. C) Infected needle at the late stage showing black fruiting bodies

Dothistroma needle blight symptoms are characterised by brick red bands (1-3 mm wide) that appear some weeks after infection and persist long after the green

needles have withered (Figure 1.2). The red colour is due to production of dothistromin by the fungus (Bassett et al., 1970). Small black fruiting bodies erupt in the red infected band at the later stage. The area beyond the red band dies, sometimes causing premature defoliation of needles (Edwards and Walker, 1978; Kershaw et al., 1988). Depending on host susceptibility and disease severity, the degree of needle death may be extremely high, as needles of all ages can be affected (Brown and Webber, 2008).

1.2.2 *Dothistroma septosporum* life cycle *in planta*

Recently Kabir et al (2014) studied the *D. septosporum* life cycle *in planta* using microscopic, histological and molecular tools in artificially inoculated *P. radiata* seedlings (Figure 1.3). The life cycle was divided into four stages: 1) Epiphytic: spore germination and surface growth 2) Penetration 3) Mesophyll colonisation and early lesion development 4) Late lesion development and fruiting body maturation.

In this study, it was demonstrated that *D. septosporum* completes its life cycle as a hemibiotroph fungus. In stage 1 spores germinated with single or multiple germ tubes and an extensive network of hyphae grew over the needle during the 2-4 week post inoculation period. However, no evidence of directional growth toward stomatal pores was shown nor any visible needle blight symptoms. Penetration into stomatal pores was seen during stage 2 which started 2 weeks post inoculation, then fungal hyphae grew in the epistomatal chambers. No direct penetration of the epidermis was seen. *D. septosporum* stages 1-2 were depicted as the latent or biotrophic phase with no host cell wall disintegration and minimal fungal biomass and toxin accumulation (Kabir et al., 2014).

In stage 3 which started from weeks 3-5 post inoculation, hyphae were reported in the intercellular spaces of mesophyll cells but no intracellular colonisation occurred.

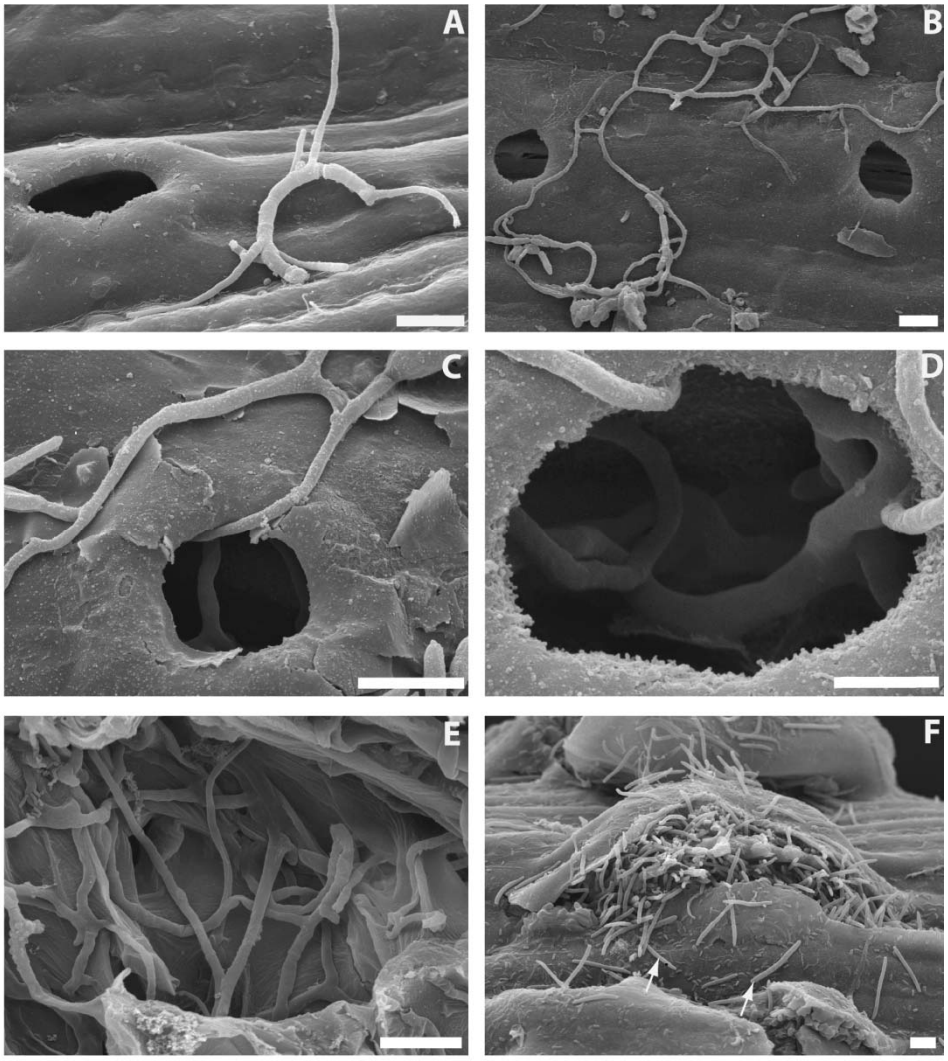


Figure 1.3: *Dothistroma septosporum* infection stages on radiata pine (from Kabir et al., 2014)

A) Stage 1: spore germination, B) epiphytic growth on needle surface; C) Stage 2: penetration of hyphae into stomata; D) fungal hyphae growing inside epistomatal chamber; E) Stage 3: mesophyll colonisation; Stage 4: F) fruiting body bursting with asexual spores.

Early water soaked necrotic lesion less than 1 mm in size that extended to girdle the whole needle were reported. In stage, 4 which started from 6-12 weeks post inoculation, spores were released from the fruiting bodies. The necrotrophic phase started when the early lesion appeared and mesophyll cells started to disintegrate (stage 3) followed by a rapid increase in fungal biomass and toxin production (stage 4) (Kabir et al., 2014).

1.3. Secondary metabolites

Secondary metabolites are complex organic compounds, which do not have a role in the normal growth and development of an organism but can play important roles in defense, communication, niche adaptation and development (Kamei and Watanabe, 2005; Keller et al., 2005; Calvo et al., 2004). Fungi produce a structurally diverse range of secondary metabolites, some of which have been exploited because of their potential usefulness to human as antimicrobials, enzyme inhibitors, antitumour, immunosuppressive, antiparasitic and anticholesterol agents (Demain, 1999; Higginbotham et al., 2014). Many secondary metabolites are found only in a specific organism or group of organisms and are not necessarily produced under all conditions (Dewick, 2002; Ochi and Hosaka, 2013).

In spite of being complex and diverse, most secondary metabolites are synthesized from one or a combination of biosynthetic pathways with precursors derived from a small set of primary metabolism intermediates such as acetyl coenzyme A (acetyl-CoA), shikimic acid, mevalonic acid and 1-deoxyxylulose 5-phosphate (Dewick, 2002). Secondary metabolites are classified according to the enzyme classes involved in their biosynthesis and include polyketides, non-ribosomal peptides, terpenes and indole alkaloids (Keller et al., 2005). In fungi, genes involved in the biosynthesis of these metabolites are usually clustered along with genes for various additional tailoring enzymes, transporters and regulatory proteins.

1.3.1 Non-ribosomal peptides

Non ribosomal peptides are a large class of natural products. They are derived from both proteinogenic and non-proteinogenic amino acids (D amino acids) by large multifunctional modular enzymes called nonribosomal peptide synthetases (NRPSs) and are synthesized independently of ribosomes (Stein et al., 1996). NRPSs are organized

into modules. The minimal NRPS module consists of an adenylation (A) domain which activates the amino acid building block, a thiolation or peptidyl-carrier protein (T or

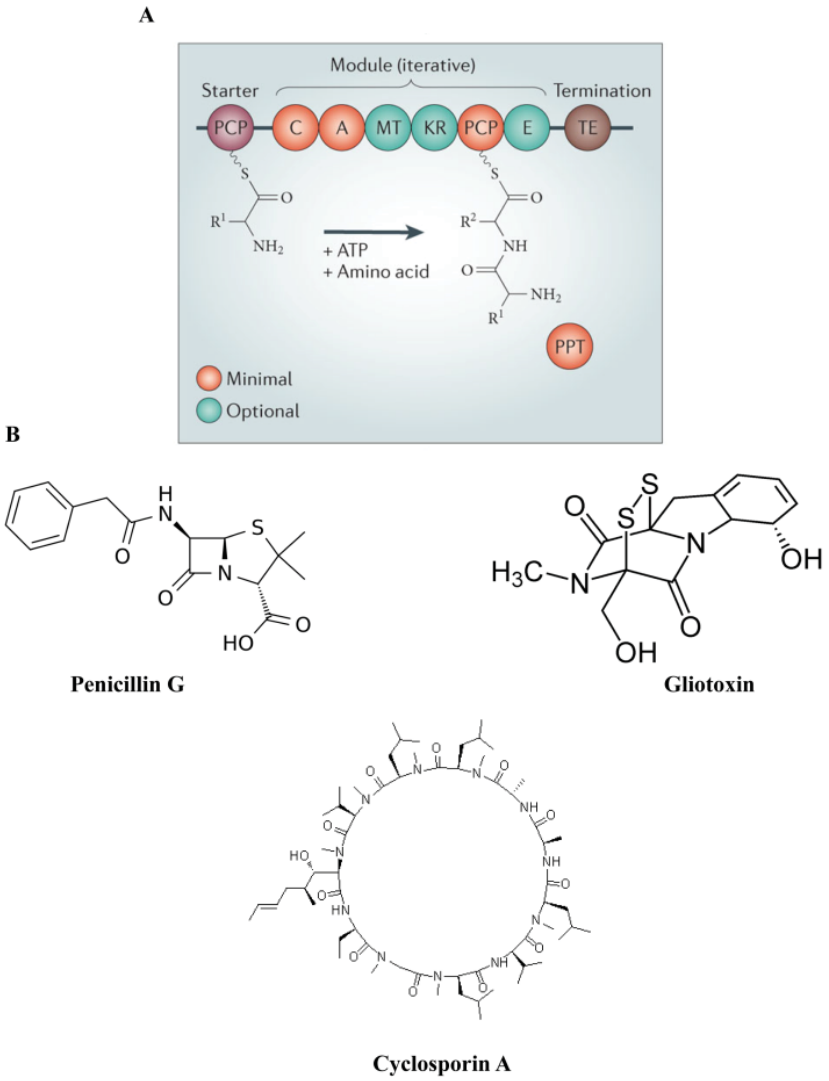


Figure 1.4: Non- ribosomal peptide synthetase (NRPS) domain organisation and some NRPS derived secondary metabolites (Adapted from Brakhage, 2013).

A) Domains of NRPS enzymes with the minimal module shown in red. It consists of domains for adenylation (A), thiolation or peptidyl-carrier protein (T or PCP), condensation (C), epimerization (E), methyltransferase (MT) and β-ketoacyl reductase (KR).

B) Examples of fungal NRPS derived secondary metabolites: antibiotic penicillin G and immunosuppressant drug Cyclosporin A are derived from *Penicillium chrysogenum and Tolypocladium inflatum* respectively. Gliotoxin is a mycotoxin produced by *Aspergillus fumigatus* and some *Trichoderma* spp.

PCP) domain which transports substrates between the active sites of the domains, and a condensation domain (C) which catalyzes the formation of a peptide bond (C-N) between amino acids to form an elongated chain. (Figure 1.4) (Gallo et al., 2013; Evans et al., 2011; Dewick, 2009). Other than the minimal core module NRPS enzymes can also contain other domains like oxidation (Ox), epimerization (E), β-ketoacyl reductase (KR), methyltransferase (MT) and cyclization (Cy) domains (Strieker et al., 2010; Evans et al., 2011; Hoffmeister and Keller, 2007, Lambalot et al., 1996; Stachelhaus et al., 1998). Penicillin, cephalosporin, and gliotoxin are common examples of NRPS metabolites (Keller et al., 2005).

1.3.2 Terpenoids

Terpenoids, commonly known as terpenes, are a diverse group of natural compounds produced by microorganisms, plants, insects and animals. Terpenes are synthesised from the monomer isoprene, which has the molecular formula C_5H_8, and the synthesis is catalysed by terpene synthases. The empirical formulae of terpenes are multiples of isoprene, $(C_5H_8)n$ where n is the number of linked isoprene units. Dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) are the two building blocks of terpenoids (Figure 1.5) and are derived from acetyl-CoA via the intermediates of mevalonic acid, or pyruvate and glyceraldehyde 3-phosphate in a non-mevalonate pathway (Eisenreich et al., 2004). Examples of terpenes produced by fungi are carotenoids, which have free-radical scavenging properties and are known to enhance the immune system (Johnson, 2002), gibberellins that are phytohormones that can alter transcriptional activity (Bomke and Tudzynski, 2009) and trichothecenes that cause health problems in humans and animals induced by their protein inhibiting activity (Alexander et al., 2009).

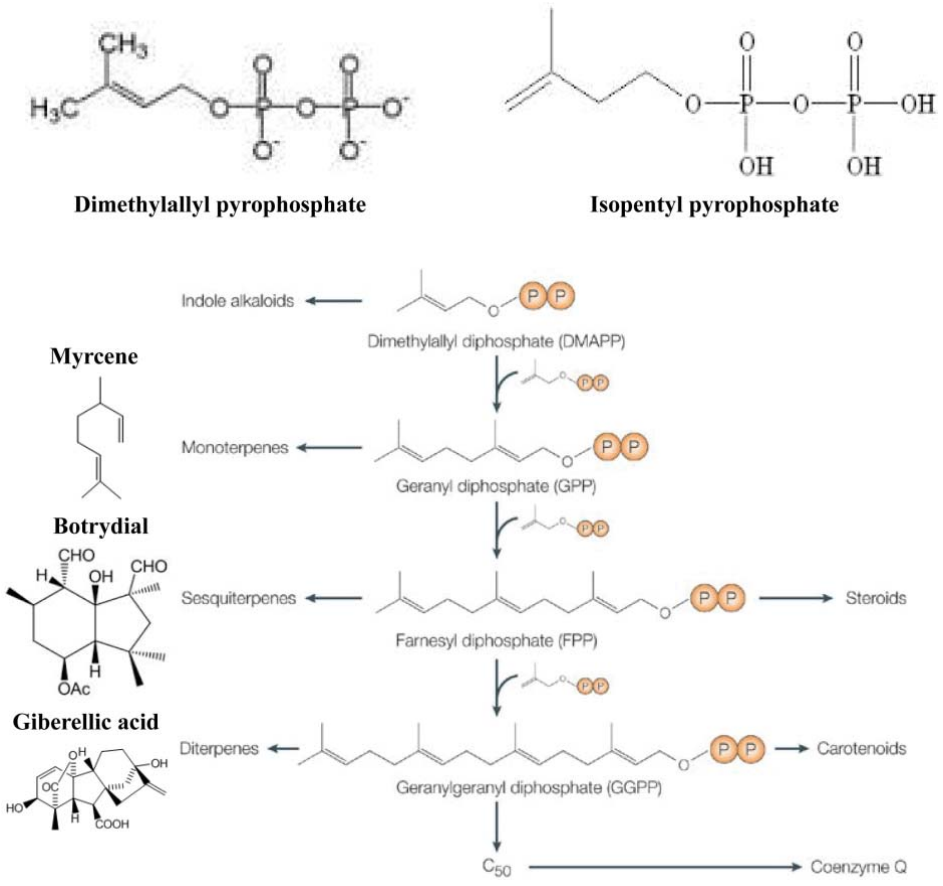


Figure 1.5: Terpene biosynthetic pathway (Adapted from Keller et al., 2005)

Isopentyl pyrophosphate and dimethylallyl pyrophosphate are building blocks of various class of terpenes. From these, isoprenyl diphosphate synthases catalyse production of geranylpyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). The terpenes are produced by cyclization of the isoprenoid unit. Monoterpenes are derived from geranyl diphosphate, sesquiterpenes from farnesy diphosphate and diterpenes from geranylgeranyl diphosphate

.

1.3.3. Alkaloids

Indole alkaloids are derived from the amino acid tryptophan or from dimethylallyl pyrophosphate. The best known are ergot alkaloids derived from *Claviceps spp.* Some other common examples include caffeine, nicotine, cocaine and morphine. These alkaloids are known to have effects on the central nervous system and have analgesic and hallucinogenic effects (Dewick, 2009). The fungal derived alkaloids that have been studied include fumitremorgin produced by *Aspergillus fumigatus* that are associated

with tremors, seizures and abnormal behavior in animals (Maiya et al., 2006 Dhingra et al., 2013). The ergot alkaloids festuclavine and fumigaclavines A, B and C are present in or on conidia of *A. fumigatus* and can cause colitis and liver damage in mammals (Coyle et al., 2010).

1.3.4 Polyketides

Polyketides are the most abundant and structurally diverse class of fungal secondary metabolites, and are synthesized from the acetyl-CoA, malonyl-CoA and methylmalonyl-CoA derived from the primary metabolite pool (Keller et al., 2005; Dewick, 2009). Fungal polyketides are synthesized enzymatically by polyketide synthase (PKS) enzymes, which are closely related to fatty acid synthetases (FAS) (Hutchinson, 2003). Polyketide synthases can be categorized into three different types (I, II and III), which have the same enzymatic functions, but differ in several respects, including chain initiation and termination (Gallo et al., 2013).

Type I PKSs are enzymes with multiple domains acting only one time (modular) or used repeatedly (iterative) during the biosynthesis. Type II PKSs are aggregates of monofunctional proteins that act iteratively, whilst type III PKSs are homodimeric enzymes that also act iteratively. Type III PKSs do not include an acyl-carrier protein (ACP) domain and are typically made by plants (Shen, 2003). Most of the fungal PKSs characterised so far are type I and iterative. In fungal PKSs, the ketoacyl CoA synthase (KS), acyltransferase (AT) and acyl carrier (ACP) domains are essential for polyketide synthesis, whereas the ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains are optional (Khosla et al., 1999; Chan et al., 2009). Thus, the enormous diversity of polyketides is partly explained by the optional use of those three domains (Turgeon and Bushley, 2010). Further, the diversity is achieved by additions or modification of various functional groups after the polyketide backbone is synthesised.

This is achieved by other enzymes whose genes that are usually clustered with PKSs, such as monoxygenases, reductases, oxidases and dehydrogenases. Examples of polyketides are shown in Figure 1.6.

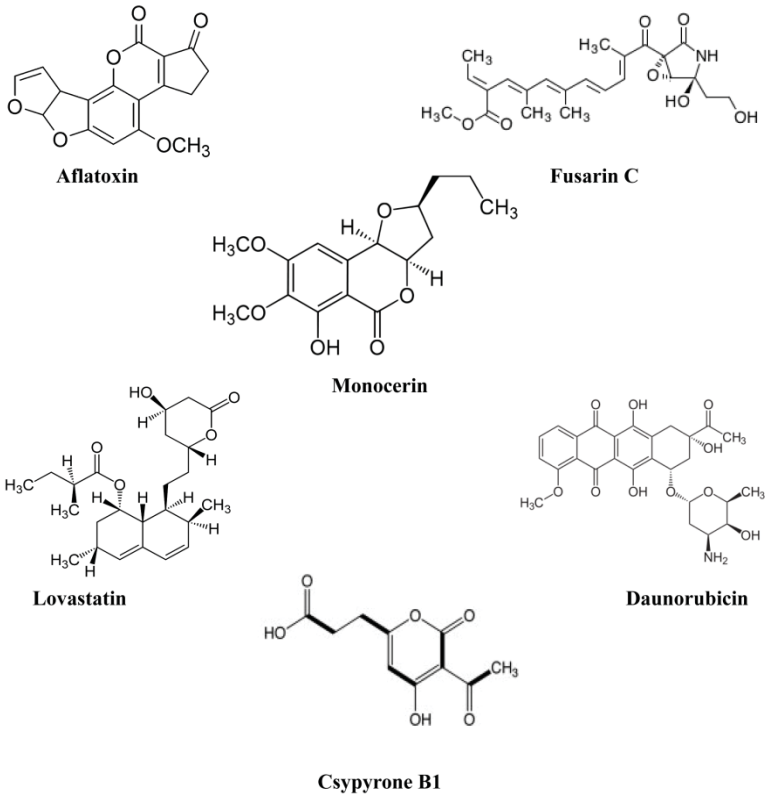


Figure 1.6: Examples of polyketide derived secondary metabolites

Type I polyketide includes toxic secondary metabolite aflatoxin produced by *Aspergillus flavus* (Wannop et al., 1961) fusarin c by *Fusarium spp.* (Sondergaard et al., 2011), clinically important lovastatin from *Aspergillus terreus* (Hajjaj et al., 2001) and antifungal and insecticide compound monocerin produced by *Exserohilum turcicum* (Cuq et al., 1993). Chemotherapeutic drug daunorubicin isolated from *Streptomyces peucetius* (Creutzig et al., 2013) belongs to type II. Csypyrone B1 is a novel product of *Aspergillus oryzae* type III polyketide synthase CsyB. Figure from Seshime et al., 2010.

1.3.5 Secondary metabolite gene clusters

In prokaryotes, clustering of co-regulated genes is common, with those genes arranged as operons and expressed as a polycistronic mRNA, thus facilitating co–ordinate gene expression (Rocha, 2008; Koonin, 2009). Clustering of genes with related functions is

also seen in eukaryotes such as mammals, worms and plants (Semon et al., 2006) although each gene has its own promoter unlike the prokaryotic system. Similarly, in filamentous fungi, genes involved in certain metabolic processes or functions have been found to be clustered (Figure 1.7) and most often subtelomerically located (Perrin et al., 2007; Palmer and Keller, 2010, Keller and Hohn, 1997; Hoffmeister and Keller, 2007). The products of many of these gene cluster pathways are of major interest by virtue of their toxic properties and/or pharmaceutical applications. The availability of genome sequences for a growing number of fungi, advances in bioinformatics and recombinant DNA tools led to rapid progress in identifying the genes that are putatively responsible for secondary metabolite production and regulation, as well as in discovery of new secondary metabolite clusters. The analysis of fungal genomes has revealed many more gene clusters than were originally thought to exist (Brakhage, 2013). Some examples of unclustered secondary metabolites genes are those involved in T-toxin biosynthesis in *Cochliobolus heterostrophus* (Kodama et al., 1999), trichothecene biosynthesis in *Fusarium* spp. (Kimura et al., 2007), aflatrem biosynthesis genes in *Aspergillus flavus* (Nicholson et al., 2009). The additional clusters suggested most biosynthesis gene clusters are silent under standard laboratory culture conditions, posing questions on regulation and roles.

Another important question is why secondary metabolite genes are clustered at all? One reason postulated to keep genes clustered together on a chromosome is that gene clustering could facilitate horizontal gene transfer and prolong the existence of secondary metabolite traits in nature (Khaldi and Wolfe, 2011). Another reason is that clustering can facilitate co-regulation of the genes, particularly those located in the sub telomeric regions (Palmer and Keller, 2010). Location of gene clusters in such a position can provide another tier of regulation by telomeric position effect. Telomeric

position effect (TPE) is a phenomenon commonly present in eukaryotic organisms and involves gene repression in areas adjacent to telomeres through heterochromatic silencing (Barrales et al., 2008) and this extends at least 30 kb from the telomere in *A. nidulans* (Palmer et al., 2010). Regulation of AF genes has been shown to be affected by their position in the cluster. For example location of *nor1* and *ver1* outside the AF cluster showed reduced expression compared to their normal cluster location (Chiou et al., 2002; Liang et al., 1997) while moving the ST pathway specific regulatory gene *aflR* in and out of the cluster in *A. nidulans* was correlated with gain or loss of its

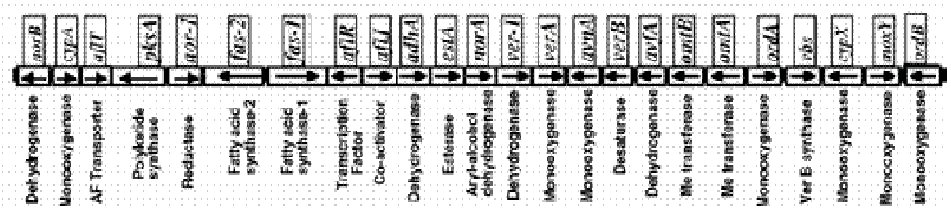


Figure 1.7: Aflatoxin gene cluster in *Aspergillus flavus* (adapted from Yu et al., 2002)

Figure shows the aflatoxin biosynthetic pathway gene cluster in *A. flavus*. The corresponding genes and their enzymes are shown. The gene names are labeled on the side of the cluster.

regulation activity mediated by the global regulator LaeA (Bok et al., 2006). However, a recent study demonstrates that chromatin mediated regulation is not restricted to telomeric location (Gacek and Strauss, 2012).

1.3.6 Dothistromin genes and genetics

Prior to the release of the genome, identification of dothistromin genes was facilitated by knowledge of AF/ST biosynthesis genes in *Aspergillus* spp. Using hybridization probes that were designed based on AF/ST genes, putative dothistromin genes were identified in four groups (Table 1.1) that appeared to be physically separate as opposed to clustered (Bradshaw et al., 2006; Zhang et al., 2007). The *PksA, VbsA* and *Ver1*

(previously called *DotA*) genes of *D. septosporum* were experimentally validated to be involved in the biosynthesis of dothistromin (Bradshaw et al., 2002; Bradshaw et al., 2006; Zhang et al., 2007). Four other genes *HexA, CypA, MoxA* and *AvfA* were orthologs of well characterized AF/ST biosynthesis genes but functions of remaining genes were not defined.

Table 1.1: Predicted dothistromin biosynthesis genes identified prior to genome sequencing.

Gene	Putative Gene Function	ST Gene Ortholog	Identity aa (%)	AF Gene Ortholog	Identity aa (%)
Ver1 (DotA)	Versicolorin reductase	*stcU*	79.1	*aflM*	80.2
DotB	Oxidase	*stcC*	24.0	-	-
DotC	MFS transporter	-	-	*aflT*	31.2
DotD	Thioesterase	*stcA*	37.9	*aflC*	34.8
DksA	Polyketide synthase	*stcA*	57.0	*aflC*	54.8
CypA	Oxidase	*stcB*	59.8	*aflV*	59.3
AvfA	Averufin monooxygenase	*stcO*	43.7	*aflI*	47.8
EpoA	Epoxide hydrolase	-	-	-	-
MoxA	Monooxygenase	*stcW*	59.0	*aflW*	55.1
DS31	Translation elongation factor	*stcT*	41.1		
HexA	Fatty acid synthase (partial)	*stcJ*	41.3	*aflA*	48.8
HypC	Anthrone oxidase	*stcM*	47.9	*aflC*	35.2
VbsA	Versicolorin B synthase	*stcN*	69.1	*aflK*	72.0
NorB	Norsolorinic acid reductase	*stcV*	43.4	*aflF*	60.7

The predicted gene products, with amino acid identities to *A. nidulans* (ST) and *A. parasiticus* (AF) gene products and their putative functions are shown. Shading indicates the grouping of these genes into four loci; linkage of these groups was not established prior to availability of the genome sequence.

1.4. Regulation of fungal secondary metabolism

Fungal secondary metabolite gene cluster regulation occurs at several levels. Some transcriptional regulatory elements are specific for the respective pathway and called pathway specific regulators (eg. AflR, GliZ, SirZ) (Chang et al., 1995, Bok et al., 2006; Fox et al., 2008) whilst others are global in nature in that they affect expression of many genes and/or pathways (eg. VeA, LaeA, MeaB,) (Brakhage, 2013; Yin & Keller, 2011).

1.4.1 Pathway specific regulatory elements

The Class III $Zn(II)_2Cys_6$ family is the largest group of transcription factors that are found predominantly in fungi (MacPherson et al., 2006; Chung et al., 2013). One of the well characterised type III proteins is AflR in *Aspergillus* spp. that is a sequence-specific DNA-binding binuclear zinc cluster ($Zn(II)_2Cys_6$) protein. AflR is required for AF and ST activation by binding to a palindromic sequence, $TCGN_5CGR$, found in the promoters of target genes (Ehrlich et al., 1999) and is discussed in more detail in chapter 3.

Other examples of pathway-specific regulators include GliZ and SirZ. GliZ is involved in gliotoxin biosynthesis cluster regulation. Gliotoxin is a well-studied nonribosomal peptide toxin (Gardiner and Howlett, 2005), produced by the opportunistic human pathogen *A. fumigatus,* and is considered a virulence factor (Fox and Howlett, 2008). Gliotoxin genes are arranged in a cluster consisting of 13 genes (Cramer et al., 2006; Kupfahl et al., 2006) that are regulated by the Zn-finger transcription factor GliZ. The deletion of this gene results in the loss of gliotoxin production, whereas overexpression increases the gliotoxin level (Bok et al., 2006). SirZ is present in the epipolythiodioxopiperazine (ETP) sirodesmin cluster in *Leptosphaeria maculans* and is required for sirodesmin gene expression (Fox et al., 2008).

AlcR from *A. nidulans* is another important regulator, which has a role in ethanol catabolism (Panozzo et al., 1997**).** AlcR controls expression of the *alcA* and *aldA* genes (Kulmburg et al., 1992), which encode an alcohol dehydrogenase and an aldehyde dehydrogenase, respectively. *AlcR* deletion mutants were unable to grow on ethanol. Some class II Cys2His2 zinc finger proteins are also important in secondary metabolite regulation in fungi. For example Tri6 for trichothecene biosynthesis pathway

regulation in *Fusarium spp.* (Hohn et al., 1999) ScpR and AfoA for asperfuranone synthesis in *A. nidulans* (Bergmann et al., 2010).

1.4.2 Gene regulation by cross talk between gene clusters

In some cases there is regulatory cross talk between gene clusters (Figure 1.8; Brakhage, 2013). This discovery was made when screening secondary metabolite genes of the model fungus *A. nidulans*. A silent biosynthesis pathway with two non ribosomal peptide synthetase (NRPS) genes, *inpA* and *inpB* were found along with a regulatory gene named *scpR* on chromosome II.

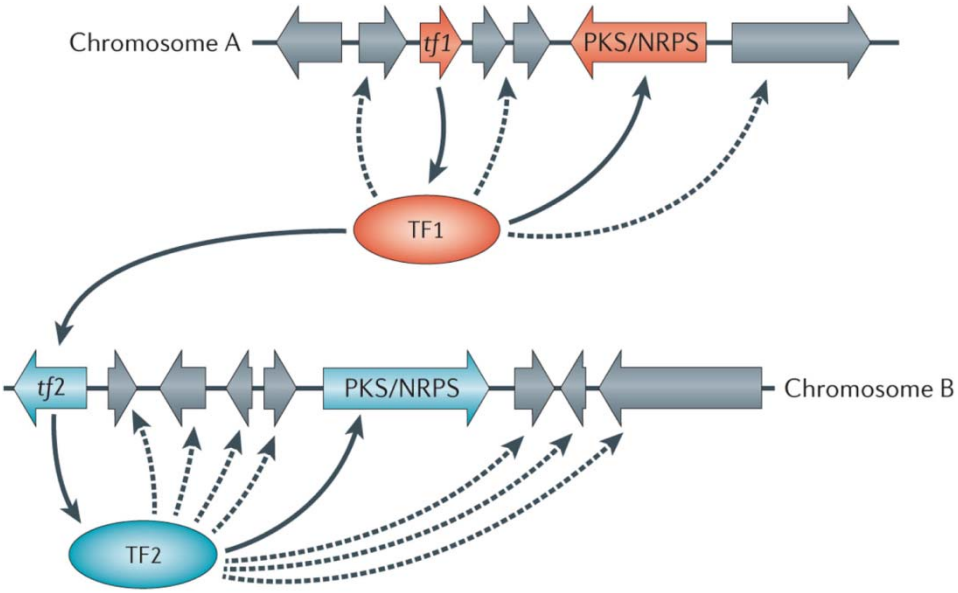


Figure 1.8: Pathway specific regulators and their cross talk between clusters (Adapted from Brakhage, 2013)

Fungal secondary metabolite gene clusters shown that contain a backbone gene (which may be PKS, NRPS or hybrid), genes encoding decorating enzymes, and a regulatory gene (*tf*) encoding a pathway regulating transcription factor (TF1 or TF2) which regulates the cluster genes. Cross talk between clusters is known to exist where the pathway regulator of one cluster (TF1) activates the transcription factor encoding gene (*tf2*) of another cluster.

Surprisingly, regulated expression of *scpR* using an inducible (*alcA*) promoter induced not only *inp* pathway genes but also genes for the polyketide asperfuranone located on a

different chromosome (chromosome VIII) (Bergmann et al., 2010). More recently it was shown in *Penicillium roqueforti* that silencing of four structural genes (*prx1* to *prx4*) involved in biosynthesis of PR-toxin (a bicyclic sesquiterpene) resulted in a reduction of 65–75% in the production of PR-toxin but also a dramatic increase in mycophenolic acid, an antitumor compound formed by an unrelated pathway, suggesting regulatory cross talk between pathways (Hidalgo et al., 2014). Other examples of cross talk between gene clusters include fumiquinazoline and fumigaclavine C biosynthesis hexadehydroastechrome (HAS) pathways, and an NRPS pathway producing anticancer drug fumitremorgins in *A. fumigatus* (O' Hanlon, et al., 2012; Yin et al., 2013). Recently FtmG, a cytochrome P450 from the fumitremorgin biosynthetic pathway, was determined to catalyze the spiro-ring formation in spirotryprostatin (Tsunematsu et al., 2013). However, the molecular mechanisms behind such cross talk are yet to be identified.

1.4.3 Global regulatory proteins

Other than pathway specific regulators, fungal secondary metabolite biosynthesis is responsive to environmental cues including carbon, nitrogen source, ambient temperature, light, and pH. This is mediated through broad domain transcription factors called global regulators, for example carbon by CreA (Dowzer and Kelly, 1989), nitrogen by AreA (Hynes, 1975; Wong et al., 2007), pH by PacC (Tilburn et al., 1995) and light by the velvet complex (Calvo et al., 2004) and has been well studied well in the model systems *A. nidulans* and *Neurospora crassa.*

1.4.3.1 Regulation of secondary metabolite biosynthesis by nitrogen source

Fungi preferentially utilise simple nitrogen sources (ammonium and glutamine) over other sources, such as nitrate (Arst & Cove, 1973; Kudla et al., 1990). Selective utilization is achieved through a global regulatory circuit known as either nitrogen

metabolite repression or nitrogen catabolite repression and is mediated by AreA, a transcription factor belonging to the GATA family. The level of AreA is controlled depending on the available nitrogen source by a positive autoregulation loop at the transcription level (Langdon et al., 1995) or at the protein level by the corepressor NmrA whose activity in turn is regulated by a B zip transcription factor called MeaB (Wong et al., 2007). AreA also positively regulates secondary metabolite clusters (Lee et al., 2012; Min et al., 2012). Nitrate as nitrogen source has been shown to reduce AF production and decrease expression of AF biosynthesis genes in *A. parasiticus* compared to ammonium source (Price et al., 2005). However, in *A. nidulans* an opposite effect is seen: nitrate induces ST while ammonia causes a reduction (Feng and Leonard, 1998) suggesting a different regulatory mechanism.

1.4.3.2 Regulation of secondary metabolism by carbon source

Fungi adjust their carbon catabolism to preferentially utilise simple sugars using a carbon catabolite repression regulatory mechanism mediated by a C_2H_2 transcription factor CreA (Dowzer and Kelly, 1989). CreA acts by binding to the consensus DNA sequence 5′-SYGGRG-3′ in promoters of glucose-repressible genes, thus switching off their expression when easily assimilable carbon source is available (Flipphi et al., 2003). Negative regulation of secondary metabolism by glucose has been demonstrated in penicillin biosynthesis in *A. nidulans* (Brakhage et al., 2004), cephalosporin in *Acremonium chrysogenum* (Espeso et al., 1994) and lovastatin in *Monascus sp.* and *Aspergillus terreus* (Lai et al., 2007; Hajjaj et al., 2001). However, the role of carbon source on AF biosynthesis is still unclear. Unlike many secondary metabolite pathways, AF production is induced by simple sugars such as glucose (Wiseman and Buchanan, 1987). Further, complex sugars caused less repression of AF genes than other factors like pH and nitrogen in *A. parasiticus* (Price et al., 2005) and this was confirmed for AF

production in *A. flavus* (Wilkinson et al., 2007) where there was lack of strong downregulation of AF genes in the absence of sucrose.

1.4.3.3 pH mediated regulation of secondary metabolite pathways in fungi

To cope with variations in pH in their environment, fungi require an adaptive regulatory system. Predominant among fungi is a pH-responsive genetic regulatory system that involves activation of PacC, a global regulatory protein (Caddick et al., 1986). The production of many secondary metabolites involves PacC regulation, as changes in pH cause differential expression of a variety of biosynthetic clusters (Keller et al., 1997). In acidic conditions a "closed confirmation" of PacC predominates in *A. nidulans* (72-kD PacC72) but in alkaline to neutral pH it undergoes two rounds of proteolytic cleavage (Diez et al., 2002) yielding the PacC27 (27-kD) form, which is a repressor of acid-expressed genes and an activator of alkaline-expressed genes. *Fusarium graminearum* and *Fusarium verticillioides* acidification is a determinant of FUM & TRI gene expression and toxin production (Merhej et al., 2010; Flaherty et al., 2003). In another study, with *Trichoderma virens,* 5% of the transcriptome was pH-dependent, of which 25% depended on PacC; genes required for secondary metabolism and ion transport were a dominant class among the PacC pH-regulated group (Trushina et al., 2013). For the biosynthesis of AF/ST, acidic conditions are more favourable than alkaline conditions. In *A. nidulans* the transcripts for ST biosynthesis genes were down regulated in alkaline pH (Keller et al., 1997). Similarly Price et al (2005) reported the downregulation of AF genes in *A. parasiticus* under alkaline conditions.

1.4.3.4 bZIP transcription factors

The Basic Leucine Zipper Domain (bZIP domain) is found in many eukaryotic transcription factors. In *A. nidulans* the bZIP transcription factor MeaB has been shown

to regulate expression of nitrogen regulated genes (Caddick and Arst, 1998) by binding to a conserved sequence in the NmrA promoter (Wong et al., 2007).

In *Fusarium fujikuroi MeaB* deletion mutants showed upregulation of gibberellic acid and bikaverin biosynthesis (Wagner et al., 2010) whereas in *MeaB* mutants of *A. flavus* AF production was reduced (Amaike et al., 2013). Another bZIP transcription factor termed RsmA (restorer of secondary metabolism A) was reported by (Shaaban et al., 2010). In *A. nidulans,* expression of *RsmA* partially restored ST production in mutants of the global regulators LaeA and VeA that were impaired in the ability to make ST. In *Magnaporthe oryzae* deletion of bZIP transcription factor MoAP1 lead to reduction in expression of several secondary metabolite genes (Guo et al., 2011).

1.4.3.5 Light mediated regulation of secondary metabolite pathways

Neurospora crassa has been used as a model system to study light responses and involves a very complex network mechanism interconnected with circadian rhythms (Brunner and Kaldi, 2008; Dunlap and Loros, 2004; Dunlap et al., 2007; Bahn et al., 2007). The major regulatory proteins involved in light signalling in this fungus are the photoreceptors WHITE COLLAR-1 (WC-1) and WHITE COLLAR-2 (WC-2) which act together as a transcription factor complex (white collar complex, WCC) (Figure 1.9). The light signal is received and transmitted by altered conformation and interactions. A photoreceptor domain (PAS) of WC-1 contains a flavin binding motif and a blue light dependent adduct is formed (Crosson and Moffat, 2002; Crosson et al., 2003, Malzahn et al., 2010). This results in the activation of the HAT (histone acetyltransferase) activity of chromatin-modifying coactivator NGF-1 (a homologue of the yeast Gcn5p acetyltransferase). This leads to acetylation of H3K14 in the promoters of light inducible genes (Brenna et al., 2012; Grimaldi et al., 2006). After prolonged exposure to blue light, a small photoreceptor (VVD) accumulates that disrupts the

interaction between WCCs, leading to a reduction in light-dependent transcription. WC-1 is phosphorylated, leading to exclusion of the WC complex from the promoter and switching off the gene (He and Liu, 2005; Schwerdtfeger and Linden, 2000).

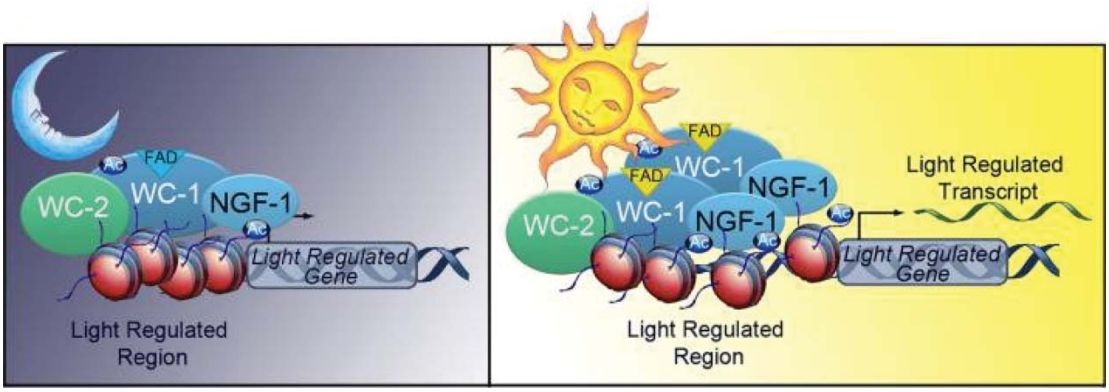


Figure 1.9: Figure showing the model proposed to explain the photoregulatory mechanism in *Neurospora crassa* (Adapted from Brenna et al., 2012)

Chromatin-modifying coactivator (NGF1) interacts with C-terminal region of WC-1 independent of light. This complex is preassembled in the promoter region of light inducible genes such that the chromatin is inaccessible to the histone acetyltransferase activitity of NGF-1. On light exposure, WCC undergoes confirmation changes, leading to acetylation of H3K14 present in the promoter of light inducible genes thus enabling them to be switched on.

1.4.3.6 Role of Velvet (VeA) as a global regulator in light perception and secondary metabolism.

Velvet is another protein involved in light perception and is present in many ascomycete fungi. Extensive work has been done in characterisation of this protein and is discussed in more detail in Chapter 5. In *A. nidulans* VeA has a light dependent role. In the absence of light, VeA accumulated in the nucleus, and subsequent exposure to light lead to decreased levels in the nucleus (Stinnett, 2007). (Figure 1.10).

In *A. nidulans* recent work has shown the existence of an equivalent photoregulatory system to that seen in *N. crassa*. This includes a white-collar complex (WCC) with LreA (WC-1), LreB (WC-2) (Purschwitz et al., 2008) and a

photolyase/cryptochrome CryA (Bayram et al., 2008b; Purschwitz et al., 2008). FphA is a phytochrome involved in red light perception, which represses fruiting body formation and induces asexual spore formation (Blumenstein et al., 2005) and interacts with VeA (Purschwitz et al., 2008). LreA and LreB are also part of the VeA complex and the interaction occurs in the nucleus (Purschwitz et al., 2008).

Bayram et al., 2008b discovered a trimeric VelB/VeA/LaeA complex (Figure 1.10). Light reduced the cellular level of VeA protein by an unknown mechanism and impaired import of VeA to nucleus. The VelB–VeA dimer is formed in the cytoplasm, and requires VeA for nuclear transport; in the absence of VeA the trimeric complex is not established. Similar trimeric complexes have also been demonstrated in *Fusarium oxysporum* (Lopez-Berges et al., 2013) and *Penicillum chrysogenum* (Kopke et al., 2013).

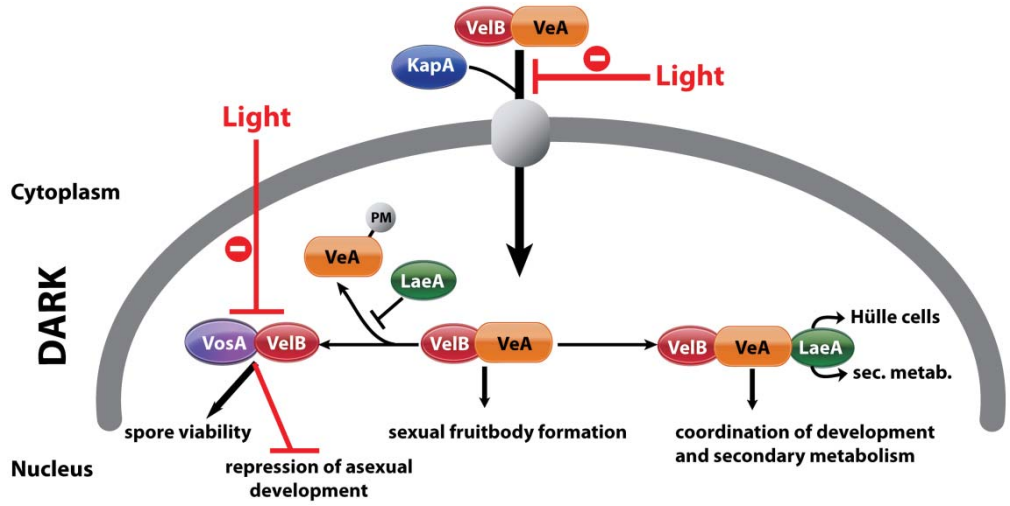


Figure 1.10: Velvet family regulatory complex (Adapted from Bayram et al., 2010)

Model proposed to explain light mediated regulation of secondary metabolite and development in *A. nidulans*. In the light VeA is predominantly in the cytoplasm along with VelB and supports asexual spore formation. In the dark VeA and VelB are imported into the nucleus where they form two types of complex: VosA-VelB dimers that repress asexual spore formation and regulate spore maturation and trehalose biogenesis; Vea-VelB-LaeA trimers that regulate secondary metabolism and sexual development.

Other than the above-mentioned protein, there is another protein, VosA, that also interacts with VeA or VelB. VosA forms a heterodimer (VosA–VelB) and inhibits asexual differentiation in the dark. Further, it has been shown that VosA–VelB regulates trehalose biosynthesis genes in the spores thus protecting from stress (Yu and Ni, 2007). VelC has recently been shown to positively regulate sexual development in *A. nidulans* (Park et al., 2014).

1.4.4 Chromatin mediated regulation

1.4.4.1 Histones and their modification

In eukaryotes, genomic DNA has to be compacted several thousand fold to fit in a cell nucleus. Histone proteins bind to and compact the DNA and this organised structure is called chromatin (Kornberg, 1974). The nucleosome is the basic repetitive unit of chromatin, consisting of 146 bp of DNA wrapped around an octameric histone core containing two copies each of histones H2A, H2B, H3, and H4 (Thomas & Kornberg, 1975). DNA is further compacted by association with the linker histone H1 and additional non-histone proteins, as well as by higher order looping and supercoiling of the chromatin fiber.

The core histones H2A, H2B, H3 and H4 are highly conserved, each consisting of a globular domain and flexible N terminal tail (Luger, 2003) which is subjected to a variety of posttranslational modifications, such as acetylation (Kuo et al., 1998; Verdone et al., 2006; Sterner & Berger, 2000), phosphorylation (Rossetto et al., 2012), methylation (Reinberg and Zhang, 2001; Zhang & Reinberg, 2001; Lilja et al., 2013), ubiquitination (Cao and Yan, 2012; Zhang, 2003), ADP-ribosylation (Zamudio and Ha, 2012) and SUMOylation (Nathan et al., 2006). Among these, acetylation and methylation have been extensively studied and both are reversible.

1.4.4.2 Histone acetylation and deacetylation

Acetylation and deacetylation of the lysine $^{\varepsilon}$-amino acid group is the most well studied post translational modification in fungi (Brosch et al., 2008) and is carried out by two sets of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HAT enzymes have activating, and HDAC enzymes have inhibitory, roles for secondary metabolite gene clusters and modulate gene expression in fungi in general.

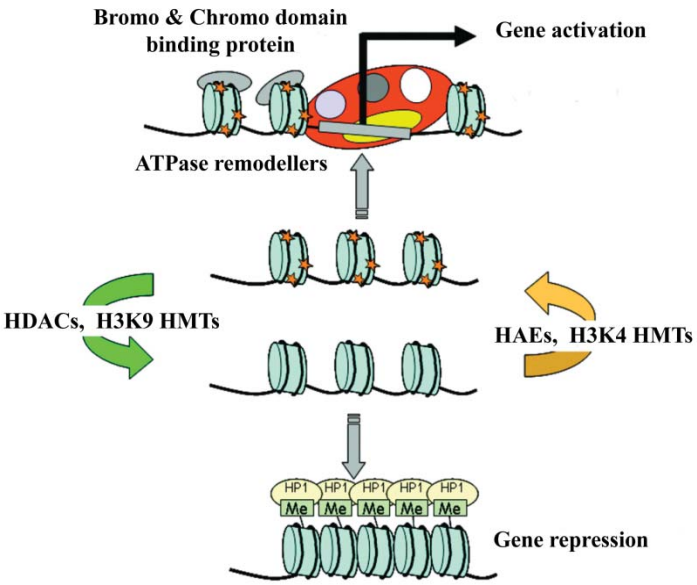


Figure 1.11: Schematic diagram showing the role of chromatin modification in secondary metabolite gene cluster regulation (Adapted from Adcock et al., 2006)

Targeted modifications of histone are involved in secondary metabolism gene regulation. Modifications such as H3 lysine-9 methylation (H3K9me) or H3 lysine-27 methylation (H3K27me) are usually repressive marks; methylation (Me) is shown to recruit heterochromatin protein 1 (HP-1). H3 lysine-9/14 acetylation (H3KAc) and H3 lysine-4 methylation (H3K4me) are activation marks that open promoters and allow further recruitment of the basal transcription machinery. Modifications shown in the figure are mediated by histone methylases (HMTs), histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylated histone tails are shown as yellow stars.

Roze et al (2007) showed that the order in which aflatoxin genes were transcribed reflected the order in which H4 acetylation occurred over time. A SAGA-ADA histone acetlytransferase complex was required for induction of the orsellinic acid gene cluster in *A. nidulans* after treatment with the bacterium *Streptomyces rapamycinicus* (Schroeckh et al., 2009; Nutzmann et al., 2011). Overexpression of a histone 4 acetyltransferase gene *EsaA* plays a role in SM cluster activation through histone 4 lysine 12 (H4K12) acetylation in four examined SM gene clusters (sterigmatocystin, penicillin, terrequinone and orsellinic acid) in *A. nidulans* (Soukup et al., 2012). In *F. fujikuroi* it has been demonstrated that expressed secondary metabolite gene clusters (e.g. gibberellin and bikaverin gene clusters) are enriched for H3K9 acetylation marks (Wiemann et al., 2013).

HDAC inhibitors (HDACi) have been extensively used to enhance the production of secondary metabolites or to activate silent gene clusters in fungi. Chemical epigenetic modifiers, such as 5-azacytidine, the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) or Trichostatin A (TSA) have been shown to be effective in increasing secondary metabolite production by chromatin modification (Willams et al., 2008; Henrikson et al., 2009).

1.4.4.3 Histone methyltransferases

The methylation of lysine is a reversible process achieved by two families of histone methyl transferases (HKMTs). The first type is represented by SET domain containing methyl transferases such as SUV39, SET1, SET2 and RIZ (Dillon et al., 2005 and Sollier et al., 2004) and the second type by non SET domain containing methyl transferases (DOT1) (Sawada et al., 2004). The methylation of arginine is carried out by protein arginine methyl transferases (PRMTs) (Gary and Clarke 1998) and is represented by two types of enzymes, classed depending on whether they add methyl

groups asymmetrically (Type I) or symmetrically (Type II) (Trojer et al., 2004; Yang and Bedford, 2013).

COMPASS (Complex Proteins Associated with Set1) is a family of conserved eukaryotic transcriptional regulators involved in repressing chromatin through methylation of lysine 4 of histone H3 (H3K4) (Sims & Rienberg, 2006). In *A. nidulans* deletion of a gene of one of these family members, *CclA,* led to production of six new aromatic compounds (Bok et al., 2009). Further analysis of the cluster genes by chromatin immunoprecipitation (ChIP) showed a strong decrease in H3K4me2 and H3K4me3 levels in all the genes analysed (Bok et al., 2009).

HP-1 homolog is non histone protein that is involved in establishment and maintenance of heterochromatin marks (Lachner et al., 2001) in eukaryotes. Deletion of *HepA, a* homolog of *HP-1.* resulted in higher expression of genes involved in SM biosynthesis in *A. nidulans* (Reyes-Dominguez et al., 2010). A similar result was shown in *hep1* deletion strains of *F. graminearum* (Reyes-Dominguez et al., 2012) where aurofusarin levels were increased.

H3K27 is known to be involved with transcriptionally silenced genes (Li et al., 2007) but its role in regulating secondary metabolite production in fungi has only recently been studied. In *F. graminearum* histone H3K27 methyltransferase KMT6 deletion caused a drastic effect on expression of SM gene clusters particularly the pigment production (Connolly et al., 2013). More recently Chujo and Scott (2014) demonstrated that deletion of *ezhB* histone H3 lysine 27 methyltransferase in *E. festucae* enhanced the levels of ergovaline and chanoclavine but not lolitrem biosynthesis *in planta*.

1.5 Hypothesis, aims and objectives

Dothistromin, despite being structurally similar to the AF/ST precursor versicolorin B, has a very different gene arrangement and biosynthesis timing. Prior work in this laboratory had established that, in contrast to AF/ST gene clusters, dothistromin genes are arranged in several 'mini clusters' or loci, and that dothistromin gene expression is highest at an early stage of growth in culture, a feature not commonly seen for secondary metabolites. The intention of this study was to understand the mechanism of regulation of the fragmented dothistromin gene cluster, with the hope of unravelling the biology behind the unusual toxin production timing seen *in vitro* and its possible implications for understanding the selective advantage this may confer to the pathogen.

Hypothesis :

Orthologs of regulatory genes *aflR*, *aflJ*, *veA*, and *laeA* that function in aflatoxin & sterigmatocystin biosynthesis in *Aspergillus* spp. are present in the *Dothistroma septosporum* genome and regulate dothistromin production.

Aim 1:

Identify and characterise orthologs of pathway specific regulators AflR and AflJ, in *D. septosporum*.

Objectives:

a) Identify genes for pathway specific regulators that are orthologs of *Aspergillus* spp. AflR and AflJ in the *D. septosporum* genome.

b) Create knockout and cross genera complementation mutants for *DsAflR* and *DsAflJ*.

c) Study the function of each regulator by analysis of dothistromin gene expression and dothistromin production in the mutants.

Aim 2:

Identify and characterise orthologs of global regulators VeA and LaeA in *D. septosporum*.

Objectives

a) Identify global regulator genes that are orthologs of *Aspergillus* spp. VeA and LaeA in the *D. septosporum* genome by BlastP and phylogenetic approaches.

b) Create knockout and complementation mutants for *VeA* and *LaeA*.

c) Study the function of each regulator by analysis of dothistromin gene expression and dothistromin production in the mutants.

d) Determine dothistromin production and gene expression *in vitro* over a time course to study the role of regulatory proteins and chromatin modification on early dothistromin gene expression.

e) Standardise a chromatin immunoprecipitation (ChIP) protocol specific for *Dothistroma septosporum* and perform ChIP-seq to determine how chromatin profiles change over time across the genome

Chapter 2: Material and Methods

2.1 Biological material

2.1.1 *Dothistroma septosporum* strain

Wild-type *D. septosporum* NZE10 (CBS128990) is a New Zealand forest isolate and is the strain sequenced by the Joint Genome Institute (http://genome.jgi-psf.org/Dotse1/Dotse1.home.html). Other *D. septosporum* strains used or developed in this investigation are listed in Table 2.1.

2.1.2 *Escherichia coli* strain and its genotype

The bacterial strain used in this study for propagation and maintenance of plasmids was *Escherichia coli* Top10 (F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Θ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G and *Escherichia coli* DB3.1™ (F– *gyr*A462 *end*A1 Δ(sr1-*rec*A) *mcr*B *mrr hsd*S20 (rB–, mB–) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(SmR) *xyl*-5 λ– *leu mtl*1) (Genotypes of Invitrogen™ competent cells).

2.1.3 Plant material

Pinus radiata seedlings used in this study were obtained from Scion (Rotorua, New Zealand). These seedlings either were raised from seeds or were rooted cuttings from a 1-2 year old trees. All saplings were re-potted in a (15 cm) plastic pot with standard potting mix and kept in a Massey University greenhouse facility until used for experiments.

Table 2.1: *D. septosporum* strains used in this study.

Fungal strain/mutants	Plasmid transformed (in house number)	Genotype	Reference
NZE10	-	Wild type single spore isolate from infected pine needle.	(Barron, 2006)
FJT2	pR209	NZE5/Δ*DotA::hph*	(Arne, 2008)
FJT96 (Δ*DsVeA* KO1)	pR297	NZE10/Δ*DsVeA::hph*	This study
FJT97 (Δ*DsVeA* KO2)	pR297	NZE10/Δ*DsVeA::hph*	This study
FJT98 (*DsVeA* C1)	pR298	FJT96 /*DsVeA, phleo*	This study
FJT100 (*DsVeA* C2)	pR298	FJT 96/OE:*DsVeA, phleo*	This study
FJT104 (Δ*DsAflR* KO1)	pR310	NZE10/Δ*DsAflR:: hph*	This study
FJT105 (Δ*DsAflR* KO2)	pR310	NZE10/Δ*DsAflR:: hph*	This study
FJT106 (Δ*DsAflR* KO3)	pR310	NZE10/Δ*DsAflR:: hph*	This study
FJT107 (*DsAflR* C1)	Nucleotides 690431 to 693567 of *D. septosporum* scaffold 12 + pR224	FJT104/*DsAflR;phleo*	This study
FJT108 (*DsAflR* C2)	Nucleotides 690431 to 693567 of *D. septosporum* scaffold 12 + pR224	FJT104/OE:*DsAflR; phleo*	This study
FJT109 (*CfAflR*)	Nucleotides 85195–88132 of *C. fulvum* scaffold 130965 + pR224	FJT104/*CfAflR*; *phleo*	This study
FJT110 (Δ*DsAflJ* KO1)	pR316	NZE10/Δ*DsAflJ:: hph*	This study
FJT111 (Δ*DsAflJ* KO2)	pR316	NZE10/Δ*DsAflJ:: hph*	This study
FJT112 (Δ*DsAflJ* KO3)	pR316	NZE10/Δ*DsAflJ:: hph*	This study
FJT113 (*DsAflJ* Ds1)	pR317	FJT112/OE:*DsAflJ*; *phleo*	This study
FJT114 (*DsAflJ* Ds3)	pR317	FJT112/OE:*DsAflJ*; *phleo*	This study
FJT115 (*DsAflJ* Ds5)	pR317	FJT112/*DsAflj*; *phleo*	This study
FJT116 (*ApAflJ* Ap2)	pR320	FJT112/*ApAflj*; *phleo*	This study
FJT117 (*ApAflJ* Ap1)	pR320	FJT112/OE:*ApAflj*; *phleo*	This study
FJT118 (*AnAflJ* An1)	pR319	FJT112/*AnAflj*; *phleo*	This study
FJT119 (*AnAflJ* An3)	pR319	FJT112/OE:*AnAflj*; *phleo*	This study
FJT120 (*CfAflJ* Cf1)	pR318	FJT112/*CfAflj*; *phleo*	This study
FJT121 (*CfAflJ* Cf4)	pR318	FJT112/OE:*CfAflj*; *phleo*	This study
FJT122 (Δ*DsOrdB* KO2)	pR321	NZE10/Δ*DsOrdB:: hph*	This study
FJT123 (Δ*DsOrdB* KO3)	pR321	NZE10/Δ*DsOrdB:: hph*	This study
FJT124 (Δ*DsDotB* KO1)	pR323	NZE10/Δ*DsDotB:: hph*	This study
FJT125 (Δ*DsDotB* KO2)	pR323	NZE10/Δ*DsDotB:: hph*	This study
FJT126 (Δ*DsDotB* KO3)	pR323	NZE10/Δ*DsDotB:: hph*	This study
FJT127 (Δ*DsNorB* KO1)	pR322	NZE10/Δ*DsNorB:: hph*	This study
FJT128 (Δ*DsNorB* KO2)	pR322	NZE10/Δ*DsNorB:: hph*	This study
FJT129 (Δ*DsLaeA* KO1)	pR324	NZE10/Δ*DsLaeA:: hph*	This study
FJT130 (Δ*DsLaeA* KO2)	pR324	NZE10/Δ*DsLaeA:: hph*	This study
FJT131 (*DsLaeA*)	Nucleotides 1802643 to 1806045 of *D. septosporum* scaffold 3 + pR224	FJT129/*DsLaeA*; *phleo*	This study
FJT132 (*CfLaeA*)	pR325	FJT129/*CfLaeA*; *phleo*	This study
FJT133 (*CfLaeA*)	pR325	FJT129/OE:*CfLaeA*; *phleo*	

hph = Hygromycin resistance gene, phleo = Phleomycin resistance gene

Ap = *Aspergillus parasiticus,* An = *Aspergillus nidulans,* Cf = *Cladosporium fulvum*

2.2 Growth and maintenance of biological cultures.

For growth media and supplements refer appendix 8.1.1 (*E. coli*) and 8.1.2 (fungal).

2.2.1 Growth and maintenance of *Escherichia. coli.*

Escherichia coli cultures were grown overnight (12 hrs) on Luria Bertani agar or in Luria Bertani (LB) broth with shaking (220 rpm) at 37ºC. For the selection of recombinant *E. coli* appropriate antibiotics (ampicillin, kanamycin, streptomycin, spectinomycin or chloramphenicol) with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and Isopropyl β-D-1-thiogalactopyranoside **(**IPTG), were used for *E. coli* harbouring plasmid with *lacZ* as a reporter gene. Plates were kept in 4ºC room for the short term. For long term storage, broth cultures were mixed with sterile glycerol to a final concentration of 15% then frozen in liquid nitrogen and stored at -80ºC.

2.2.2 Growth and maintenance of *Dothistroma septosporum*.

2.2.2.1 Growth in solid media

D. septosporum strains were grown and maintained on Dothistroma medium (DM), potato dextrose agar (PDA) or Regeneration media (RG) at 22°C. For sporulation, a 5 mm diameter plug of a culture was taken using a cork borer and ground in a 1.75 ml microcentrifuge tube. The ground mycelia were diluted in 400 µl of sterile water and 100 µl was spread on each plate of dothistroma sporulation agar media (DSM) or pine minimal salts agar media plate (PMMG) (Carsolio et al. 1994; McDougal et al. 2011), then incubated at 22°C for 7-10 days in light before spores were harvested. For storage, the plate was sealed with Parafilm M® (Bemis Neenah, WI), and then stored at 4ºC until required. Each strain was sub-cultured on fresh media every 2 months. For long term storage, a culture plug was stored in 15-30% glycerol at -80°C.

2.2.2.2 Growth in liquid media

For all growth and expression studies conidia were harvested from *D. septosporum* culture grown on DSM or PMMG plates. 5 ml of sterile water was poured into the sporulating culture and allowed to stand for 10-15 min. The 7-10 days old spores were scraped using a sterile glass spreader and collected in a 15 ml centrifuge tube. Conidia were diluted ten and hundred fold with sterile water, counted using a cytometer and the concentration adjusted according to requirements. Approximately $1x10^6$ conidia per ml were inoculated into 25 ml of DM or PMMG and incubated at 22°C in a growth room for 5-7 days with agitation at 200 rpm on a C10 platform shaker (New Brunswick Scientific, NJ, USA). For RNA extraction mycelia were collected by filtration through a nappy liner (Johnson and Johnson) weighed and divided into approximately equal parts of which one part was freeze dried on a Dura-Dry MP Freeze Dryer (Kinetics Thermal Systems, NY, USA) and weighed to calculate the dry weight of the mycelium and the second half was snap frozen in liquid nitrogen and later used for RNA extraction.

For genomic DNA extraction and transformation, fungal mycelium was obtained by inoculating 25 ml of DM broth with fungal culture in a 125 ml conical flask. Approximately 5 mm diameter plug of *D. septosporum* colony was taken using a cork borer, finely ground with a micro pestle and suspended in 400 µl of sterile water in a 1.5 ml microcentrifuge tube. 200 µl of the culture suspension was inoculated in each flask and incubated at 22°C in a growth room for 7-9 days with agitation at 200 rpm.

For time course assessment of fungal growth, dothistromin production and gene expression, approximately $1x10^5$ spores per ml were inoculated into 25 ml low Dothistromin Broth (DB) in a 125 ml conical flask and incubated at 22°C in a growth room with agitation at 200 rpm. After appropriate days, the culture was harvested by centrifugation at 3200 g in a microcentrifuge (Eppendorf 5810). The supernatant was

transferred into a new tube which was later used for toxin extraction, whilst the mycelium was collected from the bottom using a sterile spatula.

For all the above stated growth conditions, three Sylvania GRO-LUX (F30w/GRO-TB) and two Philips-lifemax (TLD-30W/840 cool white) lights, either with continuous illumination (light conditions) or wrapped in double foil (dark conditions) was used depending on experimental demand.

2.3 DNA Isolation, purification and quantification

2.3.1 Maxiprep fungal and pine needle DNA extraction

Genomic DNA from *D. septosporum* or pine needles was extracted by the following CTAB protocol (Sambrook et al., 2001) with some modifications. Fungal cultures were grown for 7 days and harvested by filtering through a nappy liner and washed with sterile water. A weighed amount of mycelium was transferred into a 15 ml centrifuge tube, snap frozen and subsequently freeze dried for 12-16 hours. Then 0.5-1 g of ground freeze dried mycelium or pine needle was transferred quickly to a 50 ml centrifuge tube containing 10 ml of CTAB pre-warmed at 55°C extraction buffer (appendix:8.2.2) with 2 µl of RNase (100 mg/ml) (Sigma-Aldrich, St. Louis, Missouri, USA) vortexed and incubated in a 37°C water bath for 5-10 min and then transferred to a 65°C bath for another 15-20 min. After cooling to room temperature, an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed by inverting, then centrifuged at 5,600 x g in a F21J-8x50y rotor (Thermo-Sorvall, Waltham, Massachusetts, USA) for 10 min at 4°C. The supernatant was carefully taken into a fresh tube, the above step was repeated once, then double the volume of ice cold ethanol was added to the supernatant and incubated at -20°C for 10-15 min. DNA was pelleted

by centrifugation at 5,600 g in F21J-8x50y rotor (Thermo-Sorvall Waltham, Massachusetts, USA). The pellet was washed with 70% ethyl alcohol, air-dried and then finally dissolved in 500 µl of $T_{10}E_1$ (Appendix 8.2.1).

2.3.2 Miniprep DNA extraction from fungal colony for mutant screening

For screening recombinant colonies of *D. septosporum,* a small area of the colony was cut from a plate and macerated using a sterile micro pestle in a 1.75 ml microcentrifuge tube. The tissue was suspended in CTAB lysis buffer (Appendix 8.2.2) with 2 µl of RNase (10 mg/ml) (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 65°C for 10 min. After cooling to room temperature an equal volume of phenol: chloroform:iso amyl alcohol (25:24:1) was added and vortexed for 5 sec followed by centrifugation at 15,682 g for 5 min (Eppendorf 5415 R). The upper aqueous phase was transferred to a fresh tube and DNA was precipitated by adding double the volume of chilled ethanol followed by incubation at -20°C for 15 min. After another centrifugation, the pellet was allowed to dry at room temperature and then dissolved in 25 µl of $T_{10}E_1$ buffer (Appendix 8.2.1).

2.3.3 Agarose gel electrophoresis

All genomic DNA and PCR products were analysed on an agarose gel prepared by melting an appropriate amount of agarose (Gold Bio, St Louis, USA) based on the DNA fragment size to be separated (Sambrook and Russel, 2001). The electrophoresis gel was prepared in 1x TBE buffer (Appendix 8.2.1) and set in a 50 ml mini unit or a larger 300 ml unit for overnight run for Southern analysis, and was run at 80 volt or 40 volt respectively. 20 µl of DNA was mixed with 3 µl of 6x loading dye (Appendix 8.2.1) and loaded alongside 1 Kb Plus DNA molecular weight marker (Invitrogen, Carlsbad,

CA, USA). The gels were stained in ethidium bromide solution (1 µg/ml in reverse osmosis water) for 20 min, rinsed in water and then visualized on a UV-transilluminator and documented using Gel- Doc™XR documentation system (Bio-Rad, Hercules, CA, USA) and Image Lab™ software.

2.3.4 Gel elution of the nucleic acid.

The specific PCR amplicons or restriction digestion products were run on a 1% agarose gel. DNA bands were cut using a sharp sterile scalpel by visualising the gel on UV transilluminator (Alpha Innotech, Johannesburg, S.A) and collected in sterile pre-weighed 2 ml microcentrifuge tubes. A QIAquick Gel Extraction Kit gel extraction kit (Qiagen, Hilden, Germany) was used to elute the DNA fragment from the agarose block as described in the user's manual.

2.3.5 Purification of PCR products

PCR products were purified using a High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

2.3.6 Plasmid DNA extraction

Plasmid DNA was extracted from overnight (12 hrs) grown *E. coli* culture using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) or High Pure Plasmid Isolation Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

2.3.7 Nucleic acid quantification

Nucleic acid quantification was done using NanoDrop® ND-1000 UV-Vis spectrophotometer with software version 3.1.0 (Nanodrop Technologies Inc, Wilmington) or fluorometric quantification using a Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences, UK) according to the manufacturer's instructions.

2.3.8 Restriction digestion of DNA

For the confirmation of constructs and subcloning, sequential digestion of vector and insert was done in a separate tube using 1 unit of enzyme per µg of DNA and the buffer provided by the manufacturer. The mixture was incubated at the appropriate temperature for 3 hours followed by inactivation by incubating at 65°C for 15 min. For Southern assay, 2 µg of genomic DNA was digested with 10 units of restriction endonuclease and incubated overnight at the appropriate temperature recommended by the manufacturer. A small aliquot was loaded on a gel and visualised to confirm the efficiency of the restriction reaction.

2.3.9 Ligation

For ligation, an optimal molar ratio of ends of vector: insert (1:3) was computed and added to a ligation mixture containing 1µl each of 10x ligation buffer (Roche) and T_4 DNA ligase (1 u/µl) and volume made up to 10 µl by adding sterile water, then incubated overnight at 4°C.

Gateway® ligation was performed using pDONR and pDEST vectors (Life Technologies, CA, USA) (Appendix 8.3.1). For creation of entry clones, 50 fmole of each pDONR™ P4-P1R, pDONR™ P2R-P3 or pDONR™ 221 vector and 5' or 3' flanking insert of targeted gene was recombined using 1 µl of BP Clonase® plus enzyme mix and the volume made up to 5 µl by TE buffer. The reaction mix was incubated overnight at 25°C. The enzyme was inactivated by adding 1 µl of proteinase K (2 µg/µl) and incubated at 37°C for 10 min. To create a Gateway® expression clone LR reactions were performed using 10 fmole of each 5' and 3' entry clone of the targeted gene and hph cassette with 20 fmoles of pDEST™ R4-R3 vector recombined using 1µl of LR Clonase® II Plus enzyme (Life Technologies).

2.3.10 Vector construction

2.3.10.1 Three way GATEWAY based construction of gene replacement vectors

The design of the PCR primers is critical for recombination cloning using MultiSite Gateway® Technology. The primer design must incorporate sequences required to facilitate MultiSite GatewayR cloning. In order to generate attL4 and attR1, attR2 and attL3 and attL1 and attL2 flanked entry clones containing the 5′ and 3' elements of the targeted gene, PCR primers were designed with appropriate sites to produce attB4 and attB1r, attB2r and attB3 and attB1 and attB2 flanked PCR products that were targeted for BP Clonase® (appendix 8.3.1**).** The three entry clones (appendix 8.3.2) prepared by amplification and BP ligation reaction were a) 5' region of the targeted gene b) a hygromycin resistance (*hph*) selectable marker gene cassette and c) 3' region of the targeted gene. The flanking regions were designed so that approximately 1.2 kb- 1.5 kb of the targeted gene open reading frame (ORF) would be replaced by the *hph* gene following recombination of the entry clones. Finally, the arrangement of the three fragments in the gene replacement plasmid, pDEST was carried out by a MultiSite Gateway® LR recombination reaction as stated above (2.3.9). The construct was transformed into *E. coli* One Shot® TOP10 cells and recombinants were confirmed by PCR and sequencing. The primers were designed using Vector NTI v 11.1.09 (Life Technologies).

2.3.10.2 One Step Construction of Agrobacterium Recombination-ready plasmids (OSCAR).

Agrobacterium mediated transformation (ATMT) requires the generation of deletion constructs in binary vectors. For the generation of knockout constructs a modified gateway method called "OSCAR" or One Step Construction of Agrobacterium-Recombination-ready-plasmids was used (Paz et al., 2011).

For the generation of deletion constructs, two primer pairs were designed to amplify about ~1 kb of the 5' and 3' flanks of the targeted gene. Each of the primer sets had different *attB* recombination sites at the both ends. Primers to amplify 5' flank and 3' flank contained *attB2r* and *attB1r* and *attB4* and *attB3* sequences respectively. After PCR amplification, both flanks were co-purified as stated above in 2.3.5. For OSCAR deletion constructs a BP reaction was set up to include 50 fmoles of each co-purified PCR flank products, 25 fmoles of pA-Hyg-OSCAR (Appendix 8.3.2) and 50 fmoles of pOSCAR (Appendix 8.3.2) with 0.5µl of BP clonase (Life technologies, CA, USA), then incubated overnight at 25°C. The BP reaction was terminated using 0.5 µl proteinase K (20 µg/µl). The reaction mixture was then used to transform *E. coli* competent cells as stated in section 2.3.10. Bacterial colonies were recovered on LB plates supplemented with 100 µg/ml spectinomycin following overnight incubation at 37°C. Analysis of the *E. coli* transformants was done to verify deletion construct by PCR using a different set of primers (Table: 2.2), and finally the construct was verified by sequencing. Before fungal transformation all constructs generated by the above procedure were either linearised with *NheI* or *NarI* or transformed as circular plasmid.

Table 2.2 Plasmids used in this study

Gene knockout plasmids						
Plasmid In house number	**Purpose**	**Protein ID**	**5' flank From - To**	**3' Flank From - To**	**Replaced region by hph cassette**	**Vector**
pR297	Δ*DsVeA*	69562	1695316 - 1694296	1692045 - 1691370	CDS	Gateway
pR310	Δ*DsAflR*	75566	692748 - 693934	690012 - 691253	CDS	Gateway
pR316	Δ*DsAflJ*	57214	693996 - 695096	696580 - 697680	CDS	Gateway
pR321	Δ*DsOrdB*	75648	1111178 - 1112396	1112833 - 1113561	434 bp of 800 bp CDS	OSCAR
pR323	Δ*DsDotB*	75412	83333 - 84248	85493 - 86242	CDS	OSCAR
pR322	Δ*DsNorB*	75044	154243 - 153018	151844 - 150994	CDS	OSCAR
pR324	Δ*DsLaeA*	148869	1802642 - 1803893	1805158 - 1807009	CDS	OSCAR

Gene complementation plasmids				
Plasmid In house number	**Purpose**	**Protein ID**	**CDS with 5' & 3' flank From - To**	**Vector used and selection marker**
pR298	*DsVeA*	69562	1695111 - 1691800	pBCPhleo
pR317	*DsAflJ*	57214	694196 - 697572	pBCPhleo
pR318	*CfAflJ*	197013	83315 - 84315	pBCPhleo
pR320	*ApAflJ*	AAS66019.1	34139 – 36841 of locus AY371490	pBCPhleo
pR319	*AnAflJ*	ANID_10021	4457926 - 4461008	pBCPhleo
pR325	*CfLaeA*	186126	11268 - 14348	pBCPhleo
pR224+ Nucleotides 690431 to 693567 of *D. septosporum* scaffold 12	*DsAflR*[a]	75566	690431 - 693567	pBCPhleo
pR224 + Nucleotides 85195–88132 of *C. fulvum* scaffold 130965	*CfAflR*[a]	197014	85195–88132	pBCPhleo
pR224 + Nucleotides 1802643 to 1806045 of D. septosporum scaffold 3	*DsLaeA*[a]	148869	1802643 to 1806045	pBCPhleo

Note: [a] Complementation by co transformation was done for complementation of these gene mutants.

Dothistroma septosporum genomic DNA was used as a template.

2.3.11 Transformation of *E. coli* and confirmation of the clones

2.3.11.1 Preparation of competent cells

E. coli Top10 transformation competent cells were prepared by following the protocol mentioned in Sambrook and Russell (2001) with minor modifications. An isolated colony from an *E. coli* Top10 culture plate was inoculated to 5 ml Luria broth and incubated at 37°C overnight at 200 rpm. The following day, the culture was diluted 100 fold using Luria broth *i.e.*, 0.5 ml of culture was added to 50 ml of Luria broth. It was incubated for 2 to 3 hours until an OD of 0.3 to 0.4 at 600 nm was attained. The culture was chilled in ice for 30 min and 25 ml of culture was dispensed into two sterile centrifuge tubes. The cells were pelleted at 4025 g in an F21J-8x50y rotor (Thermo-Sorvall Waltham, Massachusetts, USA), for 5 min at 4°C. The supernatant was discarded, and the pellet was suspended in 12.5 ml ice-cold 0.1 M $CaCl_2$. The centrifuge tubes were again kept in ice for 30 min and later centrifuged at 1788 g for 5 min, then the pellet dispersed in 1 ml of ice-cold 0.1 M $CaCl_2$ made with 10% glycerol. Aliquots of approximately 75 µl of the competent cells were distributed to pre-chilled 1.5 ml micro centrifuge tubes and snap frozen in liquid nitrogen, then stored at -80°C or used immediately for transformation.

2.3.11.2 Transformation into *E. coli* Top10

To 75 µl of the competent cells 3 µl of ligation mixture was added, mixed gently and chilled on ice for 30 min. A heat shock was given by shifting the chilled mixture to a 42°C water bath for 2 min, then immediately chilled on ice for 5 min. 900 µl of Luria broth was added and incubated at 37°C at 200 rpm for 1 hour to allow bacteria to recover and express the antibiotic marker encoded by the plasmid. The cells were pelleted at 10,000 g in (Eppendorf 5415 R), for 1 min, 500 µl of supernatant discarded

and the pellet was dissolved in the remaining 500 µl of supernatant. Then 100 µl was spread on each Luria agar plate containing appropriate antibiotics and incubated overnight at 37°C.

2.3.11.3 Confirmation of the clones by sequencing

PCR amplicons or recombinant plasmids were confirmed by sequencing using appropriate primers at the Massey Genome service, Massey University Palmerston North using ABI PRISM® BigDye® Primer Cycle Sequencing Ready Reaction Kit in an ABI 3730 DNA Analyzer (Applied Biosystems, Foster city, CA). The sequences were analysed using BioEdit v7.2.3 (Hall, T.A. 1999) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Polymerase chain reaction (PCR) analyses

2.4.1 Primer design and cycling condition

All PCRs were performed in 100 µl PCR tubes (Axygen, CA, USA) with a PCR model Eppendorf Gradient Mastercycler® (Eppendorf, Hamburg, Germany). Primers were designed using vector NTI v 11.1.09 (Life Technologies) and synthesized by Integrated DNA Technologies (Coralville, IA, USA) or Invitrogen. All primers were diluted to the stock concentration of 200 µM using TE buffer and stored at -20°C freezer. For regular PCR use 5 µM dilutions were prepared from the stock. Primers used in this study are listed in Table 2.3.

Table: 2.3: Primers used in this study

Primers for qRT PCR			
Primer	**Target gene**	**Lab ref**	**5' to 3' sequence**
DsHsp2F	DsHsp2	1092	GTGTATGAGTCGTTGGAGTC
DsHsp2R	DsHsp2	1093	GCCCAAGGATCAAGTTAGAG
DsHsp1F	DsHsp1	1094	GATTATCGTGACATCCAGAACC
DsHsp1R	DsHsp1	1095	CGCACTAGGCATGGTAATTC
DsNrps1F	DsNrps1	1096	GTTGCTATGCTGAGTGTCA
DsNrps1R	DsNrps1	1097	GGCATACGATACGAAGTCAT
DsNrps3F	DsNrps3	1098	AGCAATTCCAGAGCGATAATC
DsNrps3R	DsNrps3	1099	CTGTCGGCACATTGAGTC
DsNrps4F	DsNrps4	1101	CCAGCAGATCCCACGGCAGAG
DsNrps4R	DsNrps4	1102	CGAGTGCAGAATCCAGGCCAGTT
DsPks1F	DsPks1	1103	CACTACATTGGCTACTTCTCA
DsPks1R	DsPks1	1104	TAGTTGATACGGCACTGATG
DsPks2F	DsPks2	1105	GCTCGAACACCCATATTGTGA
DsPks2R	DsPks2	1106	GATGTGTCAAGGAACTGCCA
DsPks3F	DsPks3	1107	GATGTACCTAAGCTGGCGACG
DsPks3R	DsPks3	1108	TCTCCAGTAGGGGCCACTTC
DsNrpsYF	DsNrpsY	1123	CGTCTTCTGCGTTCGTAA
DsNrpsYR	DsNrpsY	1124	TGCCTGGTACTCTTCATATT
DsPks4F	DsPks4	1125	TGGACGCAATTACGACATC
DsPks4R	DsPks4	1126	ATACCGTAGCCAGCAGAG
rtTUB fwI	Dsβtub	709	CCGGCGTGTACAATGG
rtTUB revI	Dsβtub	710	CATGCGGTCTGGGAAC
rtpksAF1	DsPksA	695	CATTATGTCGTCCGAGCAC
rtpksAR1	DsPksA	696	CGAACAGAACTACCGACC
rtdotAF1	DsDotA	691	CTGGTGATGAATTCGACCG
rtdotAR1	DsDotA	692	AAGCACCACCGTCAATAC
DsAflR exF	DsAFlR	1013	GGAAGAGTAGTGTTACCATTGT
DsAflR exR	DsAFlR	1022	CATCTATTCAACGACCTCACA
DsAflJ exF	DsAflJ	1023	GACCATTGCGGCATTCTG
DsAflJ exR	DsAflJ	1024	GCTGTAGTGTACGGAATCCA
DsVeA exF	DsVeA	974	AACGGATACACTCAGACC
DsVeA exR	DsVeA	975	GTGGTGGTGCTGGATAA
DsAvfAexpF	DsAvfA	1207	CTGGTCTACAACATCGTCTC
DsAvfAexpR	DsAvfA	1208	TTGCAGGAGATTAGCTTGTG
DsAvnAexpF	DsAvnA	1209	ACAAGCAATACGGTTCAGTT
DsAvnAexpR	DsAvnA	1210	TGTGGAATGGTGGAAGGT
DsCypAexpF	DsCypX	1211	CTCCGTTCTATCGTCTCCT
DsCypAexpR	DsCypX	1212	AGCCTCTTCCTTCATCTTCT
DsEpoAexpF	DsEpoA	1213	GTTGGAGGAGGTGGAGAA
DsEpoAexpR	DsEpoA	1214	AGAGACGGCTGAGAAGAG
DsEstAexpF	DsEst1	1215	CATCTGCTTTGTGGTGTTATTG
DsEstAexpR	DsEst1	1216	GCCGATTGTGTAGAACTTGT
DsHexAexpF	DsHexA	1217	GCCGATACTGAGCATTCC
DsHexAexpR	DsHexA	1218	AACGCCGATGAAACTCTG
DsHexBexpF	DsHexB	1219	AGCCTGAGCGATGATGAT
DsHexBexpR	DsHexB	1220	GTTGAAGAGAATGCGTGACT
DsMoxAexpF	DsMoxY	1221	GTAATTGGATCTGGTGCTTCA
DsMoxAexpR	DsMoxY	1222	TCGCTCTTCTTCGCTGTA
DsNor1expF	DsNor1	1223	CCACAACATTCGACACATAGA
DsNor1expR	DsNor1	1224	GAAGAGGAGAAGGACTGAGT
DsNorBexpF	DsNorB	1225	AAGAAACTCCAAACGAACTACA
DsNorBexpR	DsNorB	1226	AGATATAGCACTTGACCAGAGA
DsOrdBexpF	DsOrdB	1227	ATCAGAGCAGAGAACGAGAA
DsOrdBexpR	DsOrdB	1228	GAATGTTGAGGAGATCCAGTG
CfAflJ exp F	CfAflJ	1419	GCTTATTGAATCGGCTGCTA
CfAflJ exp R	CfAflJ	1420	AACATTCGTCAAGAGTTCTACC

Table: 2.3: Primers used in this study continued

Primers for qRT PCR			
Primer	**Target gene**	**Lab ref**	**5' to 3' sequence**
DsVerBexpF	DsVerB	1229	GGAGATGGTCAACTACTACAAC
DsVerBexpR	DsVerB	1230	GAGATAGCGACAGACTTCAAG
DsOrdAexpF	DsOrdA	1231	GAAGATGTCTAAGGCTGTGATT
DsOrdAexpR	DsOrdA	1232	AACTGGAACGCTGTTTGAAA
DsVbsAexpF	DsVbsA	1233	CTTGATTCGTTCGGCTTCT
DsVbsAexpR	DsVbsA	1234	GCGGAGGTATGATGAGGA
RtTUBfwI	DsTub1	709	CCGGCGTGTACAATGG
RtTUBrevI	DsTub1	710	CATGCGGTCTGGGAAC
RtpksAfw1	DsPksA	695	CATTATGTCGTCCGAGCAC
Rt pksArev1	DsPksA	696	CGAACAGAACTACCGACC
RtdotAF	DsVer1	691	CTGGTGATGAATTCGACCG
RtdotAR	DsVer1	692	AAGCACCACCGTCAATAC
AflRexpF	DsAflR	1013	GGAAGAGTAGTGTTACCATTGT
AflRexpR	DsAflR	1022	CATCTATTCAACGACCTCACA
AflJexpF	DsAflJ	1023	GACCATTGCGGCATTCTG
AflJexpR	DsAflJ	1024	GCTGTAGTGTACGGAATCCA
Ds31 F	PID 66854	1239	CTGGTTCCTTCCTCTAATTGG
Ds31 R	PID 66854	1240	CCGTCAGGTATCGAGTCTTA
Ds29211 F	DS29211	1241	CTGTCTGCTCCTTGATGAAC
Ds29211 R	Ds29211	1242	GTAACGAAGTCCCTGGTAGT
Ds160897 F	Ds160897	1243	GTTTCCACCTTCCTCCTTTC
Ds160897 R	Ds160897	1244	CGTGATGATGTTGGGATCTG
Cbx F	Cbx	1247	GCGCGTTGCTGCCTTTTCCA
Cbx R	Cbx	1248	ACGCGAGTCGGCCATCCATC
DsAdhA expF	DsAdhA	1249	CAGTACCAGAGCTAGAACGA
DsAdhA expR	DsAdhA	1250	CCTGAACACAATCCTCCATC
DsHypC expF	DsHypC	1251	CTTCCCAACTTCTCAACCAAT
DsHypC expR	DsHypC	1252	ATCATCGCCACTGTCACT
DsTef1A F	DsTef1A	1377	CCACATCAATGTCGTCGTTAT
DsTef1A R	DsTef1A	1378	ATACCACCGCACTTGTAGAT
Dsgpdh F	Dsgapdh	1379	GAGTACAAGTCCGACATTCC
Dsgpdh R	Dsgapdh	1380	GACCCTCAACAAGAGTGAAC
DsStuA F	DsStuA	1399	CACTCGCCTCATCAGAAC
DsStuA R	DsStuA	1400	GTCGCTGCTCATAACATAGT
DsSteA F	DssteA	1401	GGCGAATATGTGTCTTGTGT
DsSteA R	DssteA	1402	TTGCGGAGGTCAGAGAAG
DsNsdD F	DsNsdD	1403	GCCGTTATGGAGATCGTATG
DsNsdD R	DsNsdD	1404	GCTTGTTGCTCGTAGACC
CfveA F	CfVeA	1437	CTCCACCTTCTGCCGCCTAGCT
CfveA R	CfVeA	1438	CCATTCCGCTCCCGCTAAGTTA
CfAflr expF	CfAflR	1455	TACACAGTTGACCATAGACAG
CfAflr expR	CfAflR	1456	CCTTCTCCGATACCTCTTCTT
AflR CfDs1F	Cf&Ds AflR	1421	GCGCGGAATTCCARCAYATHGCIAC
AflRCfDs1R	Cf&Ds AflR	1422	GCGCGCAAGCTTTTIACDATYTGKTC
AflR CfDs2F	Cf&Ds AflR	1423	GCGCGGAATTCGAMCARATHGTIAA
AflR CfDs2R	Cf&Ds AflR	1424	GCGCGCAAGCTTTGKTCKTTIGTIGG
HexA 1F	DsHexA	1425	AAGCGCAAAGTCCAGTCCG
HexA 1R	DsHexA	1426	TGCCGTTCGTCAGGTCTGTG
HexA 2F	DsHexA	1427	GCCATCGTCCACTGCCAAG
HexA 2R	DsHexA	1428	CGCCCCGGTAGTGTTGAGA
HexA 3F	DsHexA	1429	CTTGGGAGTACGGGGCCGATC
HexA 3R	DsHexA	1430	ATAGCCACGGCGATCAAACAGT
Cf-DsHexA1F	Cf&DsHexA	1431	GCGCGGAATTCATGGGICARAARACIAT
CfDsHexA 1R	Cf&DsHexA	1432	GCGCGCAAGCTTGCRTTCCACCAIGCRTTRTA
CfDsHexA 2F	Cf&DsHexA	1433	GCGCGGAATTCATGGARATGGCITGGATGATG
CfDsHexA 2R	Cf&DsHexA	1434	GCGCGCAAGCTTTCYTCIGGDATICCRTA
CfDsHexA 3F	Cf&DsHexA	1435	GCGCGGAATTCTTYGAYGAYTTYCAYGARGA
CfDsHexA3R	Cf&DsHexA	1436	GCGCGCAAGCTTAAIGCCATYTTYTGRTC
Tub4	DsTub	227	TTGCGGAGATCACTGTTGAGCTG
RtTUB fw II	DsTub	728	CGGTATGGGTACGCTCT
ApAflJ exp F	ApaflJ	1405	GGAATATGGCTGTAGGAAGTG
ApAflJ exp R	ApaflJ	1406	GGAAACGGAGTGATGGAAAT
AnAflJ exp F	AnAflJ	1413	CTCCAACATAACGATCCAACA
AnAflJ exp R	ApaflJ	1414	ATCTTATTCACCACCACCAAC

Table: 2.3: Primers used in this study continued

Primers for knockout confirmation and creating complementation constructs			
Primer	**Target gene**	**Lab ref**	**5' to 3' sequence**
AflJF 5'INT	DsAflJ	1439	CGCCAAGACCTCGATGCTCAGT
AflJR 3'INT	DsAflJ	1440	CTGCGCACCCTCCTAGCCACT
1225INT5' F	Ds1225	1449	GCGACACTATGCGACCTCAGG
1225 INT 3' R	Ds1225	1450	GCCGCCTCCGCTCCGAC
OscHPH F 5'OUT	hph	1411	AGAGCTTGGTTGACGGCAATTTCG
OscHPH R 3'OUT	hph	1412	GCCGATGCAAAGTGCCGATAAACA
DotB 5'INT F	DsDotB	1407	TTTCCAGGTATTCGGTGCGTTTC
DotB 3'INT R	DsDotB	1408	GTTGCCCGCCCACACTGATC
NorB 5' INTF	DsNorB	1385	GCCGTTCTGTGGAGCGTGCT
NorB 5' INTR	DsNorB	1386	CTCGCTCGTCCCTGCAACTCA
OrdB 5'INT F	DsOrdB	1387	CCCCGCCCTCATACCGTACC
OrdB 3'INT R	DsOrdB	1388	GGCCTCTGGGTAAGCGGTGTC
Hph 5'out	Hph out	35	GAATCTCCGGTGTGGAAGA
Hph 3'out	Hph out	36	TCCTTGAACTCTCAAGCCTACAG
DsAflR INT F	DsAflR	1245	CCGCCGCGTCCCACTTC
DsAflR INT R	DsAflR	1246	CCGGTAGCTGGTATGGCA
DsAflJ Comp F	DsAflJ	1381	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGGCCCCGGTATGGTCGATGGCCT
DsAflJ Comp R	DsAflJ	1382	GGGGACCACTTTGTACAAGAAAGCTGGGTATCTAGACGTTGTCAGGACCGAGGA
A.par AflJ F	ApAflJ	1409	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGGCCCAGATGCCCGGGGGGAGAT
A.par AflJ R	ApAflJ	1410	GGGGACCACTTTGTACAAGAAAGCTGGGTATCTAGAAGATCTCGGCTGGCGTAA
DsAflJ ApaI F	DsAflJ	1415	GCGCGGGGCCCCGGTATGGTCGATGGCCTTAGC
DsAflJ XbaI R	DsAflJ	1416	GCGCGCTCTAGACGTTGTCAGGACCGAGGAGCA
Cf AflJApaI F	CfAflJ	1417	GCGCGGGGCCCCGGATCTTGGTGCCTTAGGTGG
CfAflJ XbaI R		1418	GCGCGCTCTAGACTGCGGGCTTGACATTTGCTC
Cf.AfljF	CfAflJ	1397	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGGCCCGCTGACGGCCAGGCACG
Cf.AfljR	CfAflJ	1398	GGGGACCACTTTGTACAAGAAAGCTGGGTATCTAGACATGCAATCTGGACCTTG
AflRApaIF	DsAflR	1235	GCGCGGGGCCCTGCGGATTTGCCAGACACTCG
AflRXbaIR	DsAflR	1236	GCGCGCTCTAGACGCGCTTGAAACGGGATGATT
DspksA64	DspksA	934	CTGTCTTCCTCGACCTGTT
DspksA164	DspksA	871	AAGCACACCTGGAAAGAATGA
CAD918	Cad	935	CAGCAAGAGGATTTGGACCTA
CAD1019	Cad	936	TTCAATACCCACATCTGATCAAC
A.nid AflJ F	AnAflJ	1383	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGGCCCATAATCAGTGCTGACGGTAC CA
A.nid AflJ R	AnAflJ	1384	GGGGACCACTTTGTACAAGAAAGCTGGGTATCTAGAAGACCCGCGCCGTCTGGC
CfaflRattB1F	CfAflR	1237	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGCGGGGCCCCGGATCTTGGTGCCTTA GGTGG
CfaflRattB2R	CfAflR	1238	GGGGACCACTTTGTACAAGAAAGCTGGGTGCGCGCTCTAGACTGCGGGCTTGACATT TGCTC

Table: 2.3: Primers used in this study continued

Primers for creating knockout constructs			
Primer	**Target gene**	**Lab ref**	**5' to 3' sequence**
AflR 5' Flank *att*B4F	DsAflR5flank	1001	GGGGACAACTTTGTATAGAAAAGTTGCTACCGCCATCATATGTCTC
AflR5' Flank *att*B1rR	DsAflR5'flank	1002	GGGGACTGCTTTTTTGTACAAACTTGCGCAAGATCGGAAGTCCTC
AflR3' Flank *att*B2rF	DsAflR3 'flank	1003	GGGGACAGCTTTCTTGTACAAAGTGGCTAGTGATGACGGTGATCGA
AflR3' Flank *att*B3R	DsAflR3' flank	1004	GGGGACAACTTTGTATAATAAAGTTGCGACGGGGTGGTGTCCCGC
OrdB5'Flnk attB2r F	DsOrdB 5'Flank	1389	GGGGACAGCTTTCTTGTACAAAGTGGCTAAAGCAGTGTCTACCGCAG
OrdB3'Flank attB3R	DsOrdB 3' Flank	1392	GGGGACAACTTTGTATAATAAAGTTGCCGTTCTTTCTCGGACTTTGTACG
DotB5'Flank attB2r F	DsDotB 5' Flank	1393	GGGGACAGCTTTCTTGTACAAAGTGGCTTATTCACGGGCTCTGCAT
DotB5'Flank attB1r R	DsDotB 5' Flank	1394	GGGGACTGCTTTTTTGTACAAACTTGCGCTGGGTTAGATGTCGGC
DotB3' Flank attB4 F	DsDotB 3' Flank	1395	GGGGACAACTTTGTATAGAAAAGTTGCTGTACTCCAACCAAGCAGC
DotB3' Flank attB3 R	DsDotB 3' Flank	1396	GGGGACAACTTTGTATAATAAAGTTGCTCGGCAAACGCGACATCA
Ds LaeA5'F	DsLaeA 5' Flank	1441	GGGGACAGCTTTCTTGTACAAAGTGGAACG GGTTCCTCGCAGGGTT
Ds LaeA5'R attB1r	DsLaeA 5' Flank	1442	GGGGACTGCTTTTTGTACAAACTTGTTCGAGG CCGTGTGTGGTC
Ds LaeA3'F attB4	DsLaeA 3' Flank	1443	GGGGACAACTTTGTATAGAAAAGTTGAATTGCGTATGCACGCATCG
Ds LaA3'R attB3	DsLaeA 3'Flank	1444	GGGGACAACTTTGTATAATAAAGTTGTCTGGTCACGGAGGCTACT
122225 5'F attB2r	Ds12225 5' Flank	1445	GGGGACAGCTTTCTTGTACAAAGTGGAA GTTCTTGGCAGCTTCTTT
12255'RattB1r	Ds12225 5' Flank	1446	GGGGACTGCTTTTTTGTACAAACTTGT TCTGTATGTTTCTCAGTC
12253FattB4	Ds12225 3 Flank	1447	GGGGACAACTTTGTATAGAAAAGTTGTT TGATGGCCATATATTGGAC
122225 3'R attB3	Ds12225 3 Flank	1448	GGGGACAACTTTGTATAATAAAGTTGT GCAGGTGGACTCTCGCGC
norB5'F attB2r	DsNorB 5' Flank	1451	GGGGACAGCTTTCTTGTACAAAGTGGAAGCGCGCGCGTGTATCGCTG
NorB5'R attB1r	DsNorB 5' Flank	1452	GGGGACTGCTTTTTTGTACAAACTTGTTGTGCTGAAAGTGTTGATGGAG
NorB3'F attB4	DsNorB 3' Flank	1453	GGGGACAACTTTGTATAGAAAAGTTGAAAAAGACATGCTCTGAAGA
NorB3'R attB3	DsNorB 3' Flank	1454	GGGGACAACTTTGTATAATAAAGTTGTCGATACCCACGATCCTGG
Ds AflJ 5'Flank*att*B4F	DsAflJ5' Flank	1005	GGGGACAACTTTGTATAGAAAAGTTGCTACAGGAATCGGTCGCTGC
Ds AflJ 5FlaK *att*B1r F	DsAflJ5' Flank	1006	GGGGACTGCTTTTTTGTACAAACTTGCCGCCTGACGGTCCACAACCA
Ds AflJ 3' Flank*att*B2r	DsAfl35' Flank	1007	GGGGACAGCTTTCTTGTACAAAGTGGCTAATGTCTTGTTCCTCAAA
Ds AflJ3' FlNK*att*B3 R	DsAfl35' Flank	1008	GGGGACAACTTTGTATAATAAAGTTGCGTCGAGCCAGGGTGCAGT

2.4.2 Standardisation of PCR conditions

PCR conditions were standardized to amplify a single specific amplicon by varying primer annealing temperature and keeping other parameters as per the polymerase manufacturer instructions. PCR were made up of 20 µL volumes containing: 20 ng genomic DNA, 100 µM dNTPs, 1.5 mM $MgCl_2$, 2.0 µL 10x PCR buffer 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.0), 5 pM of each primer and 1 U FIREPol® *Taq* DNA polymerase (Solis BioDyne, Estonia); sterile water was added to complete the final volume of the reaction. Cycling conditions were: initial denaturation step at 94°C for 5 min, followed by 30 cycles, each consisting of 94°C for 30 sec, annealing temperature depending on primer for 30 sec, and 72°C for 1 min/kb of amplicon, and with a final extension at 72°C for 10 min. For construction of vectors DNA polymerase with proofreading activity was used. In this case each PCR was made to a 50 µL volume

with the above constituents except $MgSO_4$ (1.5 mM) was used and 1U HiFi Platinum Taq DNA polymerase (Invitrogen, CA, USA).

2.4.3 Colony PCR for screening of clones

For confirmation of recombinant *E. coli,* cells were picked from colonies using sterile tips and suspended in 20 µl sterile water in a 1.75 ml microcentrifuge tube. The suspension was boiled for 10 min then transferred to ice, then centrifuged at 16,500 g for 5 min; and 2 µl of the supernatant was used in a PCR reaction.

2.5 Quantitative Real time PCR (qPCR) analyses

2.5.1 Primer design

All quantitative Real time PCR primers were designed using AlleleID® (Primear Biosoft) using default parameters for SYBR green except the primer annealing temperature was changed to 60-62°C.

2.5.2 PCR Cycling conditions

Relative quantification or transgene copy number prediction was performed using a LightCycler 480 SYBR Green 1 DNA Master kit (Roche, Penzberg Germany). 2 µL of 10-fold serially diluted cDNA were added to 8 µL PCR mix and subjected to 45 cycles of PCR (10 sec at 95°C, then 30 sec at each of 60°C and 72°C) with an acquisition temperature of 72°C. For melting curve analysis PCR products were heated to 95°C and then rapidly cooled to 65°C for 10 sec and then gradually increased to 97°C at the rate of 0.2°C/sec with continuous measurement of fluorescence at 520 nm. The Ct value is the cycle number at which the fluorescence emission of the PCR amplicon could be distinguished from the background. For all the expression analysis $C_t = 35$ cycles, were considered as the threshold: any amplification with a larger number of cycles was not

considered for analysis. Real time PCR was carried out using a LightCycler® 2.0 and all the data were analysed using RealQuant® version 1.1.1 (Roche, Basal, Switzerland)

2.5.3 Standard curves and gene expression analyses

For Real time PCR, a standard curve was prepared for each of the primer pairs when required, total RNA was extracted and cDNA synthesized as mentioned in section 2.7.1. A standard curve was constructed by plotting the logarithm of six 10-fold serial dilutions of *D. septosporum* cDNA or genomic DNA against the cycle threshold (Ct) values with three replications for each dilution. All data were processed using Real Quant 1.1.1 (Roche) and the standard curve was saved as an external standard in the database.

Expression analysis of all the dothistromin biosynthesis genes in various mutants and wild type strain of *D. septosporum* was done relative to the 18s rRNA gene or β-tubulin. Three technical replicates of each of three biological replicates were used for calibrator normalized relative quantification analysis, and the values were normalized by comparing to expression levels in the NZE10 wild type.

The results were expressed as a normalized ratios.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta\text{Ct target } (control\,-\,sample)}}{(E_{\text{ref}})^{\Delta\text{Ct ref } (control\,-\,sample)}}$$

Example: To study *AflR* gene expression in *LaeA* knockout strain:

Target gene is AflR;

Reference gene is *βtubulin* or 18s rRNA gene (stable constitutive expression).

Control is Wild type (WT) strain

Sample is Δ *LaeA* strain

E_{target} is the PCR efficiency of target gene primer ; E_{ref} is the PCR efficiency of reference gene primer.

For target gene; ΔCt target = Ct of *AflR* expression in WT – Ct of *AflR* expression in Δ *LaeA* strain.

For reference gene; ΔCt ref = Ct of *βtubulin* or 18s rRNA expression in WT – Ct of *βtubulin* or 18s rRNA expression in Δ *LaeA* strain. (Pfaffl, 2001).

The expression studies were done using β-tubulin or 18s rRNA as reference genes and the results obtained were consistent but only the β-tubulin data are presented.

2.5.4 Quantification of fungal biomass in infected pine needle

To estimate the biomass of *D. septosporum* in the infected pine needles, DNA was extracted from lesions of infected needles for each strain as mentioned above in 2.3.1 and vacuum-dried if necessary to yield a minimum concentration of 500 ng/μL. Primer and TaqMan probe sets for real-time PCR assays (Table 2.3) were designed to detect *D. septosporum* polyketide synthase *PksA* (as target gene) and *Pinus radiata* cinnamyl alcohol dehydrogenase *CAD* (as reference gene). The *PksA* probe was labelled with 6-carboxyfluorescein (FAM) at the 5' end and quencher dye BHQ-1 at the 3' end. The CAD probe was labelled with HEX (5') and BHQ-1 (3'). qPCR was carried out as mentioned in 2.5.2 with slight modification, 0.2 μM of each FAM-labeled and HEX-labeled probe and 0.4 μM forward and reverse primer for each target and reference gene, in a reaction volume of 10 μL was used. To estimate fungal biomass, standard curves were prepared as mentioned above in 2.5.3 with serial dilutions of pure genomic DNA of *D. septosporum* and *P. radiata* in distilled water (1000, 200, 40, 8, 1.6, 0.32, 0.064 ng for pine and pg for *D. septosporum*). The relative amounts of fungal biomass

in the samples were calculated as fungal target/plant reference and normalised by dry weight of the samples.

2.5.5 Predicting the copy number of transgene

Transgene copy number prediction was done following the strategy outlined by (Solomon, et al., 2010). To verify the copy number of the *AflJ* complemented strains by qPCR firstly a single copy reference gene (*DsAflR*) gene was selected whose copy number had been verified previously by an *in silico* genome search and Southern hybridisation. The standard curves for each of the primer pairs were prepared as mentioned in section 2.5.3. Genomic DNA from each of the *AflJ* complemented strains was extracted (2.3.2) and diluted to 10 ng/µl as this concentration was found to give optimal efficiency. qPCR was performed as mentioned above, and all concentrations were normalised to the single copy *DsAflR*. *D. septosporum* wild type strain DNA was taken as a positive control to cross check the copy number of *DsAflJ* and to validate the use of qPCR to determine gene copy number. All analysis was done using RealQuant 1.1.1 (Roche, Basel, Switzerland) software. Subsequent melting curve analysis was done to confirm the absence of any non-specific amplification.

2.6 Southern Blotting and hybridisation

Southern blot analysis was performed as described by Sambrook and Russel (2001) using DIG-dUTP labelled probes.

2.6.1 Digoxygenin-11-dUTP (DIG) labelling of probes

All probes used for Southern Blotting were labelled by a standard PCR protocol (section 2.4.3) with alkali-labile Digoxygenin-11-dUTP (Roche) where the ordinary dNTPs were

replaced by 200 µM of each dATP, dGTP and dCTP, 133 µM dTTP and 67 µM alkali-labile Digoxygenin-11-dUTP (Roche) per 25 µL reaction.

2.6.2 Optimization of the probe concentration – the "mock" hybridization

Very high probe concentration can cause significant background noise. To prevent this, the probe concentration was optimized in a mock hybridization before the actual hybridizations were performed. The mock hybridization was carried out by incubating small membrane pieces (without DNA transferred to it) with different probe concentrations. 5-fold serial dilutions of the probe were made with sterile Milli-Q water, and 2 µL of each dilution was spotted on an Amersham Hybond™-N+ membrane (GE Healthcare Ltd) and detected following standard protocol. Then the membrane was cross-linked with UV irradiation in (Cex-800 UV crosslinker, Ultra-Lum Inc). After cross-linking the membrane was rinsed briefly in sterile distilled water and air-dried and detected following standard protocol as mentioned below (section 2.6.5). The highest probe concentration that gave an acceptably low background was used for the hybridization experiment.

2.6.3 Southern blot

Southern blot analysis was performed as described by Southern (1979) with some modifications. In order to check the copy number and site of insertion, DNA from each of the PCR positive fungal strains was isolated using the CTAB method (section 2.3.1). 2 µg of restriction endonuclease digested DNA was loaded on a 0.8% large (20 cm) agarose gel and run at (1.75 V/cm) overnight. The gel was stained and documented as described in section 2.3.3. The gel was then submerged for 15 min in 0.25 M HCl to depurinate the DNA and then rinsed with water. This was followed by submerging the

gel in denaturing solution (Appendix: 8.2.4) for 2 x 15 min on an orbital shaker and then submerging in neutralisation solution for 2 x 15 min at room temperature.

To blot the DNA from the gel by capillary action the transfer apparatus was assembled as described by Sambrook and Russell (2001). Three pieces of 3 mm Chromatography Paper (Whatman, Maidstone, UK) wick larger than the gel were saturated with transfer buffer 20 x SSC (Appendix: 8.2.4) and placed on a glass plate with both ends of the wick dipping in transfer buffer. The denatured gel was placed on the top of the wick (upside down), and then Amersham Hybond™-N+ membrane (GE Healthcare Ltd), was carefully placed on the gel without any air bubbles in between the two layers. Over that 2-3 pieces of 3 mm Chromatography Paper (Whatman, Maidstone, UK) of gel size were placed and a stack of paper towels was placed above, followed by 500 g of weight above it. The sides of the gel were covered with glad wrap and allowed to stand overnight.

After blot transfer overnight, the membrane was taken out and gently rinsed in 2x SSC buffer. Then the membrane was cross-linked with UV irradiation (Cex-800 UV crosslinker, Ultra-Lum Inc). After cross-linking the membrane was rinsed briefly in sterile distilled water and air-dried.

2.6.4 Pre hybridisation and hybridisation with DIG labelled probes

Prehybridization blocks non-specific nucleic acid-binding sites on the membrane and this ultimately served to lower background noise. The membrane was prehybridised by placing the membrane in reconstituted DIG Easy Hyb Granules (Roche) for 2 hours, using a hybridisation tube, and was incubated in a Bachofer hybridisation oven at 42°C. The double stranded gene specific probes were heated in a boiling water bath for 10 min to denature the DNA and chilled directly on ice. An appropriate amount of the probe as

previously determined in section 2.6.2 was added to the hybridization solution and allowed to hybridizing overnight.

The following day the hybridization solution was poured into a 50 ml Schott Duran bottle and kept in the -20°C freezer for re-use if needed in the future. Excess probe was removed by washing the membrane twice, for 5 min per wash, in wash buffer I (appendix 8.2.4) at room temperature followed by two stringent washes for 15 min per wash, in wash buffer II (Appendix: 8.2.4) at 68°C.

2.6.5 Signal detection

After hybridization and post-hybridization washes, the membrane was washed with Buffer I (Appendix 3.13) for 2 min, followed by incubation in 30 ml of buffer II *(*Appendix 8.2.4) blocking solution for 30-45 min with agitation on an orbital shaker. At the end of the blocking period, Anti-Digoxigenin-AP antibody (Roche) was centrifuged briefly at 15,682 g for 5 min and added at the ratio of 1:10,000 in blocking solution. The membrane was incubated for 30 min in the antibody solution. The antibody solution was discarded, and the membrane was gently washed twice, 15 min per wash, in buffer I (Appendix: 8.2.4) followed by equilibration with buffer III (Appendix: 8.2.4) for 5 min. The membrane was placed on an A4 copy safe pocket with the DNA side facing up, and 0.5-1 ml of CSPD® ready-to-use chemiluminescence substrate (Roche) was applied to the membrane. The copy safe pocket was sealed and the CSPD gently spread throughout the membrane ensuring no bubbles then incubated at 37°C for 10 min. For detection of the signal, the membrane was exposed to standard X-ray film initially for 15–20 min and then the exposure time was adjusted depending on the signal strength.

2.6.6 Stripping of the probe and re-hybridisation

The luminescent signal was removed by a short wash of the membrane with water. For the subsequent removal of probe, the membrane was transferred to a tray, and 100 ml of

stripping buffer **(**Appendix: 8.2.4) was added and incubated in a rocking platform for 10 min at 37°C. Then the membrane was washed for 5 min in wash buffer I and could then be used again.

2.7 RNA extraction and manipulation

2.7.1 RNA extraction and DNase treatment

Fungal mycelium was harvested by filtration through a sterile nappy liner and then snap- frozen in liquid nitrogen and stored at -80°C until used. All fungal and infected pine needle RNA was extracted using Trizol® (Invitrogen) or Spectrum™ Plant Total RNA Kit (Sigma, MO, USA) following the manufacturer's protocol. RNA was made free of DNA contamination by treating with Turbo DNA - free™ kit, (Life technologies) and the purity and concentration was determined on a Nanodrop (Thermo Scientific, MA, USA).

2.7.2 Formaldehyde gel electrophoresis

RNA can form both secondary and tertiary structures which hinder its separation by usual gel electrophoresis, so the electrophoresis needs to be performed under denaturing conditions (Bryant and Manning, 1998). Prior to gel preparation the gel box, comb and tray were treated with 0.1 M NaOH for 30 min and rinsed with deionised water. For preparing 100 ml of formaldehyde gel, 1 g of agarose was added to 73 ml of RNase free water and boiled in a microwave oven for 2 min and then transferred to a water bath at 50°C. Once the solution cooled 17 ml of 37% formaldehyde and 10 ml of 10 x formaldehyde agarose gel buffer (Appendix:8.2.5) were added, mixed gently and poured onto the gel unit. RNA samples were prepared by the addition of four volumes of RNA to one volume of 5 x loading buffer (Appendix: 8.2.5) and incubated at 65°C

for 10 min, and then chilled on ice and loaded onto the equilibrated formaldehyde agarose gel. The gel was run at 5–7 V/cm in 1x formaldehyde agarose gel running buffer and then was stained and visualised as described in section 2.3.3.

2.7.3 DNA contamination check and cDNA synthesis

Prior to cDNA synthesis, RNA samples were checked for genomic DNA contamination. A PCR reaction was set up using rt TUB fw II and TUB 4 primers with RNA as template. These primers bind to the intron amplifying only in the presence of genomic DNA. A PCR with genomic DNA and no template were run as control. PCR products were run and visualised as discussed in section 2.4.1. RNA samples that failed to yield product in these reactions, indicating lack of gDNA contamination, were used for cDNA synthesis. The cDNA was synthesized by random hexamer primed reverse transcription using Superscript^{TM} III reverse transcriptase (Invitrogen, CA, USA) or a qScript cDNA synthesis kit (Quanta Biosciences, MD, USA) following the manufacturer's instructions. cDNA was diluted 5-10 fold and stored at 4°C until used.

2.8 Extraction detection and quantification of dothistromin

2.8.1 Extraction of secreted dothistromin from media

The culture was grown as in section 2.2.2.2. Dothistromin was extracted from broth cultures by agitation for 72 h at 30°C with an equal volume of ethyl acetate, acidified with 1% formic acid. The mixture was centrifuged at 3217 x g for 5 min in an Eppendorf A-4-62 swing bucket rotor (Eppendorf, Hamburg, Germany), then 200 µl of the ethyl acetate supernatant transferred to a microcentrifuge tube and dried overnight in a fume hood. The toxin was re-suspended in 10 µl of acidified ethyl acetate for TLC samples, or in 200 µl acetonitrile acidified with 1% formic acid for HPLC samples.

2.8.2 Extraction of intracellular dothistromin from mycelium.

For extraction of intracellular dothistromin, 100 mg fresh wet mycelium harvested from broth was placed in a 2 ml cryovial and disrupted using a Thermo Savant FastPrep® FP120 cell disrupter (Thermo Electron Corporation, MA, USA) with 500 µl ethyl acetate and 2-3 x 5 mm steel beads (Quagen, Hilden, Germany) per tube for ten bursts of 20 sec at speed level 5. The tube was then kept in a shaker at 180 rpm for 72 hrs followed by centrifugation at 3217 x g for 5 min; the supernatant containing dothistromin was transferred to a fresh tube and kept at 4°C until further use.

2.8.3 Extraction of dothistromin from infected pine needle

For extraction of dothistromin from infected needles and needle lesions, 1 ml of acidified ethyl acetate was added to ground samples in 1.75 ml microcentrifuge tubes. The tubes were then wrapped with aluminium foil and placed on a shaker table for 72 hrs. Following extraction the tubes were centrifuged at 15,682 x g for 5 min, and the ethyl acetate supernatant transferred to new tubes and kept at 4°C until further use.

2.8.4 Thin layer chromatography (TLC)

For qualitative assessment of dothistromin and other metabolites, TLC was performed using glass-backed, 200 µm analytical plates (Merck TLC Silica Gel 60). 10 µl of each of the ethyl acetate samples was spotted onto the plates along with 1 µg of purified dothistromin standard in 10 µl of acidified ethyl acetate. The TLC was run in a glass chamber containing 1 cm depth of solvent (80:20 toluene:acetone for separation of secondary metabolites or 50:3 chloroform:methanol for optimal resolution of dothistromin; each solvent acidified with 1% formic acid) until the mobile phase had travelled approximately 80% of the distance. The plate was then removed from the

mobile phase, and the solvent front was marked with pencil and air dried in the dark before imaging under UV illumination using a Bio-Rad Gel documentation system.

2.8.5 High performance liquid chromatography (HPLC)

For quantitative assessment of dothistromin, acetronitrile samples prepared as above were filtered through minisart RC4 0.2 µm syringe filters (Sartorius) into labelled septum vials. A Dionex HPLC system (Sunnyvale, CA) was used with a C-18 4.7 x 150 mm column (Phenomenex, Torrance, CA), a solvent flow rate of 1 mL/min and injection volume of 50 µL. The gradient was maintained for five minutes at 5% acetonitrile, with a rise to 75% over the next 30 min, followed by a rise to 100% at 38 min, dropping back to 5% between 42 and 45 min, and a 15 min equilibration. UV spectra were measured with a Dionex UVD340U diode array detector, and fluorescence responses (excitation wavelength 470 nm, emission wavelength 545 nm) using a Dionex RF2000. Data were processed using Dionex Chromeleon software (http://www.dionex.com). Dothistromin was quantified by fluorescence peak area at 23.1 ± 0.05 min. The standard curve was determined from a 10 fold dilution series of purified dothistromin, which was kindly provided by Dr Robert Franich of Scion (Rotorua, New Zealand). The standard in the range 0.001 to 100.0 ng/ml, and concentrations of unknowns were determined from this standard curve.

2.9 Transformation of *Dothistroma septosporum*

Dothistroma septosporum transformation was done using a protoplast based method as described by Bradshaw et al. (1997; 2007)

2.9.1 Preparation of protoplasts

For preparation of protoplasts, a fungal culture was grown as in section 2.2.2.2. Mycelium was harvested by filtering thorough nappy liners (Johnson and Johnson) followed by three washes with sterile water and two with OM buffer (Appendix: 8.2.3) and then transferred to a 125 ml conical flask. Digestion of the fungal cell wall was done by incubating 1 g of mycelium in filter sterilised (0.2 µm Minisart acrodisc) Glucanex® 200G (Novozymes) (10 mg/ml) in OM buffer (Appendix 8.2.3) to give a final concentration of 50 mg mycelium per ml and incubated for 12 hrs at 30ºC with shaking at 100 rpm on an orbital shaker. The protoplasts were filtered through a nappy-liner into 15 ml Corex tubes (5 ml/tube) followed by the addition of 2 ml ST buffer (Appendix 8.2.3) overlay. After 5 min centrifugation at 3,000 x g (F21J-8 x 50 rotor; Sorvall), the protoplasts formed a milky layer at the buffer interface. Protoplasts and the upper buffer layers were transferred into clean corex tubes, washed with 5 ml STC buffer **(**Appendix: 8.2.3) and centrifuged for 5 min at 4,274 x g (F21J-8 x 50 rotor; Sorvall, MA, USA). This wash step was repeated three times, and then the protoplasts were resuspended in 0.5 ml STC buffer. The concentration of protoplasts was determined using a haemocytometer and stock was diluted in STC buffer to a concentration of 1.25 x 10^8 protoplasts/ml.

2.9.2 Transformation procedure

In *D. septosporum* the transformation efficiency is good but high frequency transformation results from non homologous (ectopic) integration into the genome as well as homologous integration. Several attempts at transformation using circular plasmids resulted in failure to get homologous recombination so an attempt was made to transform linearised plasmid vector instead.

5 µg of circular or linear plasmid DNA with 20 µl of 40% PEG solution (Appendix 8.2.3) and 80 µl of 1.25 x 10^8 protoplasts/ml was placed in a 1.75 ml microcentrifuge tube, and vortexed briefly then incubated on ice for 30 minutes. 900 µl 40% PEG solution was added to each reaction, mixed, and then incubated at room temperature for another 20 min. 100 µl aliquots of reaction mix were transferred to Falcon tubes, mixed with 0.8% regeneration overlay medium maintained at 50°C (Appendix 8.1.3), and spread over pre-poured RG (Appendix 8.1.3) plates. The plates were incubated overnight at 22°C. For selection of transformants either hygromycin B (Roche Penzberg, Germany) or phleomycin (Apollo scientific Ltd, Stockpott, UK) at 70 µg/ml and 7 µg/ml concentration was used. Three different controls were used in each transformation. Protoplasts diluted to 10^2, 10^3 and 10^4 fold in STC buffer (Appendix 8.1.3) were plated without antibiotic selection to get the viable cell count. For a negative control, undiluted protoplasts were spread on RG media with respective antibiotics. For a positive control protoplasts transformed with 5 µg of pBC-Hygro (pR223) (Appendix: 8.3.4) or pBC-Phleo (pR224) (Appendix 8.3.5) were plated on appropriate selective media.

2.9.3 Co-transformation

In cases of co-transformation recipient cells are exposed to two different kinds of DNA simultaneously; there is a high probability that a cell that takes up one will also take up the other. This was first demonstrated in *A. nidulans* (Fincham, 1989). For the complementation of *ΔDsAflR* mutant FJT104, 2.5 µg of each PCR amplicon 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 . were used for co-transformation and the recombinants were selected on 7 µg/ml of phleomycin.

2.9.4 Pathogenicity assays

Dothistroma septosporum spores (1x 10^6 /ml) collected from 8 days old DSM cultures, were sprayed onto 1 year old *Pinus radiata* seedlings, 3 replicate trees for each strain. The pine seedlings, approx 40 cm tall, were individually covered using ventilated plastic bags and placed in a plastic container with 2 water misters to maintain high humidity under natural light conditions in a glasshouse. After 8-10 weeks the needles were collected and needle blight symptoms assessed by calculating the percentage of needles showing infection symptoms (lesions with fruiting bodies) and the numbers of lesions per needle. The experiment was repeated twice.

2.10 Phenotypic characterisation of *D. septosporum DsLaeA* and *DsVeA* mutants

2.10.1 Radial growth rate in light and dark condition

Plates were inoculated with cultures prepared as mentioned previously in section 2.2.2. 5 mm diameter agar plugs were removed with a sterile cork borer from the leading edges of colonies, and one such plug was placed in the centre of each 90 mm Petri plate containing DM agar. Three replicates were taken for each strain or condition. Plates then were wrapped with Parafilm (Bemis) and then incubated at 22°C. The colony diameter in each plate was measured along two axes perpendicular to one another at two day intervals until day sixteen, and then every three days until day 31 post inoculation, and the two measurements for each day were averaged. Daily radial growth rates were calculated to give a final value.

2.10.2 Spore production in light and dark condition

To assess sporulation, 100 µl of freshly collected spores (1 x 10^5/ml) were spread over three PMMG plates for each strain. After 10 days of growth, 5 ml of sterile water was added to the plate, spread with a sterile glass spreader and allowed to stand for 10 min with intermittent spreading. The spore suspension was poured into a 15 ml centrifuge tube and the same spore harvest steps were repeated once. The spore concentration was quantified using a haemocytometer (Weber Scientific, Middlesex, England) and expressed as number of spores per ml of water.

2.10.3 Germination rate

The germination rate was determined by spreading freshly-grown spores (from 10 d DSM light-grown cultures) at 10^5 per ml concentration onto a water agar plate overlaid with cellophane membrane. After 12 and 48 hours and incubation at 22°C 25 µl of the inoculum was taken from the water agar plate, spotted on a microscope slide and viewed under microscope (Zeiss). The spores that generated germ tubes were counted and the percentage of spore germination was calculated. Approximately 200 conidia were scored for each sample. Because some spores had germinated in the 0 h samples, the germination rate was taken as the difference in % germination between 0 and 12 - 48 hrs.

2.10.4 Hydrophobicity assay

To compare the hydrophobicity of colony surfaces, 30 µl of water or 0.2% bromophenol blue water solution was placed on the surface of a fungal colony grown on DM or PDA media and visualised at 10, 30 and 60 min. Fungal colonies with reduced hydrophobicity are more hydrophilic so the bromophenol blue water solution is readily absorbed whereas in hydrophobic colonies the spherical droplet of dye is maintained.

2.11 Microscopy

Hyphal and spore morphology was observed using a Zeiss Axiophot compound light microscope (Zeiss Axiophot, Germany) equipped with a DFC320 digital camera (Lecia Microsystem, Wetzlar, Germany).

2.12 Statistical analysis

Student t-test was conducted using Microsoft Excel to compare values such as the expression of dothistromin genes between wild type, knockout and complemented strains. Tests were conducted to consider the null hypothesis of no difference between wild type and other strains at $P \leq 0.05$.

Pearson correlation was used to analyse the qPCR data of dothistromin gene expression in the time course experiment of Ds*LaeA* KO mutant. using the on-line program at http://department.obg.cuhk.edu.hk/researchsupport/PearsonCorrelation.asp.

Chapter 3: DsAflR regulates the fragmented dothistromin biosynthesis gene cluster.

3.1 Introduction

In fungi genes involved in the biosynthesis of secondary metabolites are generally clustered and co-regulated (Keller and Hohn, 1997; Martin and Liras, 1989). Among them aflatoxin (AF) and sterigmatocystin (ST) are very well characterized and this enabled the characterization of dothistromin biosynthesis genes, based on shared characteristics with these toxins (Bradshaw, et al., 2002 ; Bradshaw, et al., 2006; Zhang et al., 2007). However, instead of being clustered dothistromin genes are arranged at six separate loci on a 1.3-Mb chromosome (Schwelm and Bradshaw, 2010; Zhang et al., 2007). Although ten dothistromin 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 dothistromin genes were difficult to locate since they are not contiguous. The *D. septosporum* genome that was sequenced by the Joint Genome Institute (http://genome.jgi-psf.org/ Dotse1/Dotse1.home.htm) was assembled to chromosome level allowing identification of more biosynthesis genes at additional loci. A similar fragmented arrangement of dothistromin genes was also found in the genome of the closely related tomato pathogen, *Cladosporium fulvum*, a species not known to produce dothistromin (De Wit et al., 2012).

Many fungal secondary metabolism gene clusters encode a single transcription factor (Bergmann et al., 2007; Chiang et al., 2009; Chiang et al., 2010) that regulates the expression of the biosynthesis pathway. 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* (*ΔaflR*) 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 *ΔaflR* strain. Eighteen of the genes differentially expressed on the microarray were AF biosynthetic genes with a putative consensus AflR binding site (5'-TCGN$_5$CGR-3') in their promoters (Price et al., 2006) but recent studies report that the promoters of almost all AF cluster genes contain AflR binding sites (Ehrlich, 2009; Ehrlich et al., 2008). Similarly the gliotoxin and aspyridone biosynthesis gene in *Aspergillus* spp. is arranged in a single cluster and regulated by a GAL4-type Zn_2Cys_6 binuclear (Bergmann et al., 2007; Bok et al., 2006) type transcription factor.

Fungal genes involved in biosynthesis of a secondary metabolite are usually co-regulated and clustering is thought to be important for co-regulation (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). A previous study from our laboratory had demonstrated that the ten dothistromin genes identified earlier showed they were co-regulated but also revealed an unusual pattern of expression (Schwelm et al., 2008). Dothistromin is produced mainly during early exponential phase in culture, instead of during late exponential and stationary phases as is normally seen for secondary metabolites such as AF/ST (Schwelm et al., 2008), leading us to question how the physically separated set of dothistromin genes is (co)-regulated in *D. septosporum*.

To address this question the objectives of this study were:

1) To identify and characterize the GAL4-type Zn_2Cys_6 type transcription factor within the dothistromin cluster and determine its role in the regulation of dothistromin biosynthesis.

2) To identify additional genes involved in the pathway and perform functional analysis of some of them.

3) To compare dothistromin genes in *C. fulvum* to those of *D. septosporum* to determine why *C. fulvum* does not produce dothistromin.

This work has been published (Chettri et al., 2013) with input from co-authors.

3.2 Results

3.2.1. Organization, re-naming and putative functions of the predicted set of *D. septosporum* dothistromin biosynthesis genes.

The sequencing of the *D. septosporum* genome enabled rapid identification of a complete set of candidate Dothistromin biosynthetic and regulatory genes. Dothistromin genes have been named according to their orthologs in the AF biosynthetic pathway. After consultation by Dr R.Bradshaw with the AF research community it was decided to use the old-style descriptive gene names (eg. *vbsA, verB*) instead of the newer *afl* letter codes (eg. *aflK, aflL*) (Yu et al., 2004). However, the Dothideomycete gene-naming format 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 3.1. In some cases there has been a change in name of dothistromin 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. To distinguish between orthologs from two species the genes will be referred to

with a prefix such as *DsAflR* for *D. septosporum AflR* and *CfAflR* for *C. fulvum AflR* when required.

Genes involved in the biosynthesis of dothistromin are scattered and present in six loci across chromosome 12 (De Wit et al., 2012; Bradshaw et al., 2013; Chettri et al., 2013). The biosynthesis steps up to formation of versicolorin A (VERA) are predicted to be similar to those in aflatoxin for which the mechanism and chemistry involved have been studied well (Yu et al., 2004; Ehrlich et al., 2009; Ehrlich et al., 2010; Wen et al., 2005; Yabe et al., 2004); we have referred to these thirteen dothistromin genes as 'core genes' Figure 3.1 and Table 3.1. In *D. septosporum* five of these genes: *PksA* (Bradshaw et al., 2006), *VbsA* (Zhang, 2007), *HexA* and *AdhA* (Chettri et al., 2013; Olson, 2012) and *Ver1* (Bradshaw, 2002) have been functionally characterized. Dothistromin loci contain several other genes that are candidate DOTH biosynthetic genes. Putative orthologs of late pathway AF/ST genes occur in locus 5 (*OrdB*) and on chromosome 11 (*NorB*). No orthologs of the late pathway AF genes of *A. flavus ordA* (*aflQ*), *omtA* (*aflP*), *omtB* (*aflO*), or *verA* (*aflN*) were found in the *D. septosporum* genomes 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 cluster 1 (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 (160897). However, there was no evidence for a sugar cluster adjacent to dothistromin genes as seen for the AF cluster in *A. parasiticus* (Yu et al., 2000); the closest matches to the four sugar cluster genes were found on chromosomes 1 (*nadA, glcA*) and 2 (*hxtA, sugR*) in *D. septosporum*.

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
Ver1[a]*	192193	264	2	7	0.03	*aflM*	*stcU*	79.1	79.2
*DotB**	75412	415	0	11	0.09	-	*(stcC)*	-	24.0
*DotC**	75413	581	3	15	0.22	*(aflT*[e]*)*	-	31.2	-
*PksA**	192192	2400	2	2	0.13	*aflC*	*stcA*	55.1	58.2
CypX[a]	139960	512	2	4	0.50	*aflV*	*stcB*	58.9	61.6
AvfA	75546	285	0	4	0.14	*aflI*	*stcO*	49.3	44.9
EpoA	57187	421	1	6	0.01	-	-	-	-
MoxY[a]	75547	627	5	11	0.21	*aflW*	*stcW*	55.4	50.6
*AflR**	75566	480	1	2	0.40	*aflR*	*aflR*	27.5	30.4
*AflJ**	57214	457	2	1	0.08	*aflS*	*aflJ*	37.1	40.8
Est1	75609	329	3	1	0.22	*(aflJ)*	*(stcI)*	29.6	27.8
*OrdB**	75648	268	0	1	0.11	*aflX*	*stcQ*	54.1	45.3
AvnA	57312	526	3	9	0.08	*aflG*	*stcF*	57.5	58.3
HexB	181128	1905	4	2	0.11	*aflB*	*stcK*	52.2	46.0
*HexA**	66976	1693	2	2	0.28	*aflA*	*stcJ*	55.4	45.5
HypC	75655	186	1	1	0.37	*aflZ*	*stcM*	35.2	47.9
*VbsA**	75656	648	1	4	0.45	*aflK*	*stcN*	72.3	73.1
Nor1	75691	269	3	2	0.32	*aflD*	*stcE*	59.7	58.5
*AdhA**	48495	307	2	2	0.06	*aflH*	*stcG*	58.1	60.6
VerB	75692	521	2	7	0.42	*aflL*	*stcL*	67.1	67.6
*NorB**	75044	392	0	4	0.18	*aflF*	*stcV*	60.7	43.6

Table 3.1: Characteristics of dothistromin genes in *D. septosporum*

Genes are listed by position (top to bottom) in loci 1–6 or on chromosome 11 (*NorB*).

[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 nu
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, (Malin Olson BSc Hons. Thesis 2012).

[d] Expression determined by qRT-PCR and expressed relative to beta-tubulin expression (see **Appendix 8.4.6** for full data).

[e] Brackets indicate orthology to *D. septosporum* gene is not supported by phylogenetic studies (Bradshaw et al., 2013)

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

*Function confirmed by gene knockout

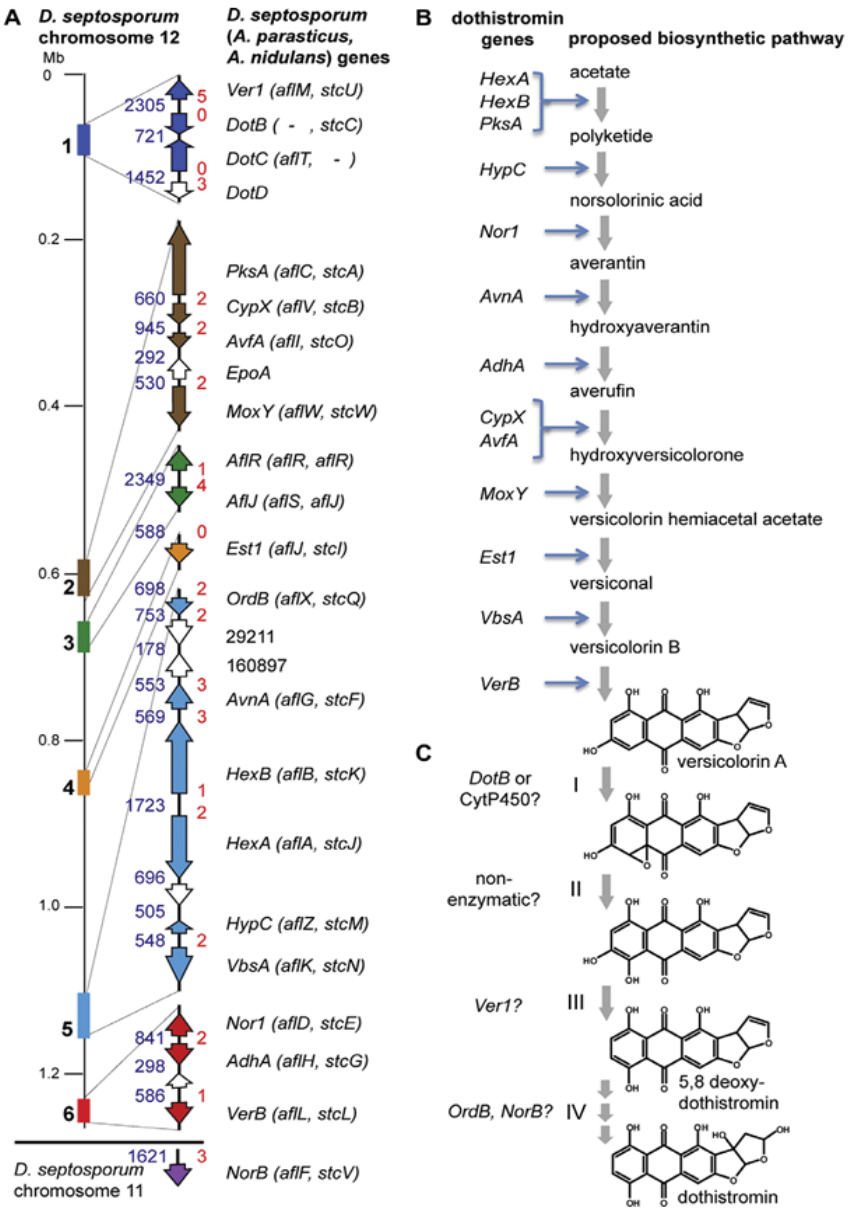


Figure 3.1: The fragmented arrangement of dothistromin genes and proposed scheme for dothistromin biosynthesis. (A) The six loci (labeled 1–6) are shown along chromosome 12, with corresponding predicted dothistromin genes shown alongside as shaded arrows indicating the direction of transcription. Unshaded arrows represent genes not thought to have a role in dothistromin biosynthesis. Sizes of intergenic regions (bp) are shown on the left. Numbers of putative AflR binding sites are shown on the right (see Table 3.2 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 dothistromin and aflatoxin have common steps as far as versicolorin A. *D. septosporum* dothistromin 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 dothistromin is shown, based on that described by Henry and Townsend (2005), with predicted gene designations as discussed in the text (Chettri et al., 2013). Figure was prepared by Rosie Bradshaw.

3.2.2 The *D. septosporum* pathway regulator AflR and its binding site

The DsAflR (*D. septosporum* PID 75566) was originally identified through homology searches of the *D. septosporum* genome sequence at (http://genome.jgi-psf.org/Dotse1/Dotse1.home.html) by using BLASTP and reciprocal BLASTP algorithm with the *A. parasiticus* (AAS66018) and *A. nidulans* (AAC49195) AflR that regulate aflatoxin/sterigmatocystin biosynthesis pathways (Ehrlich et al., 1999) as queries. Further, the phylogenetic analysis (Appendix 8.4.7) revealed that DsAflR forms a clade with known AflR proteins (aflatoxin/sterigmatocystin regulators) rather than with other GAL4-type Zn_2Cys_6 transcription factors like SirZ and GliZ that regulate sirodesmin PL and gliotoxin production in *Leptosphaeria maculans* and *Aspergillus fumigatus* respectively (Gardiner et al., 2004; Bok et al., 2006). Further analysis revealed that it was divergently transcribed from another regulatory gene AflJ, a synteny which is conserved in the AF/ST cluster.

The *DsAflR* gene encodes a predicted polypeptide of 350 amino acid residues (protein ID 75566) that is similar to AflR proteins from other ascomycetes, for example 36.6% amino acid identity to *A. nidulans* AflR (AAC49195), 32.1% to *A. parasiticus* (AAS66018) and 83.6% to *C. fulvum* AflR (Figure 3.2). Alignment of the predicted amino acid sequence to those of other AflR peptides revealed conserved features. A Cys_6Zn_2 DNA-binding motif which is involved in transcription activation (Chang et al., 1995; Ehrlich et al., 1998; Ehrlich et al., 1999b; Cary et al., 2000) is conserved in all the four fungi. The nuclear localisation signal RRARK is present only in *A. parasiticus* (Ehrlich et al., 1998) though it could not be predicted using PSORT II. The linker region (marked in green in Figure 3.2) is presumed to determine DNA-binding specificity and the arginine residue (R) has been implicated in AflJ binding in *A. parasiticus* (Chang, 2003); this is also present in all the sequences compared. However,

a PEST (proline, glutamine, serine, and threonine-rich region) motif that may be a target for ubiquitin-mediated proteolysis (Rechsteiner, 1988) is absent in DsAflR.

Transcription factors of the C6 class are, in general, positive regulators possessing a characteristic DNA-binding domain and an activation domain (Evans and Hollenberg, 1988). In *A. nidulans* and *A. parasiticus* AflR binds to the palindromic sequence TCG (N_5) CGR that is found in the promoter regions of several ST and AF genes. However, a variant, TCG (N_{10}) TCG, has also been shown to have binding affinity for AflR (Fernandes et al., 1998). Analysis of the upstream regions of putative dothistromin biosynthesis genes in *D. septosporum* and *C. fulvum* showed most of them to possess TCG (N_5) CGR sites (Table 3.2) except for *DsEst1, DsDotB* and *DsDotC* in *D. septosporum* where the N_{10} variants are present. In *C. fulvum*, however, the variants are present in *CfDotC* but are absent in the upstream regions of other genes.

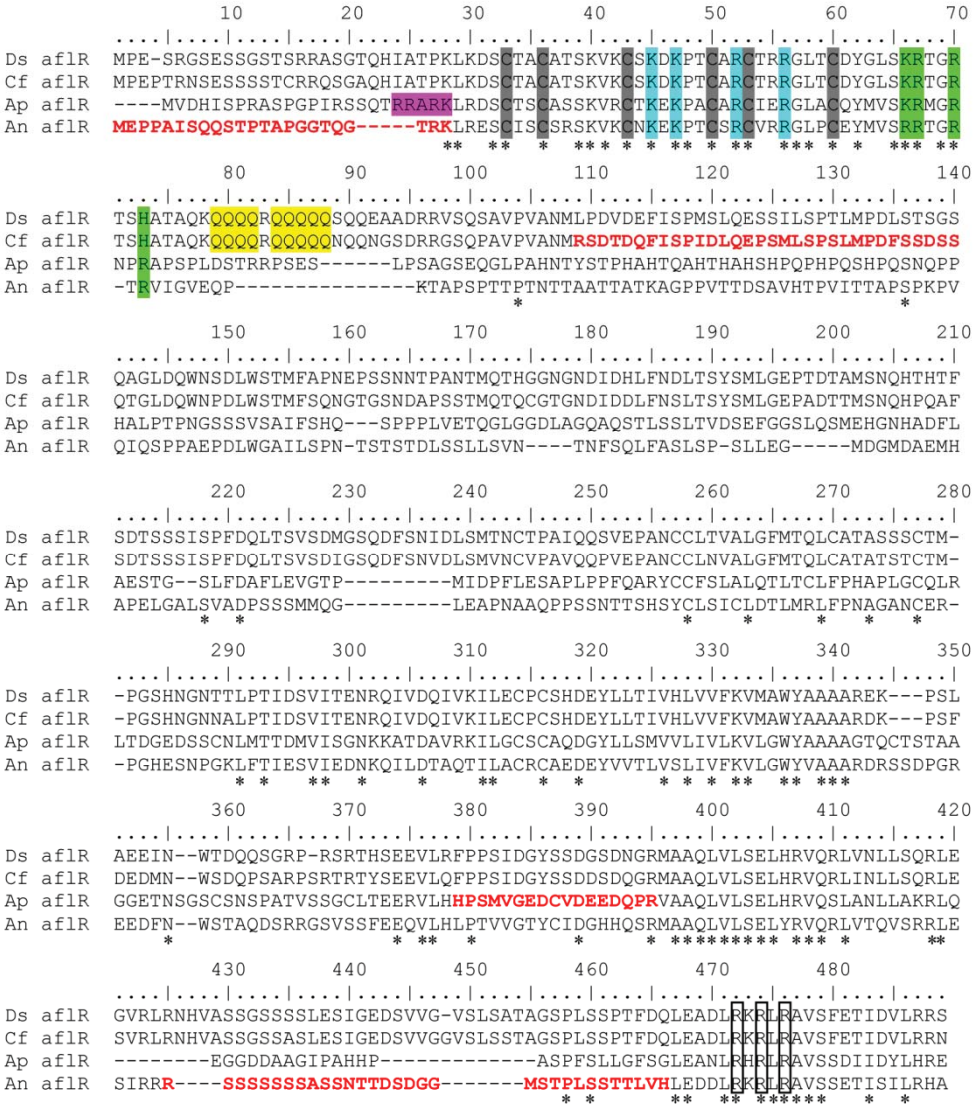


Figure 3.2: Multiple alignment of predicted AflR protein sequences.

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. The LINKER sequence, ie the basic amino acids marked in green immediately C-terminal to the C6-cluster, are thought to determine DNA-binding specificity in *Aspergillus* spp. and are present in all four fungi. PEST motif marked red is absent in DsAflR and the nuclear localization signal highlighted in pink could be predicted only in ApAflR. The conserved amino acids are marked with asterisks. C-terminal arginine residues (in blocks) implicated in AflJ binding in *A. parasiticus* (Chang, 2003) are also conserved. *D. septosporum* and *C. fulvum* proteins have a glutamine-rich motif (yellow) which is not found in the *Aspergillus* AflR proteins.

3.2.3. *D. septosporum AflR* gene regulates dothistromin biosynthesis.

To seek evidence for co-regulation of all predicted dothistromin genes by the putative pathway regulator DsAflR, gene deletion mutants were made. Protoplast-mediated transformation of *D. septosporum* with the *AflR* gene knockout construct yielded 20 hygromycin-resistant transformants. Of these, three (KO1, KO2 and KO3) were confirmed to be *DsAflR* knockout strains by PCR (Figure 3.3 A) using two pairs of primers that each targeted the hygromycin B coding region and flanking region either 5' or 3' side (Figure 3.3 D). Southern hybridization confirmed the absence of *DsAflR* in the knockout mutants (Figure 3.3 C) and that *DsAflR* is a single copy gene. The KO3 transformant had an additional hybridizing fragment of ~1kb, indicative of an additional ectopic integration site of the plasmid; this strain was not used in subsequent analyses. A complemented strain was generated by co-transformation of *DsAflR* gene knockout strain KO1 with full length *DsAflR* (methods section 2.9.3) along with phleomycin plasmid (pR224). The colonies growing on phleomycin antibiotic plates and showing restoration of pigmentation (thought to be dothistromin), were selected and confirmed by PCR (Figure 3.3 B). Southern hybridization showed that, whilst complemented strain C1 had one copy of *DsAflR*, strain C2 contained more than one copy.

Table 3.2: Putative AflR binding sites in the upstream regions of *Dothistroma septosporum* and *Cladospori* genes.

Gene name	Up stream seq (nt)	Putative AflR binding site TCG[N_5]CGR Distance from Translation start site (nt)							
		D. septosporum					*C. fulvum*		
Ver1	1000	681	506	387	365(-)	204	352		
DotB	1000						657		
*DotC**	1000								
DotD	1000	276	148(-)	24			277		
PksA	660	547	488				547	488	1
CypX	660	186	127				536	187	
AvfA	945	988	51(-)				497		
EpoA	530	275	250(-)						
MoxY	530	269	248				270	245	
AflR	1000	283							
AflJ	1000	317	166	143(-)	12(-)			730	
*Est1**	1000						451		
OrdB	1000	788(-)	364(-)				901(-)	694	3
Ds29211	753	617	34(-)				186(-)	123	
Ds160897	1000	925(-)	840(-)	39			735(-)		
AvnA	569	241(-)	186(-)	123			536		
HexB	1000	743							
HexA	1000	538	46				882	410	
HypC	548	393	386(-)				267	260(-)	
VbsA	548	358	351				389	382	
Nor1	841	198(-)	165				756	335(-)	
AdhA	841	690	657				697	276	
VerB	586	151					401	155	
NorB	1000	553	379	222(-)					

(-) indicates site is on reverse strand

Upstream regions less than 1000 bp are due to a shorter intergenic region.

* *DsDotB, CfDotC, CfHexA and CfHexB* do not have AflR binding sites but (TCG[N_{10}]CGR variants are present; *D* have neither AflR nor (TCG[N_{10}]CGR variant binding sites.

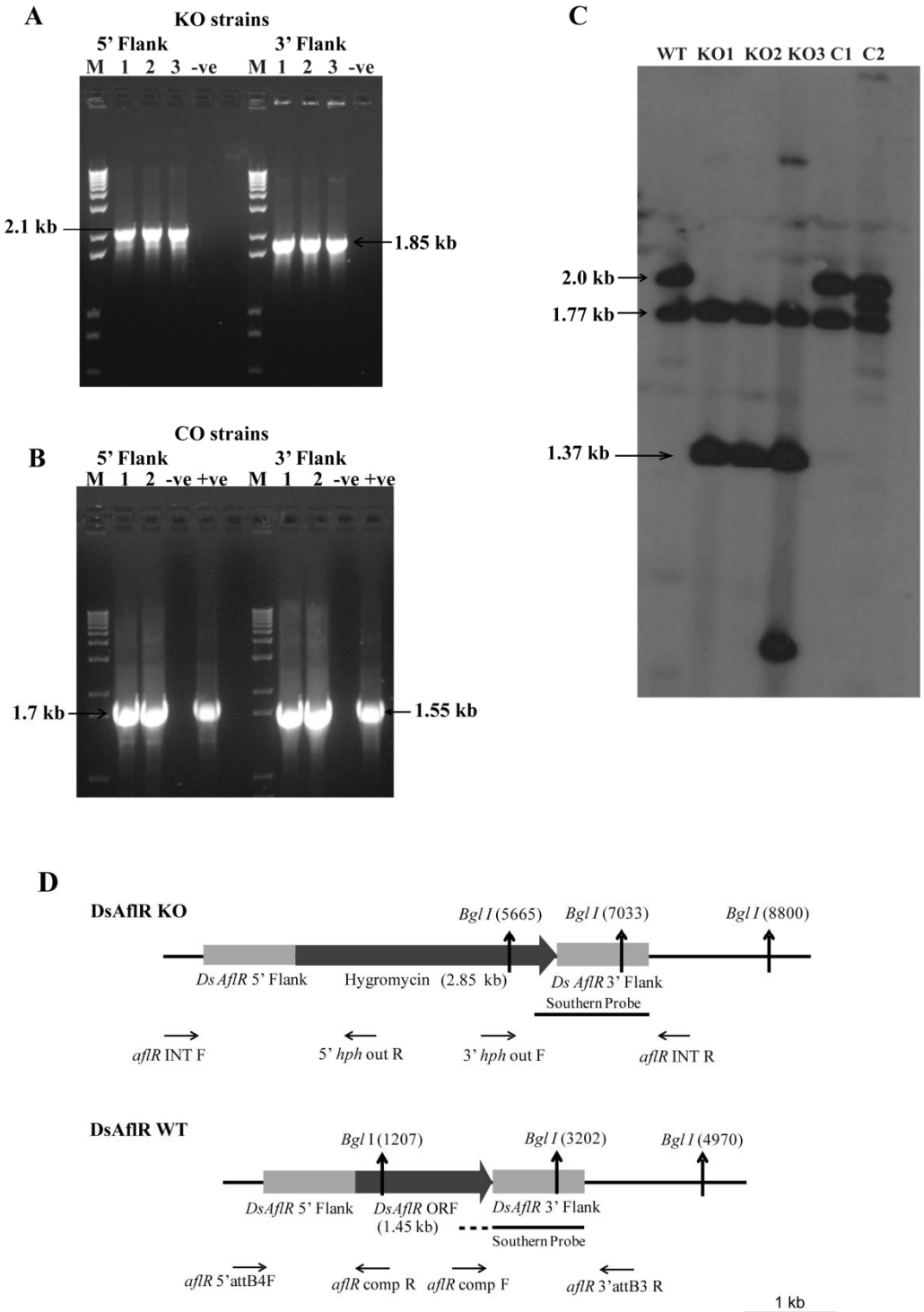

A
KO strains
5' Flank
3' Flank
M 1 2 3 -ve M 1 2 3 -ve
2.1 kb
1.85 kb
B
CO strains
5' Flank
3' Flank
M 1 2 -ve +ve M 1 2 -ve +ve
1.7 kb
1.55 kb
C
WT KO1 KO2 KO3 C1 C2
2.0 kb
1.77 kb
1.37 kb
D
DsAflR KO
Bgl I (5665)
Bgl I (7033)
Bgl I (8800)
Ds AflR 5' Flank
Hygromycin (2.85 kb)
Ds AflR 3' Flank
Southern Probe
aflR INT F
5' hph out R
3' hph out F
aflR INT R
DsAflR WT
Bgl I (1207)
Bgl I (3202)
Bgl I (4970)
DsAflR 5' Flank
DsAflR ORF
(1.45 kb)
DsAflR 3' Flank
Southern Probe
aflR 5'attB4F
aflR comp R
aflR comp F
aflR 3'attB3 R
1 kb

Figure 3.3: Molecular verification of DsAflR gene knockout and complementation.

(A) PCR-based confirmation of *DsAflR* gene knockout in *D. septosporum* transformants KO1 (1), KO2 (2) and KO3 (3) by amplification of *DsAflR* 5' flank (left; lanes 1, 2 & 3) using primer combination *aflR* INT F & 5' *hph* out R, and 3' flank (right; lanes 1, 2 & 3) using primers *aflR* INT R & 3'hph out F, according to the *DsAflR* KO scheme in (D). All three transformants had PCR products of the expected sizes for both 5' (2.1 kb) and 3' (1.85 kb) flanks, indicating deletion of the *Ds-AflR* gene. Lane M is 1 kb plus ladder.

(B) PCR-based confirmation of *DsAflR* complementation in *D. septosporum* transformants C1 (1) and C2 (2) by amplification of *DsAflR* 5' flank (left; lanes 1 and 2) using primers aflR5' attB4F & aflR-comp R and 3' flank (right; lanes 1 & 2) using primers aflR 3'attB3 R & aflR-comp F, according to the *DsAflR* WT scheme in (D). Both transformants had PCR products of the expected sizes (1.7 kb & 1.55 kb) respectively for both 5' and 3' flanks, indicating complementation of *DsAflR* deletion. Lane M is 1 kb plus ladder, lane +ve is control with wildtype *D. septosporum* DNA.

(C) Southern hybridisation of *Bgl*I-digested genomic DNA of *D. septosporum* wild type (WT), *DsAflR* knockout strains (KO1, KO2, KO3) and complementation strains (C1, C2) was performed using a probe complementary to a region of the hygromycin gene as well as to part of the 3' flanking region of *DsAflR* as shown in (D). The hygromycin cassette introduced a *Bgl*I (5665) site replacing the *Bgl*I (1207) site in *DsAflR* and resulted in 1.37 kb + 1.77 kb hybridizing fragments in knockout strains distinct from the 2.0 kb + 1.77 kb hybridizing fragments in the wild-type and complemented strains as expected. An additional hybridizing fragment of ~1kb in KO3 was indicative of an additional ectopic integration site of the plasmid. Likewise an additional fragment in C2 indicated a multi-copy integration of the complementation plasmid (pR224 & Nucleotides 690431 to 693567 of *D. septosporum* scaffold 12) , which in this case led to over-expression of *DsAflR*.

(D) Schematic diagram of *DsAflR* knockout and wild-type / complemented constructs. The locations of *Bgl*I restriction enzyme recognition sites and annealing sites of primers used to verify gene knockouts and complementation are shown.

The ability of *DsAflR* knockout mutants to produce dothistromin 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 dothistromin levels was shown in *DsAflR* mutants compared to the wild type in these respective media (Table 3.3), but low levels of dothistromin production above background levels remained (Appendix 8.4.1). The complemented mutant (C2) with additional copies of *DsAflR* produced higher levels of dothistromin than the wild type (Table 3.3), as would be expected for an *AflR* overexpression mutant (Flaherty and Payne, 1997).

Table 3.3: Dothistromin production in *Dothistroma septosporum AflR* mutants

Strain	Dothistromin production (pg/mg DW)	
	DM medium	PMMG medium
WT	45,091 ± 10,807	8,192 ± 1,437
KO1	0.15 ± 0.030*	0.16 ± 0.100*
KO2	0.12 ± 0.012*	0.36 ± 0.156*
C1	55,947 ± 7,676	6,136 ± 517
C2	150,205 ± 12,404*	16,217 ± 1,737*

Dothistromin secreted into growth media by wild type (WT), *DsAflR* knockout mutants (KO1, KO2) or mutant KO1 complemented with Ds-AflR (C1, C2). Values are means ± SD (n = 3). *Significantly different from wild type ($P<0.05$). Assay resolution limit is <0.1 pg/mg.

3.2.4 DsAflR is a key regulator of dothistromin biosynthetic genes

To assess the role of DsAflR on dothistromin gene expression, qRT-PCR assays were carried out to compare gene expression in *ΔDsAflR* mutants 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)

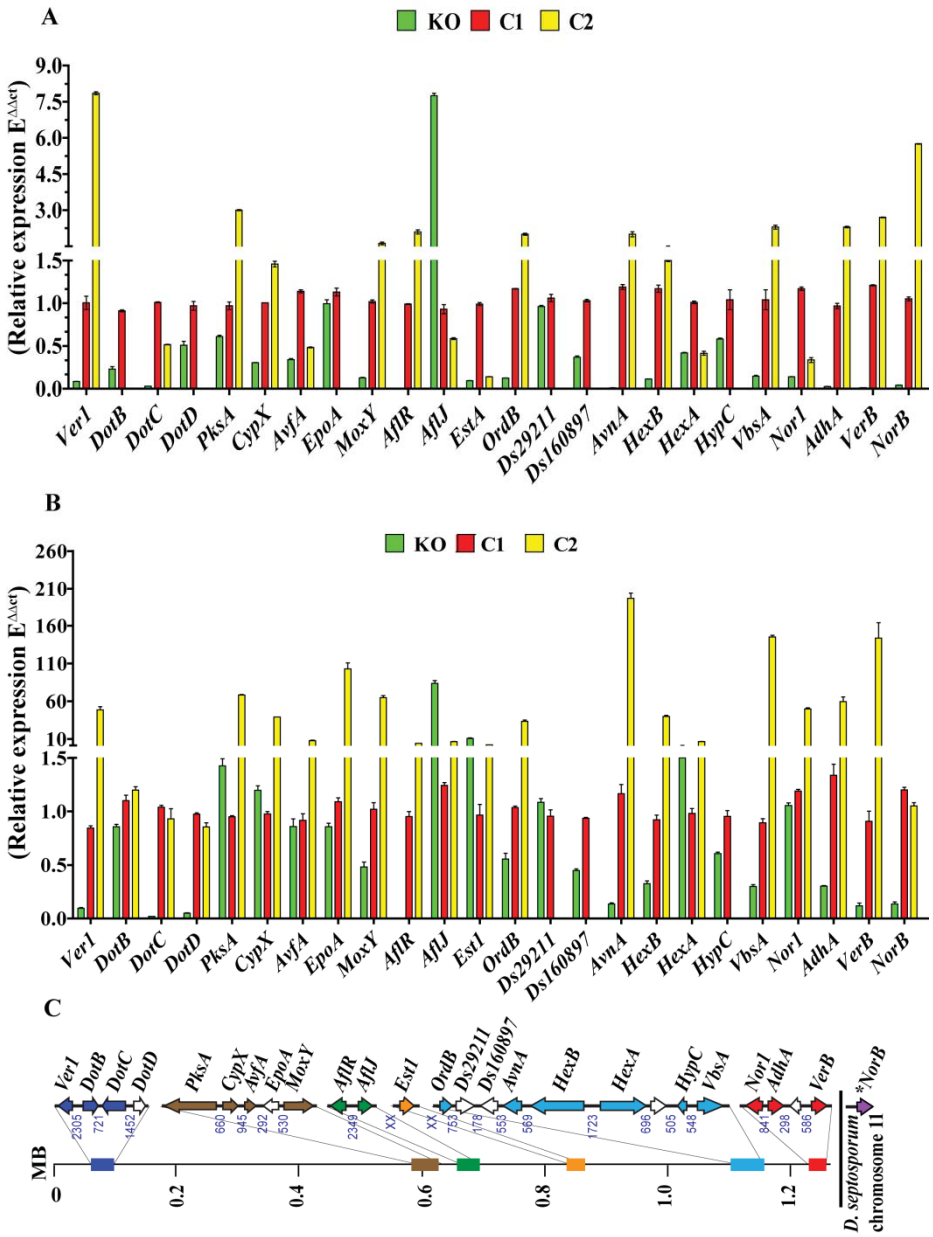


Figure 3.4: Expression of dothistromin genes in *D. septosporum* AflR mutants

Gene expression was evaluated by quantitative real-time PCR using RNA isolated from mycelium grown in (A) liquid pine minimal media (PMMG) and (B) in Dothistroma broth (DM) rich media. The expression values shown are (mean ± SEM) in *ΔDsAflR* mutant KO (green bars) and the *DsAflR* single copy complementation mutant C1 (red bars) and multicopy complementation strain C2 (yellow bar) relative to expression in the wild type (WT=1) (C) The genes are grouped according to six loci on chromosome 12, or on chromosome 11 for *NorB*.

To 90.9 fold (AvnA) down-regulation compared to the wild type (Figure 3.4 A). Down-regulation was also shown for the functionally confirmed dothistromin Ver1 gene (11.9-fold) as well as for other AF/ST orthologs with unassigned roles in dothistromin 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. Quite interestingly *D. septosporum AflJ* was 8-fold up regulated in the *ΔDsAflR* mutant.

In *D. septosporum*, the multi-copy complemented strain C2 displayed 2-fold overexpression of *DsAflR* in PMMG and correspondingly higher levels of expression for 9 out of the 13 'core' pathway genes, but 2.4-fold down-regulation of *DsAflJ* (Figure 3.4 A). Expression of dothistromin genes by cultures grown in rich DM medium showed a less consistent trend compared to those in PMMG pine extract medium but most dothistromin genes were down-regulated in a *ΔDsAflR* mutant (Figure 3.4 B). Overall the patterns of dothistromin gene expression in the *ΔDsAflR* mutant are consistent with DsAflR being the pathway-specific Zn_2Cys_6 transcriptional regulator of dothistromin biosynthesis.

Genes involved in the biosynthesis of secondary metabolites are usually clustered within the sub-telomeric region of chromosomes and it has been suggested that such location aids in epigenetic control and evolution. Although dothistromin biosynthesis genes are present in six loci, 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 (Tables 3.4, 3.5). In *ΔDsAflR* strain strongest down-regulation of dothistromin

genes compared to the wild type (>30-fold) occured within 140 kb of a telomere (*AvnA*, *AdhA* and *VerB* at loci 1, 5 and 6, respectively), whilst genes located in the more central loci 2 and 3 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 (Table 3.4).

Table 3.4: Correlations of *Dothistroma septosporum* expression data.

Genes[a]	Locus	Position on scaffold	Distance from telomere[b]	Expression in PMMG[c]	Expression in DM[c]	Fold-change WT/*AflR* KO[d]
Ver1	1	81028	81028	0.0250	0.0073	11.9
PksA	2	597907	597907	0.1309	0.0232	1.64
CypX	2	605870	605870	0.5038	0.0565	3.3
AvfA	2	608454	608454	0.1358	0.0157	3
MoxY	2	611444	611444	0.2119	0.0124	7.9
AflR	3	692747	563287	0.4012	0.1030	N/A
AflJ	3	696580	559454	0.0751	0.0181	0.13
OrdB	5	1112833	143201	0.1109	0.0053	8.1
AvnA	5	1119139	136895	0.0833	0.0039	90.9
HexB	5	1125619	130415	0.1109	0.0127	8.8
HexA	5	1132114	123920	0.2773	0.0457	2.4
HypC	5	1135224	120810	0.3724	0.0115	1.7
VbsA	5	1137537	118497	0.4513	0.0266	6.9
Nor1	6	1241528	14506	0.3207	0.0091	7.2
AdhA	6	1243421	12613	0.0640	0.0057	40
VerB	6	1246676	9358	0.4211	0.0203	83.3

[a] Dothistromin biosynthesis gene name

[b] Distances are shown in nucleotides from closest telomere; length of scaffold 12 = 1256034 bp

[c] Expression of DOTH gene in WT strain grown in PMMG or DM media; qRT-PCR gene expression was normalised to beta-tubulin (mean of 3 biological and 2 technical replicates).

[d] Fold change between expression in WT compared to *ΔDsAflR* KO (all dothistromin genes showed higher expression in WT than in the *AflR* KO mutant except *AflJ*.

Table 3.5: Pearsons correlation coefficient for continuous & normal dist data for above data

	Expression in PMMG	Expression in DM	*DsAflR* KO fold difference
Pearsons product moment correlation Coefficient (r)	0.0404	0.4372	-0.3886
95% Confidence Interval	-0.4646<= r >= 0.5256	-0.0747<= r >= 0.7667	-0.7621<= r >= 0.1789
Coefficient of determination (r^2)	0.0016	0.1912	0.1510
t-test for the significance of the coefficient	0.1514	1.8190	1.4609
Probability of t (p)	0.8818	0.0904	0.1697

Correlation between chromosomal position (in terms of distance from a telomere) gene expression and in *D. septosporum* WT grown in DM (Table 3.4; column 6) and PMMG (Table 3.5; column 7) media, and between chromosomal position and fold change in expression (WT/*AflR* KO) (Table 3.4; column 7).

* Statistical analysis was done http://department.obg.cuhk.edu.hk/researchsupport/PearsonCorrelation.asp

3.2.5 Functional analysis of candidate genes in the final steps of dothistromin biosynthesis

The genes involved in biosynthesis of dothistromin after versicolorin A (Figure 3.1 c) are not known. Based on the biochemical pathway proposed by Henry and Townsend (2005) and personal communication of Dr R. Bradshaw with Dr Kenneth Ehrlich (USDA, New Orleans) it was hypothesized that *DsOrdB*, *DsDotB* and *DsNorB* might be involved in the dothistromin biosynthesis downsteam of versicolorin A. To test this hypothesis transformation of *D. septosporum* with the *DsOrdB*, *DsDotB* and *DsNorB* gene knockout constructs pR321, pR323 & pR322 was done which resulted in three *ΔDsOrdB,* four *ΔDsDotB* and three *ΔDsNorB* knockout mutants. PCR analysis designed to confirm targeted knockouts showed products of the expected size for the respective mutants (Figure 3.5-3.7) with no amplification in wild type DNA negative control. Southern hybridization revealed gene replacement in mutants of *DsOrdB* (KO2 & KO3), *DsDotB* (KO1 - KO4) and *DsNorB* (KO1 - KO3) (Figure 3.5 - 3.7).

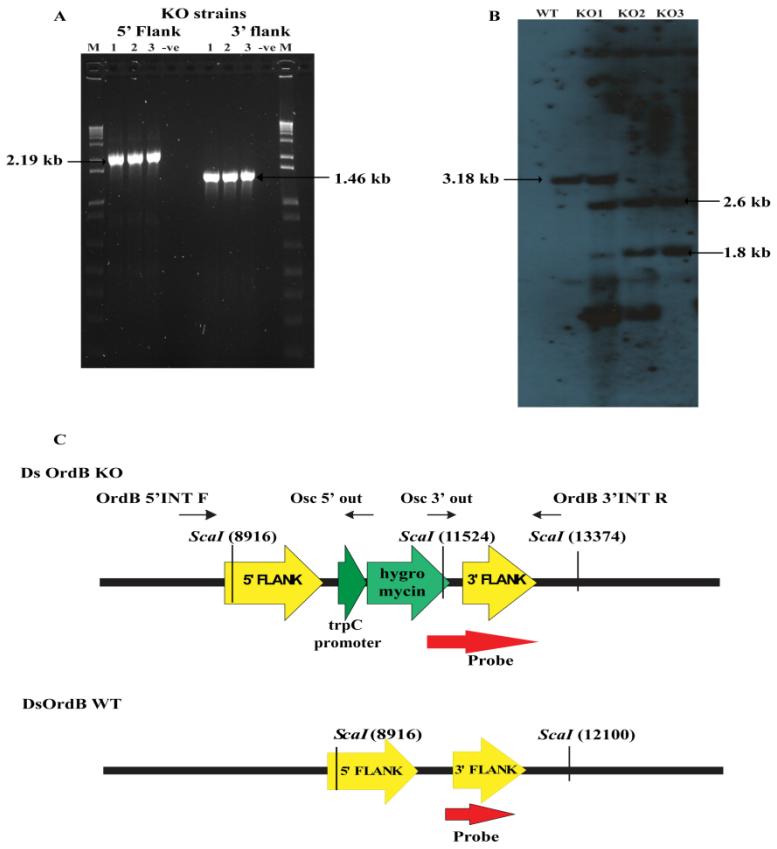


Figure 3.5: Molecular verification of DsOrdB gene knockout .

(A) PCR-based confirmation of *DsOrdB* gene knockout in *D. septosporum* transformants KO1 (1), KO2 (2) and KO3 (3) by amplification of *DsOrdB* 5' flank (left; lanes 1, 2 & 3) using primer combination *OrdB* INT F & Osc 5' out R and 3' flank (right; lanes 1, 2 & 3) using primers *OrdB* INT R & Osc 3' out F, according to the *DsOrdB* KO scheme in (C). All three transformants had PCR products of the expected size for both 5' (2.19 kb) and 3' (1.46 kb) flanks, indicating deletion of the *DsOrdB* gene. Lane M is 1 kb plus ladder.

(B) Southern hybridisation of *ScaI*-digested genomic DNA of *D. septosporum* wild type (WT), *DsOrdB* knockout strains (KO1, KO2, KO3) was performed using a probe complementary to a region of the hygromycin gene and part of the 3' flanking region of *DsOrdB* as shown in (C). *DsOrdB* does not have a *ScaI* site but the hygromycin cassette introduces a *ScaI* (11524) site and resulted in 2.6 kb + 1.8 kb hybridizing fragments in knockout strains (KO1, KO2 & KO3). In KO1 the wild type 3.18 kb band could still be detected, indicating a random integration of the knockout plasmid or possibly contamination of pure knockout with an ectopic strain thus needing more rounds of single spore purification. The KO2 transformant showed a hybridisation fragment of ~1kb, indicative of an additional ectopic integration site of the plasmid but the 3.18 kb in WT was absent. KO3 had no ectopic integration and was a clean knockout. The KO2 strain was similar to KO3 in terms of doth production and coloration.

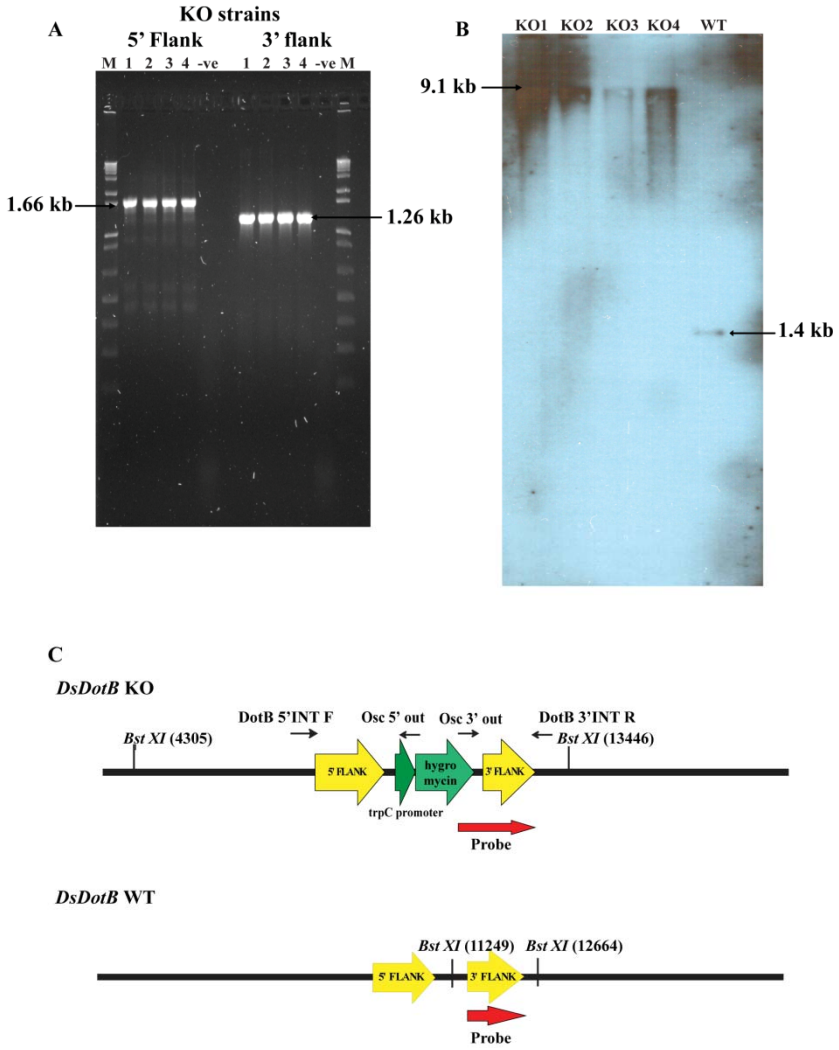


Figure 3.6: Molecular verification of *DsDotB* gene knockout .

(A) PCR-based confirmation of *DsDotB* gene knockout in *D. septosporum* transformants KO1 (1), KO2 (2) KO3 (3) and KO4 (4) by amplification of *DsDotB* 5' flank (left; lanes 1, 2, 3 & 4) using primer combination *DotB* INT F & Osc 5' out R and 3' flank (right; lanes 1, 2, 3 & 4) using primers *DotB* INT R & Osc 3' out F, according to the *DsDotB* KO scheme in (C). All three transformants had PCR products of the expected size (indicated by arrows) for both 5' and 3' flanks, indicating deletion of the *DsDotB* gene. Lane M is 1 kb plus ladder

(B) Southern hybridisation of *BstXI*-digested genomic DNA of *D. septosporum* wild type (WT), *DsDotB* knockout strains (KO1, KO2, KO3 & KO4) was performed using a probe complementary to a region of the hygromycin gene and part of the 3' flanking region of *DsDotB* as shown in (C). Introduction of the *DsDotB* KO construct abolished the *BstXI* site present in the *DsDotB* gene thus producing a 9.1 kb fragment in the knockout strain and 1.4 kb in the wild type.

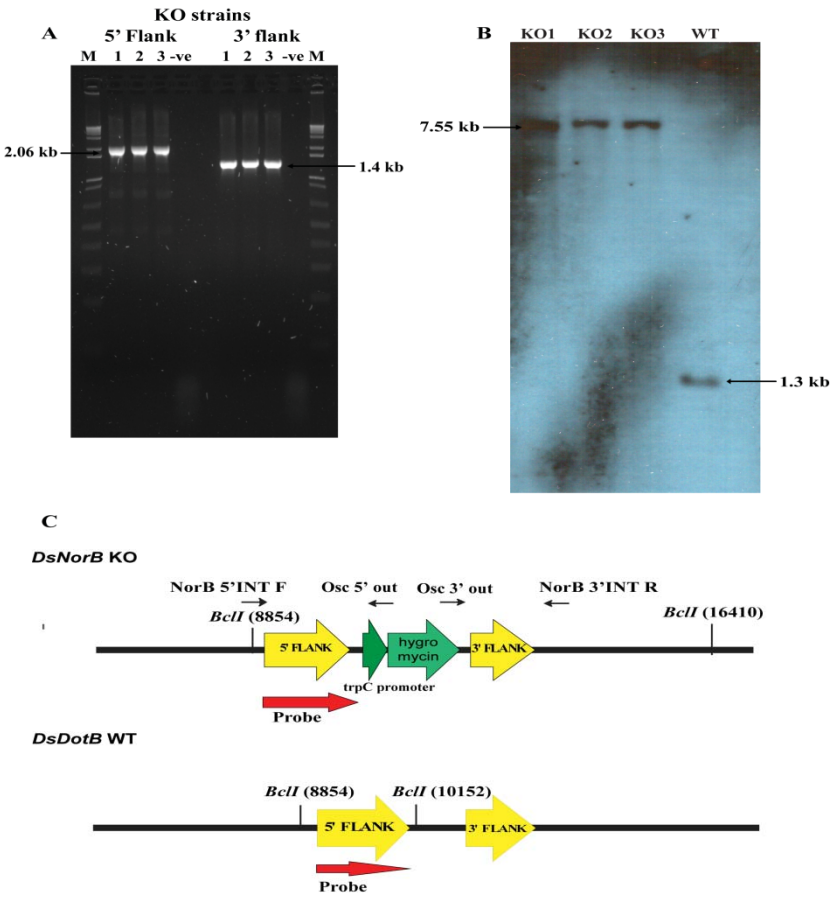


Figure 3.7: Molecular verification of *DsNorB* gene knockout.

(A) PCR-based confirmation of *DsNorB* gene knockout in *D. septosporum* transformants KO1 (1), KO2 (2) and KO3 (3) by amplification of *DsNorB* 5' flank (left; lanes 1, 2, & 3) using primer combination *NorB* INT F & Osc 5' out R and 3' flank (right; lanes 1, 2 & 3) using primers *NorB* INT R & Osc 3' out F, according to the *DsNorB* KO scheme in (C). All three transformants had PCR products of the expected size (indicated by arrows) for both 5' and 3' flanks, indicating deletion of the *DsNorB* gene. Lane M is 1 kb plus ladder

(B) Southern hybridisation of *BclI*-digested genomic DNA of *D. septosporum* wild type (WT), *DsNorB* knockout strains (KO1, KO2, & KO3) was performed using a probe complementary to a region of the hygromycin gene and part of the 5' flanking region of *DsNorB* as shown in (C). Introduction of *DsNorB* KO construct abolished the *BclI* site present in the *DsNorB* gene thus producing a 7.55 kb fragment in the knockout strain and 1.3 kb in the wild type.

An HPLC assay for detection of dothistromin in PMMG medium (minimal liquid medium) at seven days post inoculation showed that the wild-type isolate produced 8.03 ± 0.166 ng dothistromin per mg dry weight (DW) of mycelium, while each of two *DsOrdB* and *DsDotB* knockout mutants (KO2, KO3 and KO1, KO2)
produced significantly less dothistromin (Figure 3.8). However, in *ΔDsNorB* mutants KO1 & KO3 dothistromin was made at wild-type levels (Figure 3.8). *DsOrdB* mutants produced yellow coloration in the liquid media and showed extra peaks on an HPLC chromatogram compared to WT (Appendix 8.4.2) indicating accumulation of a dothistromin pathway intermediate.

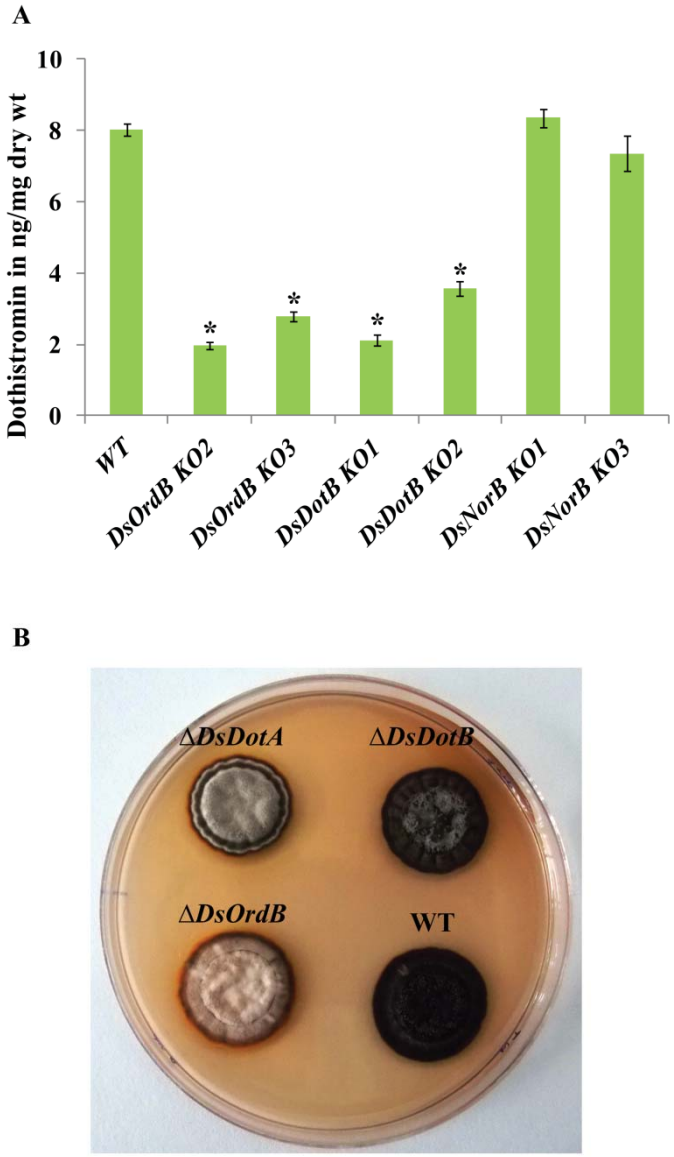


Figure 3.8: Characterisation of *DsOrdB, DsDotB* and *DsNorB* mutants.

(A) The histogram shows dothistromin production by two independent knockout mutants of *Dothistroma septosporum OrdB*, *DotB* and *NorB* genes. Values are means ± SD (n = 3). Δ*DsOrdB* and Δ*DsDotB* produced significantly less dothistromin than the wild type (WT) strain ($P<0.05$).

(B) Colony phenotypes of mutants that showed reduced levels of dothistromin (Δ*DsOrdB*, Δ*DsDotB*) compared to wild type (WT) and confirmed dothistromin mutant Δ*DsVer1* (=Δ*DsDotA*). The yellow pigment surrounding the Δ*DsOrdB* knockout mutant colony is similar to that produced by the Δ*DsVer1* mutant.

3.2.6 *Cladosporium fulvum AflR* partially complements *DsAflR* but some pathway genes are non-functional

C. fulvum contains a complete set of dothistromin genes but does not produce dothistromin (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 ΔDsAflR* 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. Dothistromin was produced in these complemented transformants at levels ~100-fold higher than those in the *ΔDsAflR* mutant. However these dothistromin levels were less than 0.1% of *D. septosporum* wild type levels (Appendix 8.4.3) The predicted CfAflR protein contains the same conserved domains as DsAflR (Figure 3.2) but whether other sequence differences between these two proteins affect CfAflR function is not yet known.

The suggested deficiency in CfAflR function led to the question whether the dothistromin biosynthetic genes are functional in *C. fulvum*. Manual annotation and, where necessary, cDNA sequencing, was carried out to ascertain structures of all the predicted dothistromin genes of *C. fulvum*, and showed predicted amino acid identities ranging from 74% (AvfA) to 98% (Ver1) compared to *D. septosporum* gene products (Table 3.6). Closer analysis of the gene models done by Jerome Collemore (Laboratory of Phytopathology, Wageningen University) revealed that *CfHexA* contains a premature stop codon and two frameshift mutations, whilst *CfNor1* has a 7 nt insertion causing immediate nonsense (Appendix 8.4.4). Both HexA and Nor1 are required for early steps of dothistromin biosynthesis (Figure 3.1), hence these mutations block the dothistromin biosynthetic pathway.

Table 3.6: Comparison of dothistromin genes in *D. septosporum* and *C. fulvum*

Gene name [a]	% ID[b] *Ds-Cf*	Dotse1protein ID[c]	Clafu1 protein ID[c]	Dotse1 aa	Clafu1 aa	Dotse1 Introns	Clafu1 Introns
Ver1	97.7	192193	197005	264	264	2	2
*DotB***	53.4	75412	197007	415	(287)	0	0
DotC	86.2	75413	194202	581	576	3	3
DotD	59.5	75414	194201	321	319	0	1
PksA	86.5	192192	194256	2400	2387	2	2
CypX	89.6	139960	194255	512	539	2	2
AvfA	74.3	75546	194254	285	293	0	0
EpoA	48.0	57187	193854	421	412	1	1
MoxY	90.9	75547	194253	627	636	5	5
AflR	83.6	75566	197014	480	481	1	1
*AflJ***	49.0	57214	197013	457	(312)	2	?
Est1	81.1	75609	194417	329	320	3	3
OrdB	94.0	75648	194437	268	268	0	0
AvnA	88.9	57312	194434[d]	526	526	3	3
HexB	86.6	181128	194434[d]	1905	1905	4	4
*HexA**	85.5	66976	194433	1693	1692	2	3
HypC	80.0	75655	194431	186	170	1	1
*VbsA**	94.4	75656	194430	648	638	1	1
*Nor1**	86.6	75691	194456	269	247	3	2
AdhA	88.6	48495	194457	307	307	2	2
VerB	93.3	75692	194459	521	524	2	2
NorB	75.3	75044	186031	392	319	0	0

[a] *C. fulvum* (Clafu1) gene names are the same as *D. septosporum* (Dotse1) names and a prefix will be added to distinguish them when required, eg. *CfVer1*.

[b] Percentage amino acid identities were computed by CLUSTALW whole sequence alignment.

[c] Protein identification (accession; PID) numbers refer to those at (http://genome.jgi.doe.gov/Dotse1/Dotse1.home.html) for *D. septosporum* (Dotse1) and (http://genome.jgi-psf.org/Clafu1/Clafu1.home.html) for *C. fulvum* (Clafu1). 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.

[d] *CfAvnA* and *CfHexB* are incorrectly annotated as one gene, hence both PID 194434.

One asterisk indicates the Cf gene is a pseudogene; two asterisks indicate the Cf gene model is not yet resolved.

3.3 Discussion

Dothistromin biosynthesis genes lack tight clustering, and not all dothistromin genes are orthologs of AF/ST genes. This study sought evidence for co-regulation of all predicted dothistromin genes by the putative pathway regulator AflR. In *DsAflR* gene knockout mutants the production of dothistromin was drastically reduced but low levels of dothistromin production (above background levels) remained. 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 dothistromin genes. Another putative fungal specific transcription factor domain containing protein (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 putative zinc binuclear cluster DNA-binding sites (Appendix 8.4.5) and is expressed in broth culture (Appendix 8.4.6 A & B). Whether this protein or another transcription factor has a role in dothistromin 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 dothistromin production in the absence of DsAflR might relate to the dispersed distribution of dothistromin genes having moderate levels of basal expression or that their expression is modulated by regulatory factors other than AflR.

Consistent with a role for DsAflR in regulation of dothistromin biosynthesis, the 13 core genes were downregulated (Figure 3.4) in the Δ*DsAflR* knockout strain consistent with that observed for *aflR* mutants in *A. parasiticus*. (Price et al., 2006). The expression was reverted to wild type levels in the complemented strain (C1). However, the complemented strain carrying multicopy *DsAflR* genes showed higher level of

expression for 9 out of 13 core genes compared to wild type; this was also reflected in the level of dothistromin produced where the *DsAflR* overexpression mutant produced three times more toxin. Similar observations were made by Flaherty and Payne (1997) where overexpression of *aflR* in *A. flavus* using glyceraldehyde-3-phosphate dehydrogenase promoter lead to constitutive transcription of the pathway genes *fas-1A*, *pksA*, *nor-1*, and *omtA* and increased aflatoxin.

AflR has been shown to bind to the palindromic sequence 5'-TCGN_5CGA-3' (also called AflR binding motif) in the promoter region of many of the structural genes in *A. parasiticus, A. flavus*, and *A. nidulans* (Fernandes et al., 1998). Consistent with a role for DsAflR in regulation of dothistromin biosynthesis, most core dothistromin genes in *D. septosporum* contain between one and five putative upstream AflR binding sites (TCGN_5CGR) (Table 3.2). The number of predicted AflR sites does not appear to be important; *DsVerB* has only one such site but was 83-fold down-regulated in the *ΔDsAflR* mutant, whilst *DsVer1* 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 dothistromin genes without a putative AflR-binding sequence TCG(N_5)CGR are *DsDotB, DsEst1* and *DsDotC*. The *DsEst1* 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* and its placement in a separate clade by phylogenetic analysis (Bradshaw et al., 2013). Despite 11-fold down-regulation in the *ΔDsAflR* mutant, *DsEst1* does not have 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

($TCGN_{11}CGR$) that was previously noted upstream of many dothistromin 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*.

Henry and Townsend, (2005) proposed a biosynthetic scheme for synthesis of dothistromin that involves epoxidation of versicolorin A and a 5,8-dihydroxyanthraquinone intermediate which is known to be produced by dothistromin producing fungi (Danks and Hodges, 1974). Using this scheme, the possible roles for AflR-regulated genes found in the last stages of dothistromin biosynthesis is discussed, according to the model shown in (Figure 3.1C). In step I, it is proposed that *DsDotB*, a predicted peroxidase with weak (24%) amino acid identity to *StcC* (McDonald et al., 2005) performs epoxidation of the A-ring of VA. This was well supported by the fact that in the *DsDotB* deletion mutants (KO1 & KO2) produced two fold less dothistromin than the wildtype (Figure 3.8 A) giving a leaky phenotype. Alternatively, a cytochrome P450 (CytP450), such as CypX or AvnA (CytP450 enzymes encoded by the dothistromin cluster and thought to function in the earlier part of the pathway) could catalyze this step. In AF biosynthesis it has been shown that a single enzyme can catalyze more than one step of the pathway and 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). Functional analysis of CytP450 genes can be problematic due to functional redundancy and similar to *DsDotB,* leaky mutants have been reported for AF genes (Ehrlich et al., 2009).

Opening of the epoxide in Step 2 could potentially be achieved by the epoxide hydrolase *DsEpoA*. However, *DsEpoA* knockout mutants produced dothistromin and this gene appears to be non-functional (Jin, 2005). It is possible that other epoxide hydrolases catalyse this step, for which there are at least 6 genes in the genome (Table 3.1). Alternatively, opening of the epoxide ring could be achieved non-enzymatically as suggested for ST and related compounds (Henry and Townsend, 2005).

Step 3 involves A-ring reduction to yield the 5,8-dihydroxyanthraquinone and could be achieved by the NADPH dehydrogenase encoded by *DsVer1* (previously called *dotA*) as suggested by Henry and Townsend (2005). This gene was functionally analysed and shown to be required for dothistromin biosynthesis, with gene knockout mutants accumulating a yellow pigment, consistent with a stable VER-type intermediate (Bradshaw et al., 2002).

The last stages of biosynthesis of dothistromin (Step 4) involve oxidation of the bisfuran rings and here it was proposed that the remaining genes, *DsOrdB* and *DsNorB* might function. Both genes are regulated by DsAflR (Figure 3.4). OrdB (aflX) is an NAD(P)-dependent oxidoreductase that functions in conversion of VER A to DMST in *A. flavus* (Cary et al., 2006). An *OrdB* ortholog is also predicted to be involved in the biosynthesis of monodictyphenone from emodin (Chiang et al., 2010). In *D. septosporum*, *DsOrdB* was shown to have a role in dothistromin biosynthesis as the *ΔDsOrdB* mutants (KO2 & KO3) produced less dothistromin compared to the wild type strain, and there was a concomitant increase of yellow coloured metabolite similar to versicolorin (Figure 3.8 B); these results were comparable to that seen in the *aflX* (*ordB*) knockout mutant in *A. flavus* (Carey et al., 2006).

The other gene considered for step 4, *DsNorB*, is a putative aryl alcohol dehydrogenase gene encoded on a separate chromosome to all the other proposed

dothistromin genes. Nonetheless it was strongly down-regulated in *DsAflR* mutants (23.8-fold) compared to the wild-type. In *A. parasiticus norA* and *norB* are predicted to encode similar NAD+ or NADP+ dependent alcohol dehydrogenases, shown to function in an oxidation step in a later stage after NadA reduction (Ehrlich et al., 2008). *A. parasiticus norA* mutants still produced AF, but *norB* mutants did show accumulation of two 362 and 326 Da yellowish green coloured metabolites along with aflatoxin (Ehrlich et al., 2008). However, in *D. septosporum* the *NorB* mutants (KO1 & KO3) did not show reduction in dothistromin production nor accumulation of any coloured intermediate suggesting that it is not needed for dothistromin biosynthesis. The *D. septosporum* genome does contain other genes similar to NorB (with >40% aa similarity and e-value <1e20; Table 3.1), but they are located on different chromosomes. The fact that the *DsOrdB* and *DsDotB* mutants could still make dothistromin suggests that the pathway involved in the biosynthesis of dothistromin may not be as linear shown in Figure 3.1. There may be a metabolic grids that provide alternate pathways to the final product dothistromin, as has been reported for aflatoxin biosynthesis in *Aspergillus* spp. (Yabe et al., 2003) and lolitrem B in *Epichloë festucae* (Saikia et al., 2012). Further analysis of the intermediates produced by these mutants needs to done to shed more light on the biosynthesis pathway.

Heterologous complementation has been used in fungal systems to study gene function (Yu et al., 1996). In an attempt to examine the function of CfAflR a deletion mutant of *Dothistroma septosporum AflR* was transformed with a plasmid carrying functional *CfAflR*. Although the expression of *CfAflR* was similar to that of wild type *DsAflR* it was not able to fully complement DOTH production in *ΔDsAflR*. This suggests that the *CfAflR* promoter is fully functional but the CfAflR protein is not Similar results were obtained in an effort to complement *A. fumigatus ΔgliZ* mutant with

Penicillium lilacinoechinulatum PlgliZ encoding a $Zn(II)_2Cys_6$ DNA binding protein (Fox et al., 2008). The predicted CfAflR protein contains the same conserved domains as DsAflR (Figure 3.2) but whether other sequence differences between these two proteins account for its inability to afford heterologous complementation is not known. Further, it is not possible to determine whether CfAflR regulates dothistromin biosynthesis in *C. fulvum* itself as *C. fulvum* does not produce dothistromin due to mutation in key genes *HexA* and *Nor1*.The biotrophic lifestyle of this fungus may have made production of dothistromin unnecessary for its adaptation (De Wit et al., 2012).

In spite of being located in multiple loci dothistromin biosynthetic genes in *D. septosporum* are co-regulated by an $Zn(II)_2Cys_6$ transcription factor similar to that seen in other fungal genera. These expression data helped to find additional genes involved in the pathway. The results in this study also show that location of the gene clusters at subtelomeric chromosome regions is not required for co-regulation as suggested previously (Palmer and Keller, 2010; Yu and Keller, 2005). Here we reported that *DsAflJ* is regulated differently in *D. septosporum* compared to in *Aspergillus* spp. and suggest that *DsAflJ* may have a role in biosynthesis. A hypothesis implying the role of *DsAflJ* in dothistromin biosynthesis will be tested in the next chapter.

Chapter 4: DsAflJ regulates dothistromin biosynthesis but does not show functional conservation across genera.

4.1 Introduction

In the previous chapter, it was demonstrated, that despite being located in six loci, dothistromin biosynthesis genes are co-regulated by a pathway specific transcription factor DsAflR. In addition to the genes in the cluster whose functions have been characterized, there are additional genes of unknown function whose transcription was found to be regulated by *DsAflR*. One such gene, *DsAflJ,* is divergently transcribed to *DsAflR* and shares a common intergenic region of 2.35 kb. In *Aspergillus* spp. AflJ has been shown to be involved in the regulation of clustered AF and ST genes; *ΔaflJ* mutants were impaired in their ability to make toxin compared to wild type and they showed some differences in the expression of pathway genes between aflatoxin-producing species (Meyers et al., 1998; Chang et al., 2003). A recent study in *A. parasiticus* by Ehrlich et al (2012) suggests a role for AflJ in aflatoxin biosynthesis by mediating both the availability of AflR for transcriptional activation and the biogenesis and transport of aflatoxisomes/endosomes.

The shared synteny of *DsAflR* & *DsAflJ* with corresponding orthologs in *Aspergillus* spp. & *C. fulvum*, the presence of four putative AflR binding sites in the promoter region of *DsAflJ,* and enhanced expression of *DsAflJ* in the Δ*DsAflR* mutant (Chapter 3) led to the hypothesis that *DsAflJ* is involved in the regulation of dothistromin biosynthesis. The objectives of this study were to characterize *DsAflJ,* determine its role in dothistromin biosynthesis and to ascertain whether AflJ is functionally conserved in different ascomycetes.

4.2 Results

4.2.1 DsAflJ shows some common and distinct features to other AflJ proteins

The *DsAflJ* 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. nidulans* (ANID_07819) and *A. parasiticus* (AAS66019.1) AflJ as query sequences. The JGI DsAflR protein identification number (PID) is 57214. Synteny analysis revealed its conservation across the AF/ST biosynthesis cluster.

The gene model of *DsAflJ* revealed an open reading frame (ORF) of 1371 bp interrupted by two introns of 56 and 58 bp. The gene encodes a predicted protein of 310 amino acid residues and was designated *DsAflJ* (PID 57214). Pairwise alignments indicated that DsAflJ shares amino acid identities with AflJ proteins from different ascomycetes of 39.1% (*A. parasiticus*; AAS66019.1 aflatoxin cluster), 30.9% (*A. nidula ns*; ANID_07819 sterigmatocystin cluster), 41.9% (*A. nidulans*; ANID_10021 monodict yphenone cluster) and 49.5% (*C. fulvum*; PID 197013).

A multiple alignment was done using Bioedit v. 7.0.9 (Hall et al., 1999) and showed conserved regions between the proteins (Figure 4.1). By using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) to predict membrane-spanning regions (Hofmann and Stoffel 1993), DsAflJ was found to have a single helical membrane spanning domain (Appendix 8.4.7), in contrast to two, three or none in AflJ orthologs of *A. parasiticus*, *A. nidulans* and *C. fulvum* respectively. A BlastP search of the GenBank database revealed partial hits similar to some proteins with class 2 methyltransferase domains similar to that of *A. parasiticus* and *A. nidulans* AflJ proteins (Ehrlich et al., 2011). The EMBOSS program "epest find" (http://emboss.bioinfortamics.nl.cgibin/emboss/epestfind) identified proline (P),

glutamic acid (E), serine (S), and threonine (T) (PEST) motifs. This sequence is associated with proteins that require a rapid turnover and it is hypothesized that the PEST sequence acts as signal for proteolysis (Rogers et al., 1986). A PEST region (292HNGLSTPPESDTGPAAR308) was present only in DsAflJ of the protein sequences aligned (Figure 4.1).

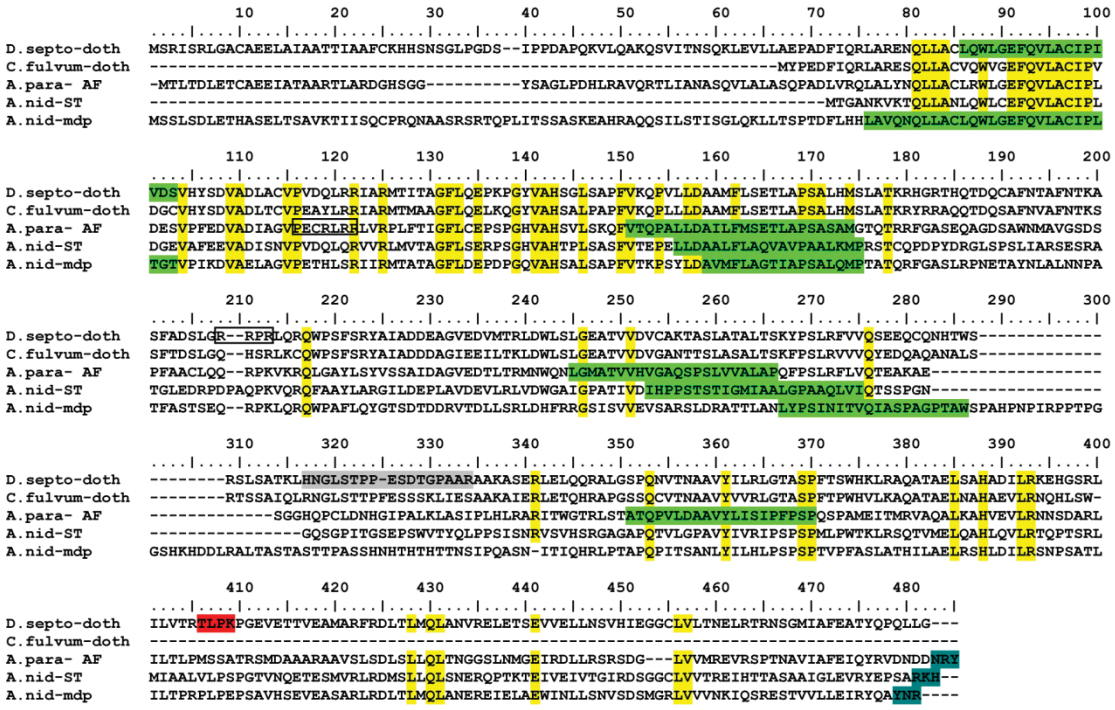


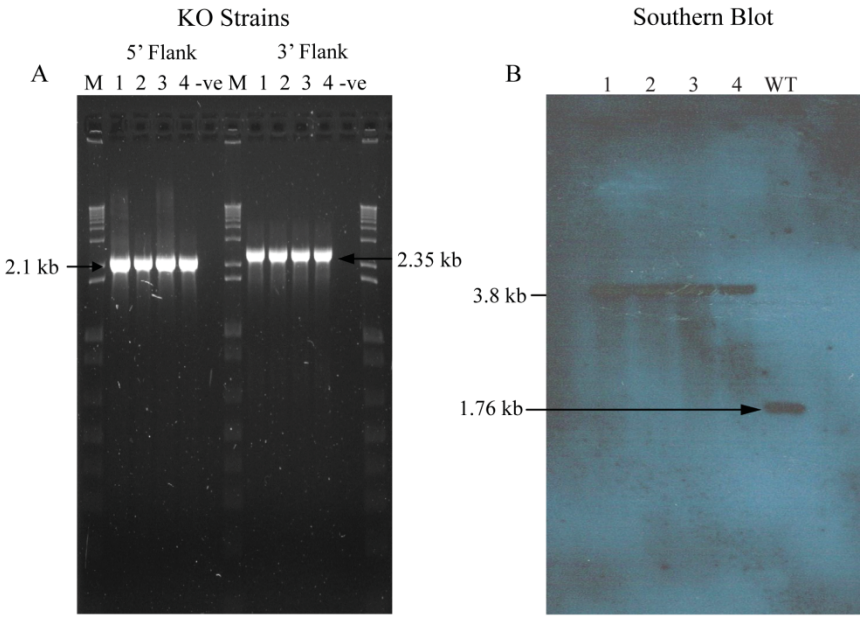
Figure 4.1: Multiple Alignment of putative AflJ orthologs from ascomycetes.

Amino acids marked in yellow are conserved in all species. Membrane spanning regions are marked in green shade. The boxed sequences in DsAflJ and ApAflJ were identified as putative nuclear localization sequences. A PEST motif (grey shade) and vacuolar localization signal (red shade) were identified only in DsAflJ. Putative microbody targeting sequences are marked in dark green shade at the C terminal. Abbreviations are: D. septo-doth, *D. septosporum* dothistromin cluster AflJ (PID 57214); C. fulvum-doth, *C. fulvum* dothistromin cluster (PID 197013); A. para-AF, *A. parasiticus* aflatoxin cluster AflJ (AAS66019.1); A. nid-ST, *A. nidulans* sterigmatocystin cluster AflJ (ANID_7819); A nid-mdp, *A. nidulans* monodictyphenone cluster AflJ (ANID_10021).

Using WolF PSORT (http://www.wolfpsort.org) (Horton et al., 2007) to determine protein subcellular localization, *A. parasiticus* AflJ was predicted to contain two pat7 nuclear localization signals (NLS): PECRLRR at 105 and PKVKRQL at 109. In contrast DsAflJ contains a predicted pat4 NLS, RRPR at 206, that is not present in the other AflJ proteins (Figure 4.1). A possible vacuolar targeting motif TLPK (Zhang et al., 2002; Wang et al., 2006) was present only in DsAflJ but the three *Aspergillus* AflJ, shown in Figure 4.1 had putative microbody targeting sequences at the C terminal.

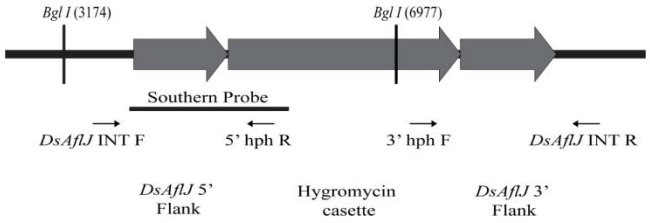
4.2.2 Disruption of the *DsAflJ* locus in *D. septosporum* NZE10.

Protoplast mediated transformation of *D. septosporum* NZE10 with a linearised *DsAflJ* gene knockout construct pR316 (methods 2.9 and 2.3.10) resulted in four knockout mutants along with several ectopic transformants. Analysis by PCR using primers that bind outside the 5' and 3' flanking regions of *DsAflJ* showed products of the expected size for the *DsAflJ* gene knockout mutants KO1 - KO4 (Figure 4.2 A). Clean targeted integration in all four mutants was confirmed by Southern hybridization of *Bgl*I digested genomic DNA using a labelled probe that hybridized to a 3.8 kb fragment in the knockout mutants but a 1.76 kb fragment in the wild type (Figure 4.2). As all four knockout Δ*DsAflJ* mutants exhibited a similar phenotype (sporulation; Appendix: 8.5.2), KO1 and KO2 were chosen for further work.



C

DsAflJ KO



DsAflJ WT

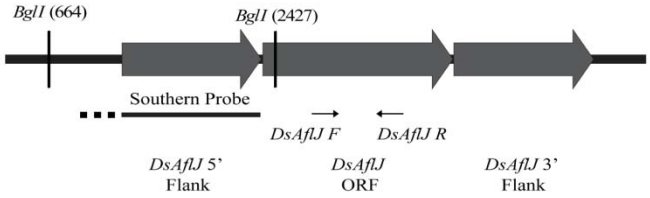


Figure 4.2: Molecular verification of *DsAflJ* gene knockout.

(A) PCR-based confirmation of *DsAflJ* gene replacement in *D. septosporum* transformants KO1 – KO4 (lanes 1-4 respectively) by amplification of *DsAflJ* 5' flank (left) using primer combination *DsAflJ* INT F & 5' *hph* R and 3' flank (right) using primers *DsAflJ* INT R & 3'hph F, according to the *DsAflJ* KO scheme in (C). All four gene replacement mutants had PCR products of the expected size (indicated by arrows) for both 5' and 3' flanks, indicating deletion of the *DsAflJ* gene. Lane M is 1 kb plus ladder.

(B) Southern hybridisation of *Bgl*I-digested genomic DNA of *D. septosporum* wild type (WT), and *DsAflJ* knockout strains (KO1 – KO4 in lanes 1-4 respectively) was performed using a probe complementary to a region of the hygromycin gene as well as to part of the 5' flanking region of *DsAflJ* as shown in (C). In the knockout mutants, a *Bgl*I site (2427) present in the wild type strain was replaced by a hygromycin cassette that introduced a new *Bgl*I site at 6977, thus producing a single band of 3.8 kb distinct from the 1.76 kb fragment in the wild type strain.

(C) Schematic diagram of *DsAflJ* knockout and wildtype/complementation constructs. The locations of *Bgl*I restriction enzyme recognition sites and annealing sites of primers used to verify gene knockouts and complementation are shown.

4.2.3 Dothistromin production and dothistromin gene expression are reduced in the Δ*DsAflJ* mutant

The production of dothistromin and gene expression by the Δ*DsAflJ* mutants was determined following their growth in liquid PMMG (pine minimal broth) media. The production of dothistromin was significantly reduced by almost 25-fold compared to wild type in both the Δ*DsAflJ* strains (Figure 4.3 A).

Quantitative PCR was used to quantify the expression of dothistromin biosynthesis genes (Figure 4.3 B). The expression of *DsHexA*, *DsHexB*, *DsPksA*, *DsAdhA* & *DsVbsA* decreased significantly *DsVer1*, *DsDotB* and *DsOrdB* were also significantly reduced in the Δ*DsAflJ* mutants compared to wild type (Figure 4.3 B). Expression of *DsAflR* was not affected by the deletion of *DsAflJ* (Figure 4.3 B) similar to the situation for AF regulation in *A. parasiticus* where disruption of *aflJ* did not affect the expression pattern of *aflR* or *vice versa* (Chang et al., 2003). However

interestingly the converse was not true for *D. septosporum* as in Chapter 3 it was shown that the pathway specific regulator *DsAflR* negatively regulated *DsAflJ*. To determine whether the up-regulation of *DsAflJ* was due to a mutation in its regulatory region introduced by the *DsAflR* knockout procedure, the shared *DsAflR-AflJ* intergenic region was sequenced, but no mutations were found (not shown)

4.2.4 Complementation of the *DsAflJ* gene knockout mutant

To confirm that the observed reduction in dothistromin production was due to the *DsAflJ* gene deletion, one of the mutant strains (KO1) was transformed with the *DsAflJ* complementation construct pR317 harbouring a functional *DsAflJ* gene (methods section 2.9). Five transformants were randomly selected and screened by PCR using primers specific for *DsAflJ* (Figure 4.4 A). In the native gene, the primer sites were 300 bp apart served a positive control (+ve), but in the disruption construct the primers did not amplify any product, as expected negative control (-ve). The complemented strains that produced amplicons of similar size to that of the native gene were designated Ds-1, Ds-2, Ds-3, Ds-5 and Ds-7.

Traditionally, biologists have used Southern blotting to confirm the integration and copy number of transgenes (Southern, 1975). This is quite cumbersome with multiple samples as it involves extracting a large quantity of genomic DNA, followed by restriction digestion blotting and hybridisation. Instead, a qPCR based method (Solomon et al., 2010) was adopted for determination of copy number in the various *DsAflJ* complemented strains (method section 2.2.5). *D. septosporum* NZE10 genomic DNA was used to construct a standard curve to determine the efficiency of the primers.

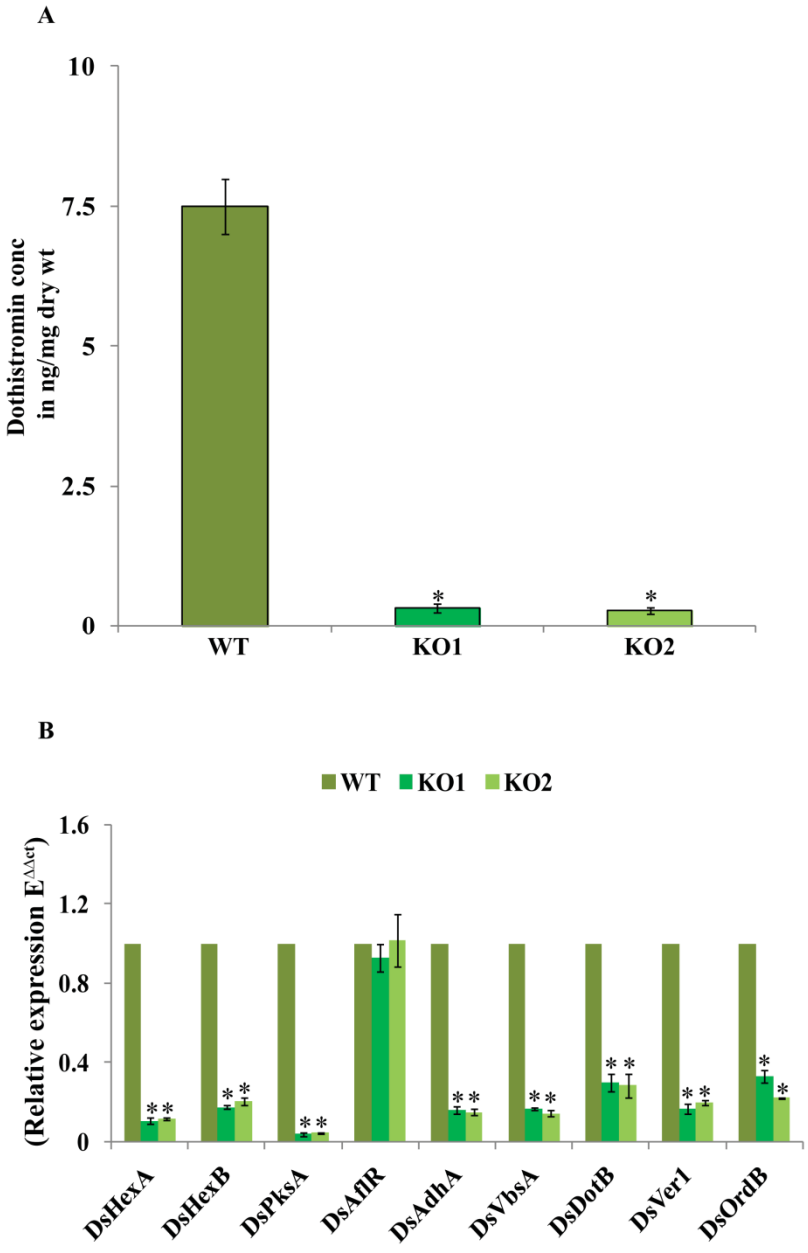


Figure 4.3: Dothistromin production and gene expression by *DsAflJ* mutant.

(A) Dothistromin production in culture. Dothistromin concentration in *DsAflJ* knockout strains KO1, KO2 (0.31 ± 0.08 & 0.28 ± 0.06 ng/mg dry wt) and wild type strain (WT) (7.5 ± 0.5 ng/mg dry wt). (B) qPCR results for relative quantification of dothistromin gene expression in *DsAflJ* mutants: 'early' biosynthetic pathway genes (*DsHexA*, *DsHexB* & *DsPksA*), 'mid' (*DsAdhA* & *DsVbsA*) and 'late' (*DsVer1*, *DsDotB* & *DsOrdB*) along with regulatory gene *DsAflR*. Normalised expression ratios for *DsAflJ* gene replacement mutants KO1 & KO2, relative to wildtype (WT) are shown as mean ± SD. Significant differences between mutant and WT ($p \leq 0.05$) are shown by an asterisk.

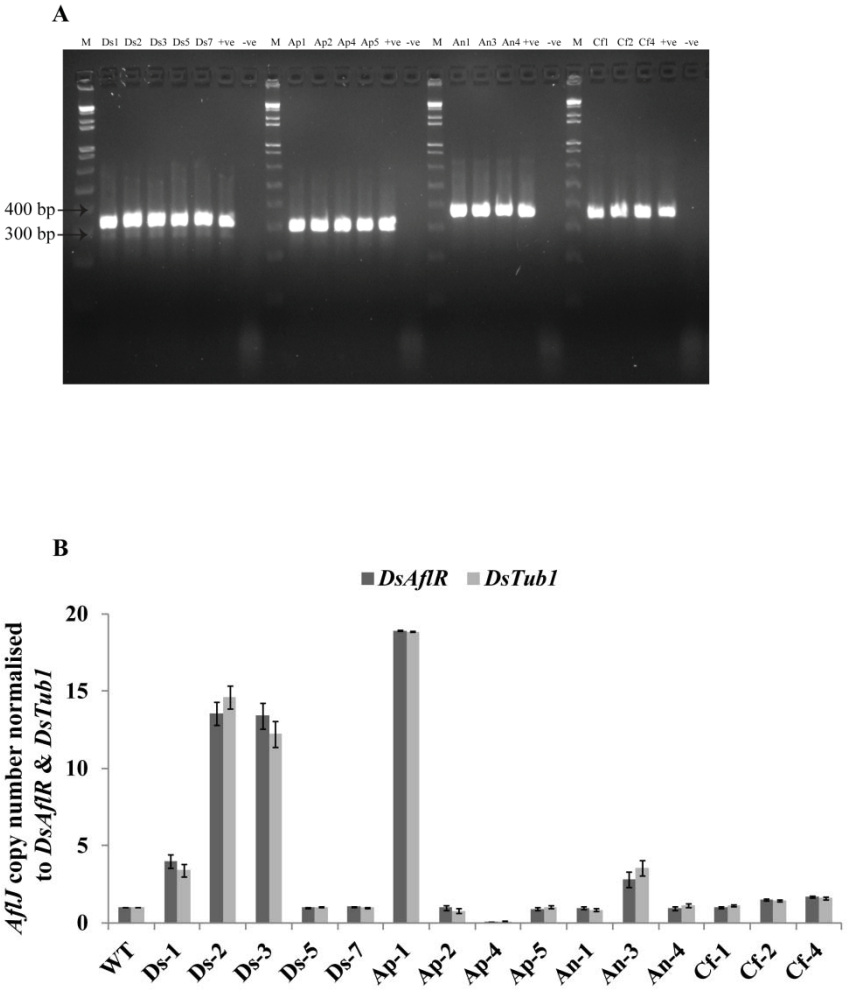


Figure 4.4: Confirmation of *ΔDsAflJ* complemented strains

(A) PCR based confirmation of *ΔDsAflJ* complemented strains. Five strains complemented with *DsAflJ* (Ds-1, Ds-2, Ds-3, Ds-5 & Ds-7), four *with ApaflJ* (Ap-1, Ap-2, Ap-4 & Ap-5), three with *AnaflJ* (An-1, An-3 & An-4) and three *with CfAflJ* (Cf-1, Cf-2 & Cf-4). All transformants had PCR products of the expected size (315 bp for *D. septosporum*, 300 bp for *A. parasiticus*, 300 bp for *A. nidulans* and 350 bp for *C. fulvum*) indicating the successful transformation of the complementation constructs.

(B) qPCR evaluation of gene copy numbers for each gene (*DsAflJ*, *ApaflJ*, *AnaflJ*, *CfAflJ*) in the complemented strains normalised to the single copy genes *DsAflR* and *DsTub1*. The control was WT *D. septosporum* genomic DNA with *DsAflJ* as target gene, the copy number of which is known. Mean and standard deviation of 3 biological and 2 technical replicates are shown.

The copy numbers of two reference genes, *DsTub1* and *DsAflR,* were determined previously by genome searches and Southern hybridisation and known to be single copy genes. The results (Figure 4.4 B) represent the copy numbers calculated for each of the *DsAflJ* complemented strains, along with wild type *D. septosporum* NZE10, using the $E^{-\Delta\Delta Ct}$ method (Methods Section 2.5.5). The single copy genes *DsAflR* and *DsTub1* were used as endogenous controls (dark and light shaded bars in Figure 4.4 B respectively). The copy number in *DsAflJ* complemented strains varied from a single copy (in Ds-5 & Ds-7) to multi copies (in Ds-1, Ds-2 & Ds-3); copy number of *DsAflJ* in the wild type strain was one, confirming the expectation from Southern hybridisation (Figure 4.2 B) and the accuracy of the assay. For further experiments single copy complemented strain Ds-5 and multicopy Ds-1 (4 copies of *DsAflJ*) were chosen; characterisation of multicopy Ds-2 was also done, and gave the same results as Ds-1, but not presented in this thesis.

qPCR was also used to determine *AflJ* transcript accumulation in the *DsAflJ* complemented strains using the $E^{-\Delta\Delta Ct}$ method with *DsTub1* as an endogenous reference gene (Pfaffl, 2001; Yuan et al., 2006). The results (Figure 4.5 A) showed that the *AflJ* gene in the complemented strains was functioning as expected, with wild-type levels of expression in single copy strains (Ds-5) but higher levels in a multi copy strain (Ds-1).

In *Aspergillus* spp. deletion of *aflJ* lead to very little or no aflatoxin production. In *A. flavus* overexpression of *aflJ* by using strong constitutive glyceraldehyde 3-phosphate dehydrogenase promoter derived from *A. nidulans* (*gpdA*) lead to eight times as much aflatoxin compared to wild type (Du et al., 2007). Thus, the production of dothistromin by *DsAflJ* complemented strains was assessed in *D. septosporum* broth cultures after 7 days growth in PMMG media. Both a single copy complemented strain

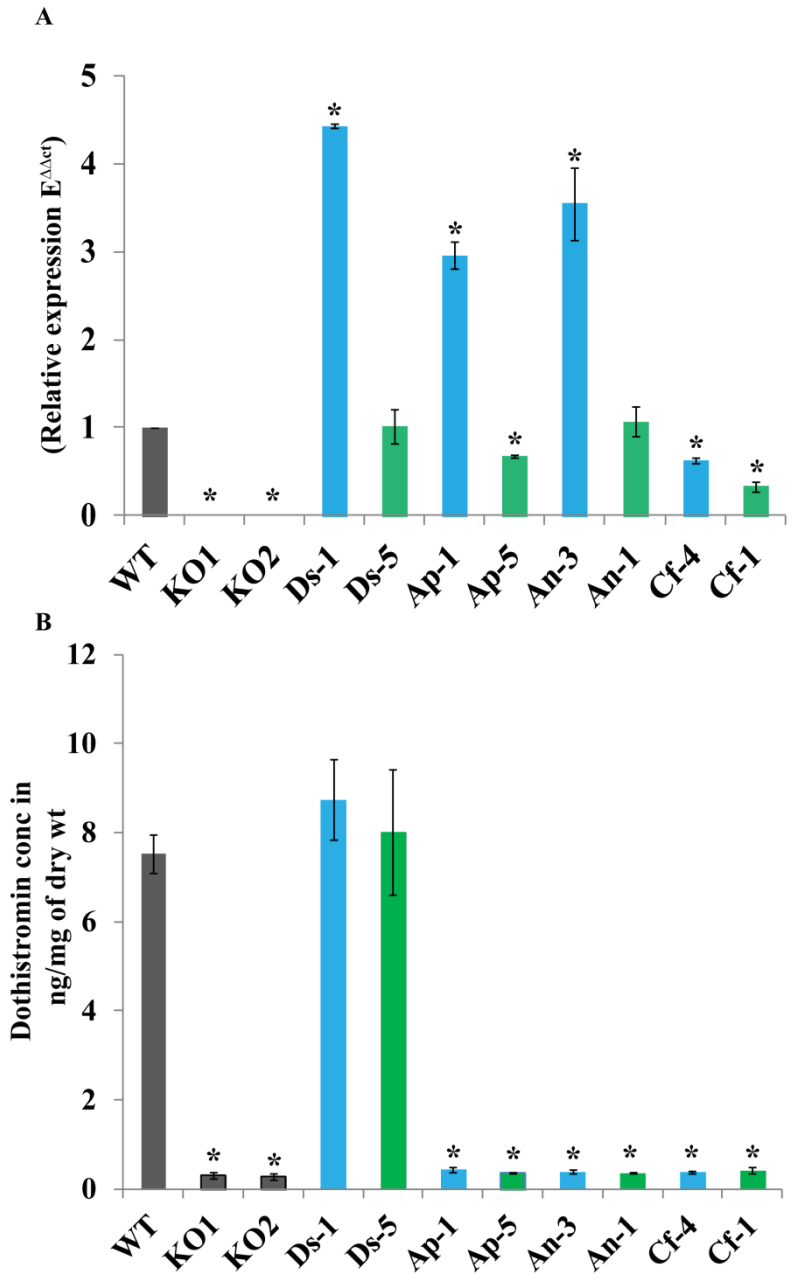


Figure 4.5: *AflJ* expression and dothistromin production in complemented strain

(A) Real time RT-PCR analysis of *aflJ* genes of heterologous fungi in the *ΔDsAflJ* KO complemented strain. Shown is the expression of *DsAflJ*, *ApaflJ*, *AnaflJ* and *CfAflJ* in single (green bars) and multicopy (blue bars) complemented strains. Mean expression and standard deviation are shown for at least three biological replicates relative to *DsTub1* expression. Here the control used was wild type *D. septosporum* NZE10 with *DsAflJ* as target.

(B) Dothistromin production in the broth culture as measured by HPLC with (n = 3). The values show the amount of dothistromin secreted into the broth by each mg (DW) of mycelium in the flask. Significant differences from *D. septosporum* NZE10 values ($P \leq 0.05$) are indicated by an asterisk.

(Ds-5) and multi copy (Ds-1 with 4 copies and Ds-2 with 13 copies (data not shown) of *AflJ*) showed similar levels of dothistromin to that of the wild type strain (Figure 4.5 B).

4.2.5 Are AflJ proteins of different ascomycetes functionally conserved?

To investigate whether the role of *DsAflJ* in regulating dothistromin biosynthesis could be accomplished by its orthologs in *Aspergillus* spp. and *C. fulvum* transformation of the *DsAflJ* knockout mutant strain KO1 was performed with *A. parasiticus aflJ* (*ApaflJ*), *A. nidulans aflJ* (*AnaflJ*) *and C. fulvum AflJ* (*CfAflJ*), with *DsAflJ* as a positive control. Integration of the heterologous genes was confirmed by PCR (Figure 4.4 A). This yielded four Δ*DsAflJ* mutants complemented with *ApaflJ* (designated Ap-1, Ap-2, Ap-4 and Ap-5), and three each with *AnaflJ* and *CfAflJ* (An-1, An-3, An-4 and Cf-1, Cf-2, Cf-4 respectively).

The copy numbers of the transgenes were determined by qPCR (Figure 4.4 B) and ranged from single copy (Ap-2, Ap-5, An-1, An-4, Cf-1) to multi copy (Ap-1, An-3, Cf-2 & Cf-4). One of the *ApaflJ* complemented strains Ap-4 did not amplify with *aflJ* primers so was excluded. *AflJ* transcript accumulation in wild type *D. septosporum* NZE10 and the complemented *ΔDsAflJ* mutants was quantified by qPCR (Figure 4.5 A). The results suggested that the promoters of the heterologous genes functioned in *D. septosporum* as transcript accumulation was proportional to the copy number, except for *CfAflJ* where it was 30% of the wild type in the single copy complemented strain. The production of dothistromin was assessed on liquid media extracts using HPLC (Figure 4.5 B). None of the orthologs of *DsAflJ* could complement the function of the *ΔDsAflJ* strain. However, function was restored to wild type levels in the positive controls Ds-1 and Ds-5 (complemented with *DsAflJ*).

4.3 Discussion

Studies in *Aspergillus* spp. have shown that *aflJ* is required for wild type levels of aflatoxin production (Meyers et al., 1998; Chang 2003, 2004). Similar to *Aspergillus* AflJ, DsAflJ does not carry any known functional domain (Figure 4.1) and its role in dothistromin biosynthesis is not clear. In the present study, a regulatory role for *DsAflJ* in dothistromin biosynthesis is demonstrated.

It is clear from the experimental data that *DsAflJ* positively regulates dothistromin biosynthesis as *DsAflJ* disruption mutants KO1 and KO2 produced significantly less (25-fold) dothistromin than the wild type strain (Figure 4.3 A). However, these residual levels are higher than what is seen in Δ*aflJ* mutants of *A. flavus* (100-fold less AF than wild-type; Meyers et al., 1998) and *A. parasiticus* (in which AF production is completely abolished) (Chang et al., 2003). In *A. flavus* the disruption of *aflJ* did not affect the transcription of *pksA* or two other AF genes tested (*nor1* and *omtA*) (Meyers et al., 1998) despite the reduced levels of aflatoxin. However in *DsAflJ* mutants the accumulation of transcripts of the dothistromin pathway genes ranged from 3.9 to 33.1 % of the wild type in KO1 to 4.5 to 28.3% of wild type in KO2 (Figure 4.3 B). Quite interestingly, the expression of the polyketide synthase gene *DsPksA* was drastically reduced in the *ΔDsAflJ* mutant suggesting that *DsAflJ* is involved in the regulation of dothistromin biosynthesis, at the level of transcription.

Complementation of the *ΔDsAflJ* mutant with the native *DsAflJ* gene reverted dothistromin production to wild type levels (Figure 4.5 B). However, additional copies of *DsAflJ* in the multicopy strain did not enhance the dothistromin level further, in contrast to *A. flavus* where overexpression using the *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter enhanced the production of aflatoxin by eight fold (Du et al., 2007). This suggested that either a stoichiometric balance of the

AflR:AflJ protein is required for wild type level of dothistromin production (Schmidt-Heydt et al., 2009) or some other factor like global regulators or chromatin modifiers are rate-limiting in *D. septosporum*.

In the previous chapter, it was demonstrated that *DsAflR* negatively regulates *DsAflJ* as deletion of *DsAflR* leads to an increase in *DsAflJ* transcript by almost ten fold. However, this study showed that *DsAflJ* does not affect the expression of *DsAflR* (Figure 4.3 B) unlike in *Aspergillus* spp. (Chang et al., 2003; Du et al., 2007). This observation might possibly be explained by the previous findings (chapter 3) that the consensus AflR binding site, $TCGN_5CGR$, commonly found in the promoters of the aflatoxin pathway structural genes (Ehrlich et al., 1999; Fernandes et al., 1998), is present in the promoter of the *DsAflJ* intergenic region; this assumes that AflR could act as a repressor in addition to its known role as an activator. Another possible explanation may be the larger intergenic region in *DsAflR-DsAflJ* (2.348 kb) as compared to *Aspergillus* spp. (0.737 kb) which could lead to competition for the same pool of polymerases and associated factors (Wei et al., 2011), or due to differences in chromatin-level regulation operating in this intergenic region.

To investigate whether the role of AflJ is functionally conserved in ascomycetes, *ΔDsAflJ* strain KO1 was transformed with heterologous genes from *A. parasiticus, A. nidulans* and *C. fulvum*. The native promoters of all the heterologous genes were functional in *D. septosporum* as revealed by qPCR assays (Figure 4.5 A). However, they could not complement the function of *DsAflJ* by restoring dothistromin levels (Figure 4.5 B), suggesting strong species specificity for AflJ function. Ehrlich et al (2012) reported a very similar story in *Aspergillus parasiticus* where he failed to demonstrate the complementation of an *ApaflJ* knockout mutant with orthologs from other ascomycetes including *DsAflJ*.

Species-specific differences in codon usage can also hinder recombinant gene expression by heterologous expression hosts. This may result in lack of expression, or inefficient expression of proteins (Angov et al., 2008). Further, different taxonomic classes can have different types of chaperone machineries operating, resulting in incorrect folding of heterologous proteins and rendering them non-functional. The co-expression and engineering of chaperones has been demonstrated to improve the production of recombinant proteins in eukaryotic and prokaryotic systems (Schroder, 2008; Rodrigo et al., 2014; Stephens et al., 2011).

. Alternatively there might be different localisation of the gene products thus making them unavailable at the required sites. In the case of *CfAflJ,* the lack of complementation might reflect the incorrect JGI gene model of *CfAflJ* (PID 197013). An improved model was predicted by manual annotation (Appendix 8.5.1) but this still shows that CfAflJ is 64 & 83 aa shorter at the N and C terminals compared to DsAflJ.

The transcript profile of the *DsAflJ* mutant suggests that DsAflJ is involved in the regulation of transcription of structural genes for enzymes of the dothistromin pathway similar to that in *A. parasiticus* but the possibility of an indirect effect on transcription cannot be ruled out. The presence of the AflJ interaction motif at the C terminal of DsAflR suggests the possibility that DsAflJ interacts with AflR (Chang et al., 2003). Ehrlich et al (2012) proposed that AflJ assists in AflR transport to or from the nucleus, thus controlling the availability of AflR for transcriptional activation of aflatoxin biosynthesis cluster genes. AflJ may also assist in directing endosomes to the cytoplasmic membrane for aflatoxin export. The presence of only a single transmembrane helix (Bitopic), with a statistically low score, suggests that DsAflJ may not have the second (directing endosomes) activity. Previously the function of such transmembrane domains in bitopic membrane proteins has been mostly limited to

membrane anchoring but recently such proteins have been shown to act as cell surface markers, receptors or adhesion factors (Albers et al., 2011).

In the *ΔDsAflJ* strain, lack of AflJ may hinder AflR reaching the site of action thus indirectly affecting dothistromin gene expression. Deletion of *DsAflJ* reduced the production of dothistromin by almost 20 fold, however, in the complemented strain it was reverted to wild type level. It has been shown that excess of mediator cannot bypass the requirement of the basal factor (Baek et al., 2006) which is consistent with the finding that overexpression of *DsAflJ* did not enhance dothistromin levels (Figure 4.5 B). Co-activators cannot bind DNA by themselves (Naar et al., 2001, McKenna and O'Malley, 2013) similarly; DsAflJ does not carry any DNA binding motif. However, further in depth study is required to elucidate its mechanism in dothistromin gene regulation. The interaction between AflJ and AflR, and their role in regulation of dothistromin, is discussed in more detail in Chapter 7, along with a model.

In *A. parasiticus* aflatoxin biosynthesis the enzymatic steps take place in vesicles/aflatoxisomes (Chanda et al., 2009, Ehrlich et al., 2012). In *P. chrysogenum* last steps of the penicillin biosynthesis pathway are localised in peroxisomes (van de kamp 1999; van der lende et al., 2002). However, such compartmentalization of dothistromin biosynthesis has not been reported in *D. septosporum* though the possibility of its existence cannot be ruled out. A preliminary cellular localisation study of the NADPH-dependent reductase DsVer1 using a GFP fusion revealed that the majority of DsVer1 to be cytoplasmic during an early stage of growth *in vitro* and then localised to vesicle-like structures and vacuoles at a later stage of growth (Bradshaw et al., 2009) similar to that seen in *A. parasiticus ver1*. The absence of a C terminal microbody targeting signal in DsAflJ cannot rule out the likelihood that dothistromin is synthesized in vesicles; the vacuolar targteing motif which is present only in DsAflJ

might serve the same function as a microbody targeting signal. Further, the localisation to microbodies may not be mandatory for biosynthesis, as shown for penicillin biosynthesis in *A. nidulans* where proper localisation of the IPN acyl-transferase (IAT) is not an absolute requirement (Sprote et al., 2009). In that study, deletion of the peroxisome targeting signal lead to mislocalised IAT protein that was partially functional and did not abolish the synthesis of penicillin but reduced it compared to the wild type strain. Further detailed studies of intracellular localization are required to determine the locations of DsAflR and DsAflJ during dothistromin biosynthesis.

Chapter 5: The *DsVeA* gene of the pine needle pathogen *Dothistroma septosporum* regulates sporulation and secondary metabolism.

5.1 Introduction

In fungi, the *veA* or "*velvet*" gene regulates several cellular processes, such as asexual and sexual development and biosynthesis of natural products. This global regulator is conserved in numerous fungal species, particularly Ascomycetes (Myung et al., 2011) and in at least some members in the basidiomycetes such as *Ustilago maydis* (Karakkat et al., 2013). Most of the studies on *veA* have been carried out in *Aspergillus* spp. particularly in the model fungus *A. nidulans*, which is considered as a founding member where this gene has been described to control the developmental balance in response to light. In 2002 the *veA* gene was cloned by multicopy complementation of the *A. nidulans veA1* mutant strain (Kim et al., 2002).

In *A. nidulans* strains with a *veA* wild-type allele, light reduces and delays fruiting body formation and the fungus develops asexually forming conidiophores; whereas in the dark, the sexual stage is induced (Champe et al., 1981; Yager et al., 1992). Deletion of *veA* prevents the formation of fruiting bodies (Kim et al., 2002) and sclerotia in *A. flavus* (Duran et al., 2007). Lack of *veA* also affects conidiation, resulting in increased conidiation in *A. nidulans* and *A. flavus* mutants (Kim, et al., 2002; Duran et al., 2007) but a decrease in *A. parasiticus* mutants (Calvo et al., 2004).

Importantly, velvet proteins have been shown to be positive regulators of various metabolite biosynthetic pathways in a range of fungal species (Table 5.1)

Table 5.1: List of secondary metabolites positively regulated by velvet (*veA*)

Species	Secondary metabolite	References
Aspergillus carbonarius	ochratoxin A	Crespo-Sempere et al., 2013
Acremonium chrysogenum	cephalosporin C	Dreyer et al., 2007
Aspergillus flavus	cyclopiazonic acid, aflatrem, and aflatoxin	Duran et al., 2007
Aspergillus fumigatus	gliotoxin and fumagillin	Dhingra et al., 2012
Aspergillus nidulans	penicillin	Kato et al., 2003
Aspergillus nidulans	sterigmatocystin	Kato et al., 2003
Botrytis cinerea	bikaverin	Schumacher et al., 2013
Cochliobolus heterostrophus	T toxin and melanin	Wu et al., 2012
Fusarium fujikuroi	GA, fumonisin and fusarin C	Wiemann, 2010
	fusaric acid	Niehaus et al., 2014
Fusarium oxysporum	triacetylfusarinine C	López-Berges et al., 2013
Fusarium verticillioides	beauvericin, ferricrocin and fusarins gibberellins, and Fusaric acid	Myung et al., 2009; Myung et al., 2011
Penicillium chrysogenum	penicillin	Hoff et al., 2010
Penicillium citrinum ·	ML-236B	Baba et al., 2012

In *A. nidulans* it is known that the VeA protein is transported to the nucleus where it interacts with other components such as the light sensing proteins, FphA, LreA and LreB (Purschwitz et al., 2008), as well as proteins influencing chromatin conformation, such as LaeA, and other members of the velvet family such as velB (Bayram et al., 2008; Wiemann et al., 2010; reviewed by Calvo 2008; Bayram and Braus, 2012). Interactions between members of the velvet family lead to the transcriptional regulation of genes involved in the synthesis of secondary metabolites, commonly found in gene clusters (Bayram and Braus, 2012) as well as the induction/repression of developmental genes.

The increase in the availability of fungal genome sequences has allowed for the identification of additional putative *veA* orthologs in other species (Myung et al., 2011). Among fungal species with a *veA* ortholog are known pathogens of plants. It has been demonstrated that *veA* is also involved in pathogenicity. For example, a *F. verticillioides veA* null mutant fails to produce disease in seedlings of maize (Myung et al., 2012). Deletion of *veA* also reduced virulence of *A. flavus* (Duran et al., 2009),

A. parasiticus (Calvo et al., 2004), *F. fujikuroi* (Wiemann et al., 2010), *F. graminearum* (Merhej et al., 2012), *Cochliobolus heterostrophus* (Wu et al., 2012), *Botrytis cinerea* (Yang et al., 2013) *and Fusarium oxysporum* (López-Berges et al., 2013); and *Magnaporthe oryzae* (Kim et al., 2014) among others. Importantly, VeA also influences pathogenicity in animals by the opportunistic pathogenic fungi *Histoplasma capsulatum* (Laskowski-Peak et al., 2012) and *Aspergillus fumigatus* (Dhingra et al., 2013). Because *veA* is unique to fungi, it is a promising target to control fungal diseases.

In the current study, the hypothesis that a putative *veA* ortholog could regulate the biosynthesis of dothistromin was tested. We examined whether the *DsVeA* gene product regulates expression of dothistromin genes and other secondary metabolite genes in *D. septosporum,* and whether *DsVeA*-deficient mutants are able to cause disease in pine needles. The possible effect of DsVeA on the development of this plant pathogen was also examined.

Part of the work described in this chapter was carried out by Yanan Guo (Massey University). Ms Guo made the *VeA* mutant and complementation strains, and compared their growth rates, sporulation, germination, hyphal morphology and hydrophobicity with the wild type strain. For coherence the work is presented as a whole and has been published with input from other co authors (Chettri et al., 2012).

5.2 Results

5.2.1 *Dothistroma septosporum* contains a *veA* gene ortholog.

Prior to availability of the *D. septosporum* genome sequence, the *DsVeA* gene was identified using degenerate PCR primers designed from alignments of the putative *Mycosphaerella graminicola* (estExt_fgen- esh2_pg.C_20216) and

Mycosphaerella fijiensis (estExt_fgenesh2_pg. C_140014) VeA orthologs identified from the JGI genome databases (http:// gen ome.jgi-psf.org/Mycgr3/Mycgr3.home.html) and (http://geno me.jgi-psf.org/Mycfi2/Mycfi2.home.html) based on similarity to the *Aspergillus flavus veA* gene (AFL2G_07468.2) from the Broad Institute Aspergillus Comparative Database. Subsequently BLASTP of the JGI *D. septosporum* genome database (http://genome.jgi-psf.org/ Dotse1/Dotse1.home.html) with the candidate *D. septosporum VeA* gene enabled confirmation of the gene sequence (protein ID 69562) and flanking regions.

The gene model of the candidate *veA* gene *DsVeA* revealed an open reading frame (ORF) of 1891 bp interrupted by one 55 bp intron. The gene encodes a predicted protein of 611 amino acids (protein ID 69562) that shows identity to velvet proteins from other ascomycetes, for example 37.8% to *A. nidulans* VeA (AAD42946), 48.2% to *Penicillium chrysogenum* PcVeA (CAP92389), 38.3% to *F. verticillioides* FvVeA (ABC02879) and 75.7% to *M. graminicola* MVE1 (Mycgr3, 37276). Alignment of the predicted amino acid sequence to those of other *veA* gene products revealed several conserved regions (Figure 5.1). A PEST domain, thought to act as a signal peptide for protein degradation, was found in the C terminal region. Proteins that function in the nucleus are transported through nuclear pore complexes and contain nuclear localization signals (NLS). The best characterized NLS consists of either one (monopartite) or two (bipartite) stretches of basic amino acids (Lange et al., 2007). Given the role of VeA in the nucleus and the presence of a NLS in *A. nidulans* VeA the WolF PSORT NLS programme was used for NLS prediction. The known bipartite *A. nidulans* VeA NLS was detected correctly but it did not predict an NLS in either MVE1 or DsVeA. However a sequence similar to the putative NLS was identified in the *M. graminicola* MVE1 protein (Choi and Goodwin, 2011), but this was not evident in DsVeA.

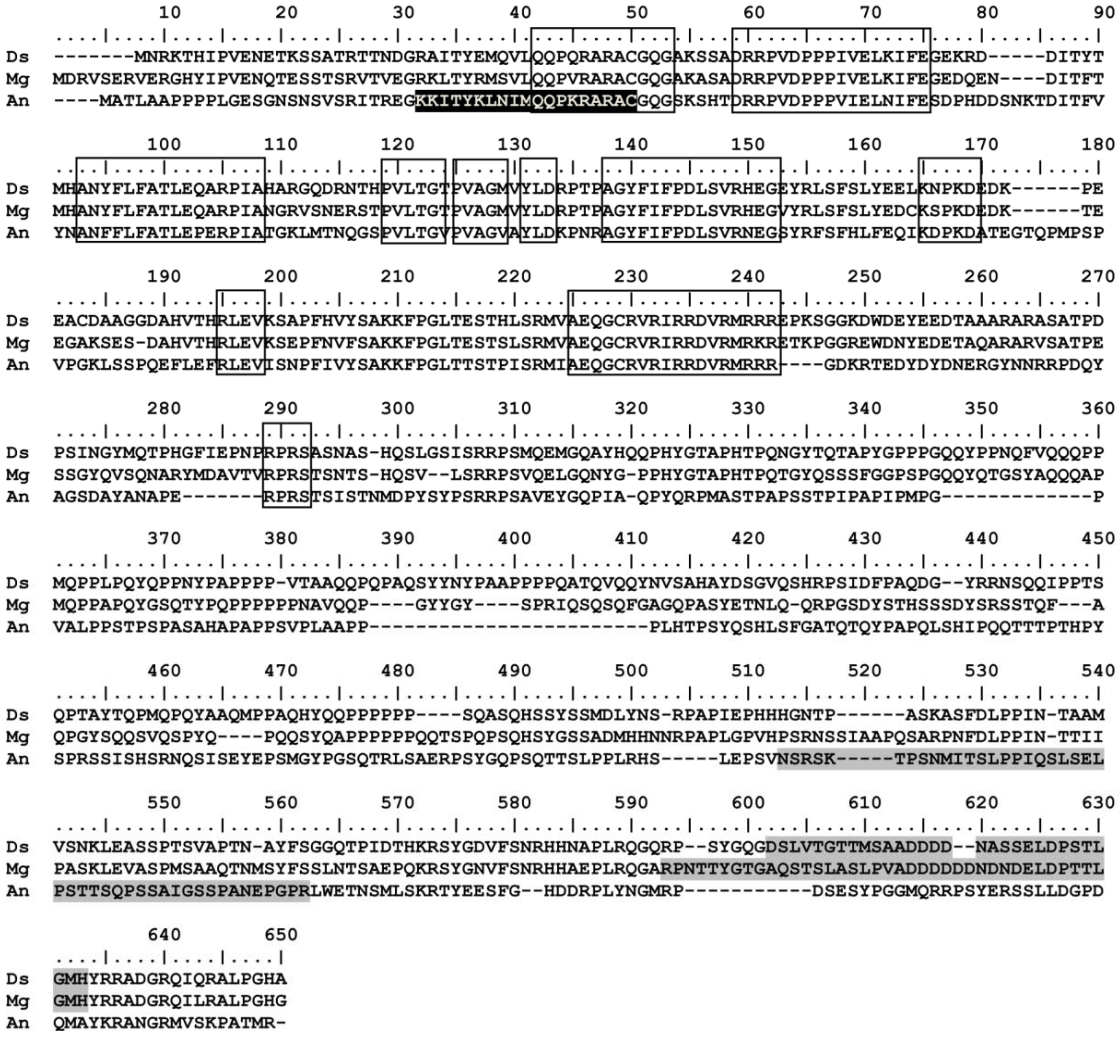


Figure 5.1: Multiple alignment of VeA sequences.

Multiple sequence alignment of predicted DsVeA amino acid sequence with that of other characterized VeA homologs. Alignment was generated using Bioedit. Putative PEST domains are marked in gray. Putative NLS predicted using WoLF PSORT is marked in black. Highly conserved regions are boxed. Sequences aligned with DsVeA (PID 69562) from *D. septosporum* (Ds) are MVE1 (PID 37276) from *M. graminicola* (Mg) and VeA (AAD42946) from *A. nidulans* (An).

5.2.2 *DsVeA* knockout and complemented mutants were made

To test the hypothesis that DsVeA regulates the expression of dothistromin biosynthetic genes and dothistromin production, *D. septosporum* mutants lacking DsVeA were obtained by transformation of *D. septosporum* with the knockout construct pR297 (method section 2.9). The transformation yielded 18 hygromycin-resistant transformants, of which 7 showed lighter colour compared to WT colonies. Of these, two transformants (KO1 and KO2) were confirmed to be *DsVeA* knockout strains by PCR using two pairs of primers that each targeted the hygromycin B coding region and flanking region (either 5' or 3' side) as shown in (Appendix 8.6.1 C). Southern hybridisation confirmed absence of a *veA*-hybridising DNA fragment in these strains (Appendix 8.6.1 C) and confirmed that *DsVeA* is a single-copy gene, as suggested by analysis of the NZE10 *D. septosporum* genome.

Complementation strains were produced by transformation of KO1 with a full-length *DsVeA* gene (plasmid pR298) and those showing restoration of pigmentation (thought to be dothistromin), were selected for analysis. Southern hybridisation confirmed restoration of a *DsVeA* gene in these strains, and suggested that C2 contains more copies of the *DsVeA* gene than C1 (Appendix 8.6.1 B). The above transformations and confirmation were done by Yanan Guo at IFS Massey University. RNA transcript levels of *DsVeA* were approximately twice as high in C2 than in C1 (C1 0.97 ± 0.01; C2 1.96 ± 0.01; both normalised to wild-type levels (WT=1), suggesting that C2 is a DsVeA over-producing strain and may have two copies of the *DsVeA* gene.

5.2.3 *DsVeA* knockout mutants are impaired in asexual sporulation

Deletion of *veA* genes has been shown to affect growth and sporulation in some fungi. Therefore the effect of *DsVeA* deletion on these morphological features in *D. septosporum* were determined, although observations of sporulation were confined to the asexual stage. Sporulation was significantly decreased in *ΔDsVeA* strains, in both light and dark conditions, with less than 10% of spore numbers compared to the wild-type and complemented strains (Figure 5.2 A). No sporulation differences were seen between wild-type and *DsVeA* complemented strains. The absence of light also had a significant effect on sporulation. Approximately twice as many spores were produced by the wild-type and complemented strains in dark conditions compared to light conditions at the selected time point (Figure 5.2 A). The same effect of light was also seen with both gene knockout strains, hence although sporulation levels were considerably lower in *DsVeA* mutants, inactivation of this gene did not affect responsiveness to light.

Expression of *D. septosporum SteA* (PID 72706) or *NsdD* (PID 68310) genes, putative orthologs of genes involved in sexual development in *A. nidulans*, did not require DsVeA (Figure 5.2 B), although the over-expression C2 strain expressed significantly higher levels of both of these genes. In contrast expression of the *DsStuA* (PID 45737) gene, an ortholog of the *SnStuA* gene that is required for normal sporulation, mycotoxin production and pathogenicity in *Stagonospora nodorum* (Ipcho et al., 2010), was reduced in *DsVeA* mutants (Figure 5.2 B).

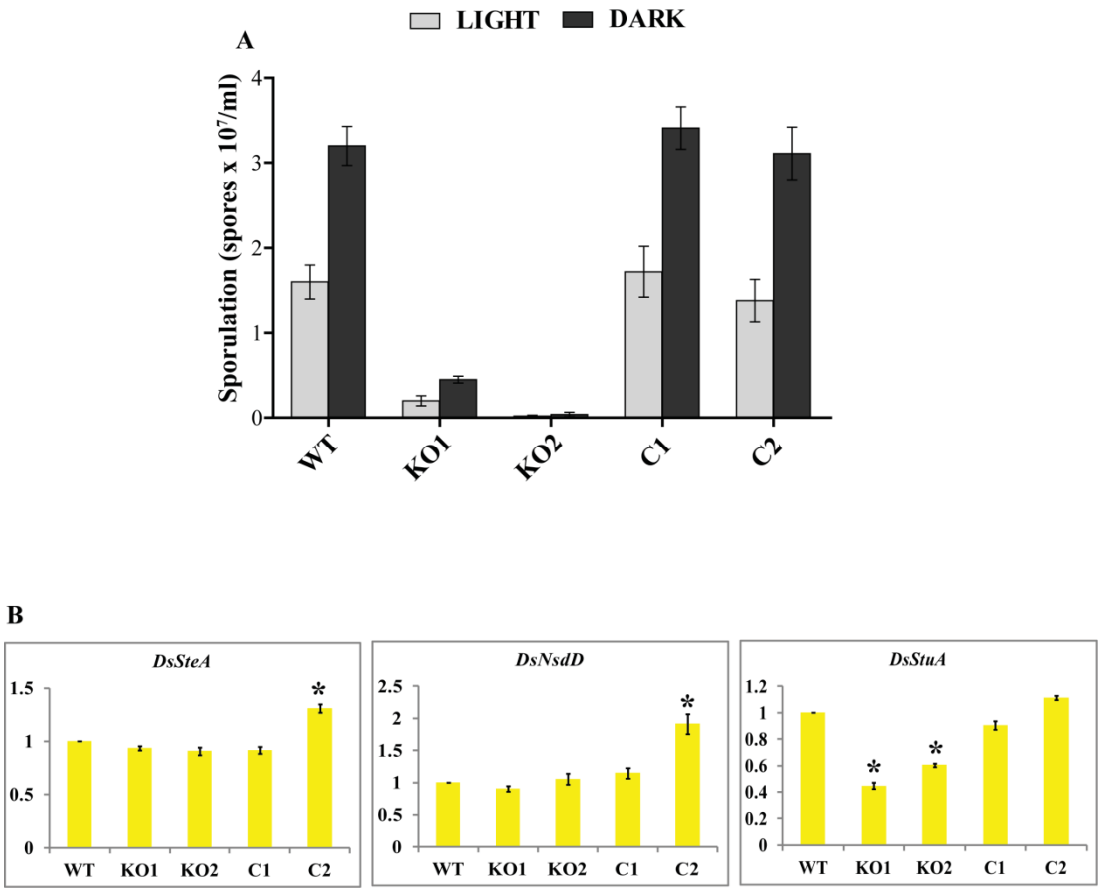


Figure 5.2: Sporulation and developmental gene expression in *DsVeA* mutants.

(A). Sporulation of *DsVeA* knockout and complemented strains. Sporulation of wild type (WT), *DsVeA* knockout (KO1, KO2) and complemented (C1 and C2) strains in *D. septosporum* in light (grey) and dark (black) conditions (mean ± SE). Significant differences ($P < 0.05$) were seen between sporulation of KO and WT strains and also between light and dark conditions for all strains. (This sporulation test was done by Yanan Guo)

(B) Expression of putative developmental regulatory genes *DsSteA*, *DsNsdD* and *DsStuA* in velvet mutants grown in light conditions. Normalized gene expression ratios (method section 2.5.3) are shown for *DsVeA* knockout (KO1 and KO2) and complemented (C1 and C2) strains, relative to wild type (WT); mean ± SE. Significant differences from WT ($P \leqslant 0.01$) are shown by an asterisk.

In contrast to the situation with sporulation, radial growth rates, spore germination rates, and hydrophobicity assay and morphology analyses performed by my colleague Yanan Guo showed no significant differences in either light or dark conditions for any of the *DsVeA* knockout or complemented strains compared to the wild type, in two independent experiments (results not shown). All strains grew slightly faster in the dark than the light, but this difference was only significant ($P<0.05$) for KO2, which showed a 30% faster growth rate in the dark. While germination rates were generally slightly lower in dark conditions, this was not a consistent effect (Appendix 8.6.2). Taken together, these results suggest that DsVeA is required for normal levels of asexual sporulation but is not required for hydrophobicity or normal hyphal or spore morphology under the conditions tested.

5.2.4 Dothistromin production and dothistromin gene expression is reduced in *DsVeA* mutants.

Because VeA is known to regulate secondary metabolite biosynthesis in many fungi, the production of dothistromin by *DsVeA* mutants was assessed in broth cultures. Dothistromin production was reduced to less than 10% of wild-type levels, but not completely suppressed, in the mutants, and restored to wild-type levels in the complemented strains (Table 5.2). There was no significant difference between dothistromin levels in light and dark in the wild-type but, somewhat unexpectedly, both *DsVeA* deletion mutants produced 2-3-fold more dothistromin in the dark than the light ($P<0.05$). In contrast, one of the complemented strains, C1, produced more dothistromin in the light however in C2 strain no significant difference in dothistromin level was observed for cultures grown in either light or dark conditions. This finding, along with high variability between replicates that is often seen with dothistromin production in *D.*

septosporum, sheds doubt on the reliability of the light/dark effect seen in the *DsVeA* mutants.

Table 5.2: Dothistromin production in culture under light and dark conditions

Strain[a]	Dothistromin[b] (ng/mL) in Light	Dothistromin[b] (ng/mL) in Dark
WT	1562.6 ± 217.2	1262.8 ± 58.3
KO1	63.0 ± 1.5[c]	103.3 ± 13.2[c]
KO2	17.7 ± 7.9[c]	59.5 ± 7.7[c]
C1	1585.8 ± 409.7	690.0 ± 62.4[c]
C2	1613.3 ± 679.6	2771.2 ± 515.2[c]

[a]WT, wild-type; KO1, KO2, *DsVeA* knockout mutants; C1, C2, complementation mutants; differences in fungal biomass between each of the strains were not significant (data not shown)

[b]Dothistromin (mean ± SD); n = 3.

[c]Significant difference to WT ($P < 0.05$)

Strains showing no significant difference in dothistromin production between light and dark conditions are highlighted.

Quantitative PCR was used to quantify the expression of dothistromin genes as shown in Figure 5.3. Two functionally-characterised dothistromin biosynthetic genes, *DsPksA* and *DsVer1* (Bradshaw et al., 2002; Bradshaw et al., 2006) were characterised, along with the two regulatory genes *DsAflR* (Chapter 3) and *DsAflJ* (Chapter 4). Expression of *DsVeA* was also assessed, along with a constitutively expressed beta-tubulin control gene. As expected, the *DsVeA* disruption mutants KO1 & KO2 did not show *DsVeA* expression above background, as this single-copy *DsVeA* gene was disrupted. Expression was restored to wild-type levels in complemented strain C1 but approximately doubled in strain C2 as mentioned above. Expression levels of a control beta-tubulin gene, *DsTub1*, were constitutive throughout, as expected.

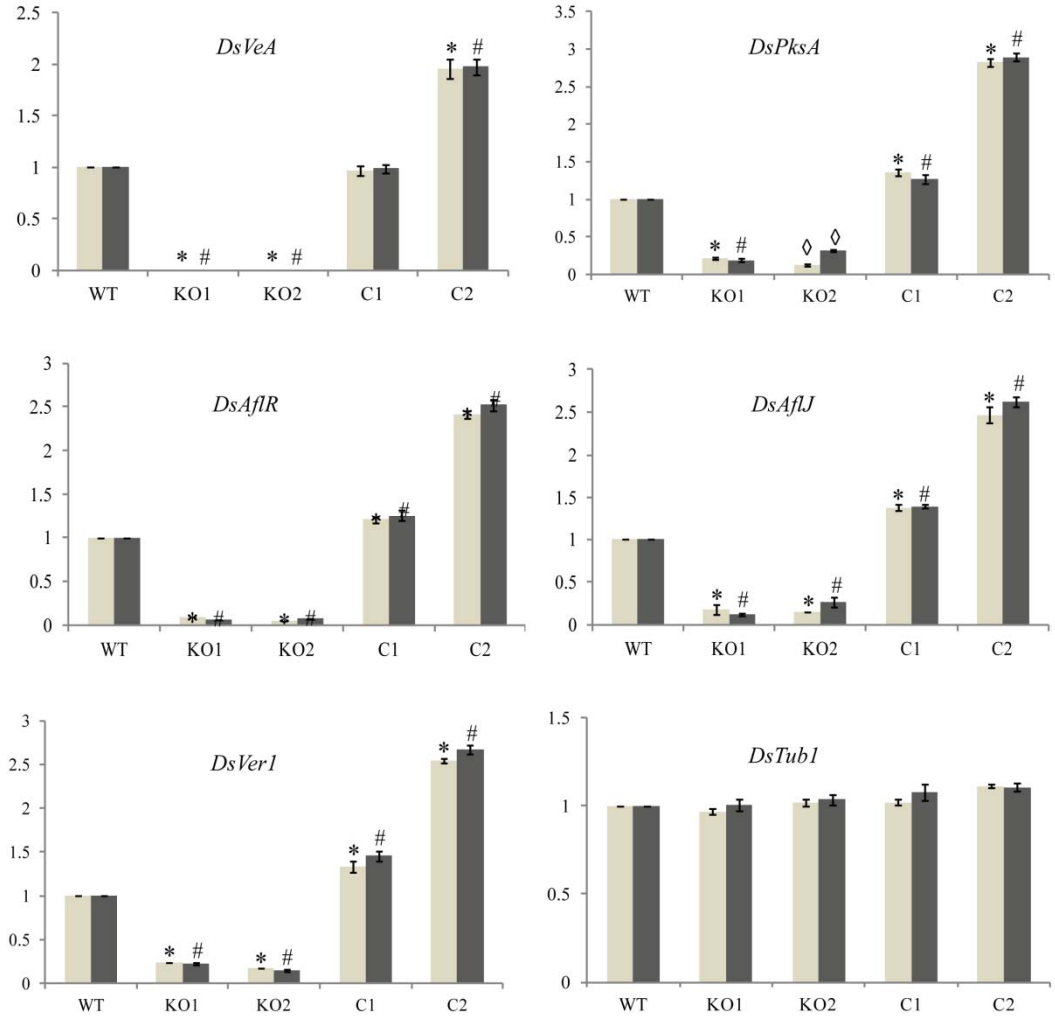


Figure 5.3: Expression of secondary metabolite genes in *DsVeA* mutants.

Normalized gene expression ratios for *DsVeA* knockout (KO1 and KO2) and complemented (C1 and C2) strains, relative to wild type (WT); mean ± SE. Significant differences from WT ($p \leqslant 0.01$) are shown by an asterisk or hash. Expression of *DsVeA* and dothistromin biosynthetic genes *DsPksA*, *DsVer1* regulatory genes *DsAflR*, *DsAflJ* and beta tubulin (*DsTub1*, constitutive control) in cultures grown either in light (grey bar) or dark (black bar) conditions. The rhombus indicates a significant difference between light and dark conditions.

Expression of the putative dothistromin biosynthesis regulatory gene *DsAflR* was reduced by almost twenty-fold in the *DsVeA* mutants compared to wild-type levels. Similarly *DsAflJ* also had reduced expression in the *DsVeA* mutant in *D. septosporum*. Expression levels of dothistromin biosynthetic genes *DsPksA* and *DsVer1*were significantly down-regulated by five- to eight-fold in the *DsVeA* mutants (Figure 5.3). This is possibly due to the strong down-regulation of *DsAflR* which regulates the expression of other pathway genes (Chapter 3), as is the case for aflatoxin and sterigmatocystin biosynthetic genes in *Aspergillus* spp. (Cary et al., 2006). In the complemented strains, expression levels of *DsAflR, DsAflJ, DsPksA* and *DsVer1* were all significantly higher in the C2 *DsVeA* over-expression strain compared to C1 that showed expression levels closer to wildtype.

Levels of gene expression were compared between light and dark growth conditions for all strains (Figure 5.3). No significant differences were seen for any of the genes except *DsPksA,* which was expressed at slightly higher levels in the dark, but only in the KO2 strain. Considered together with the results of dothistromin assays, these results suggest that DsVeA is required for wild-type levels of dothistromin gene expression and dothistromin production. However, there is no consistent evidence for an effect of light on dothistromin production.

5.2.5 Production of other secondary metabolites is reduced in *DsVeA* mutants

The *veA* gene is known to affect production of several different types of secondary metabolites in fungi. In thin layer chromatography experiments designed to provide a qualitative assessment of the effects of *DsVeA* mutation on dothistromin

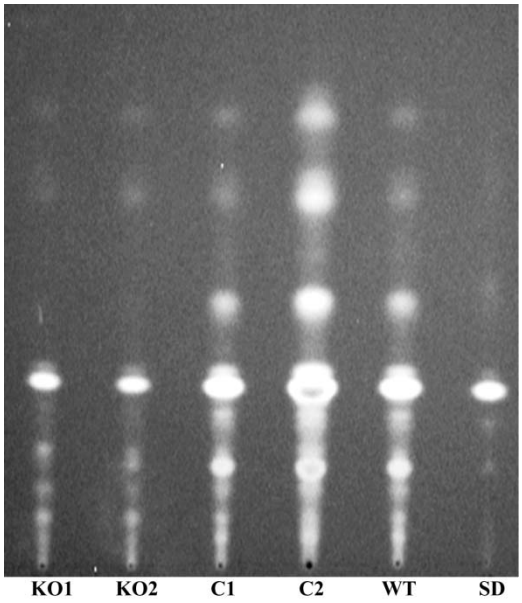


Figure 5.4: Thin layer chromatography of *Dothistroma septosporum* extracts.

TLC of extracts from *DsVeA* knockout strains (KO1 and KO2) complemented (C1 and C2) or wild-type (WT) along with a dothistromin standard. The position of dothistromin is indicated by the bright spot in the standard (SD).

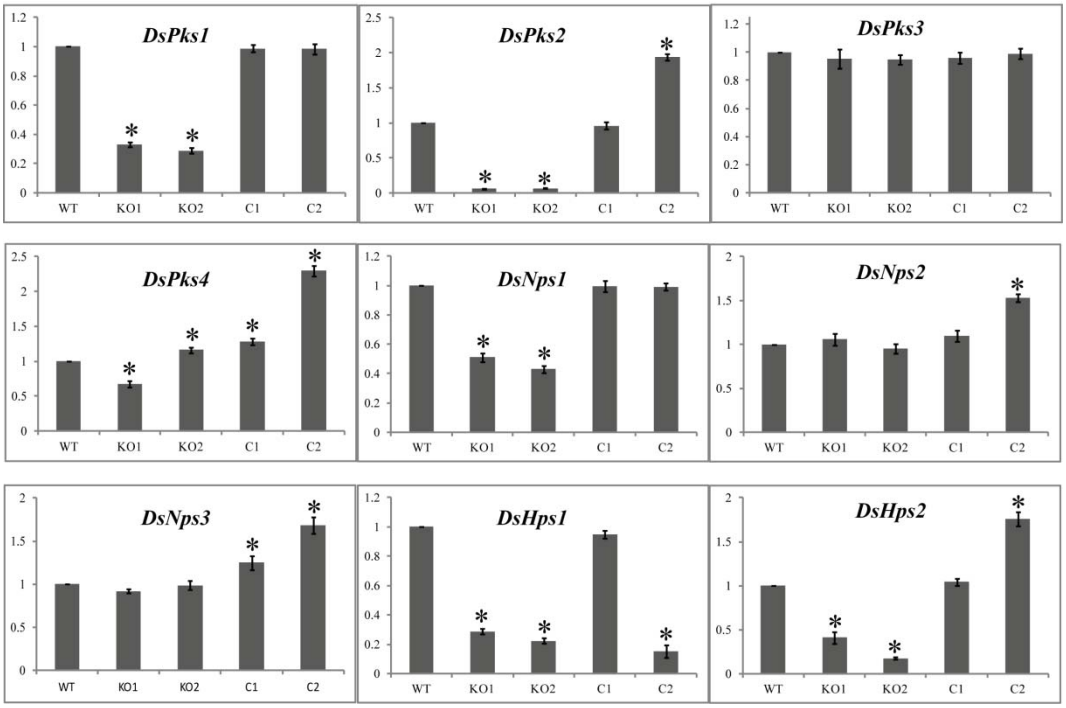


Figure 5.5: Expression of secondary metabolite genes in velvet mutants.

Normalized gene expression ratios for *DsVeA* knockout (KO1 and KO2) and complemented (C1 and C2) strains, relative to wild type (WT); mean ± SE. Significant differences from WT ($p \leqslant 0.01$) are shown by an asterisk. Expression of polyketide synthase (*DsPks1*, *DsPks2*, *DsPks3*, *DsPks4*), non-ribosomal peptide synthase (*DsNps1*, *DsNps2*, *DsNps3*) and PKS-NRPS hybrid (*DsHps1*, *DsHps2*) genes in light conditions.

production (method section 2.8) it was noticed that other metabolites also appeared to vary in concentration. By alteration of solvent from a chloroform: methanol system (optimised for dothistromin) to a toluene: acetone system (method section 2.8.4), better resolution of these additional products was seen (Figure 5.4) and mutants appeared to have lower levels of other metabolites as well as dothistromin, although the identity of these additional products is not known.

The *D. septosporum* genome contains putative polyketide synthase (*Pks*), non-ribosomal peptide synthase (*Nps*) and hybrid PKS-NRPS (*Hps*) genes that may be involved in secondary metabolite biosynthesis (De Wit et al., 2012). Four putative PKS genes (*DsPks1, DsPks2, DsPks3, DsPks4*), three NRPS (*DsNps1, DsNps2, DsNps3*) and two hybrid PKS-NRPS (*DsHps1, DsHps2*) were selected for analysis. Expression of these genes in *DsVeA* mutants, wild type and complemented strains was assessed by quantitative real-time RT-PCR as for the dothistromin genes (Figure 5.5).

The different genes showed different patterns of expression and fell into five phenotypic groups. Groups 1, 2 and 3 all showed significantly reduced expression in *DsVeA* mutants compared to the wild-type and complementation strain C1, but differed in the response of the *DsVeA* overexpressing strain C2. Group 1 genes, *DsPks2* and *DsHps2,* showed approximately two-fold higher expression in strain C2 than WT, consistent with the increased levels of *DsVeA* transcript accumulation in this strain. The Group 1 phenotype is the same as that seen for all four dothistromin genes shown in Figure 5.3. Group 2 includes genes *DsPks1* and *DsNps1*, in which expression was only restored to wild-type levels, not any higher, in the DsVeA overproducing C2 strains.

Group 3 contains a single gene, *DsHps1*, which is expressed at very low levels in C2, despite restoration to wild-type levels in strain C1. Group 4 contains just *DsPks3* which is expressed constitutively, as per the *tub1* control gene. Group 5 genes, *DsPks4,*

DsNps2 and *DsNps3* shared the unexpected phenotype of increased expression, compared to wild-type, in both complemented strains, but in each case the over-expression strain C2 had higher levels than C1.

Overall, it appears that five of the nine genes tested were down-regulated in *DsVeA* mutants (genes in groups 1-3) although the reduction in expression varied between the genes. The most severe effect was seen with *DsPks2* that was reduced to less than 10% of wild-type levels in both KO1 and KO2. Of the four genes that did not show the down-regulation phenotype, three of them (Group 5 genes) had higher levels of expression in complemented strains compared to the wild type, particularly in the over-expressing C2 strain. Overall, these results indicate that DsVeA is important in the regulation of biosynthesis of other secondary metabolites in *D. septosporum* in addition to dothistromin.

5.2.6 The *DsVeA* gene is not required for pathogenicity

Earlier studies showed that dothistromin is not required for pathogenicity (Schwelm et al., 2009), but Kabir et al (2014) reported that dothistromin is a virulence factor required for normal sporulation, mesophyll colonization and lesion expansion. However the small lesions produced in the absence of dothistromin suggest the possibility that other metabolites, possibly regulated by DsVeA, may be important in this process. Pathogenicity assay was done as described in section 2.9.4. Due to difficulties in obtaining mutant spores for inoculum, especially for KO2, only two seedlings were inoculated for KO2 in this study instead of three. After a ten-week incubation period the numbers of needles with disease lesions, and numbers of lesions per needle, were counted. Using these parameters as a measure of disease levels, no effect of *DsVeA* mutation was seen (Table 5.3).

The fungal biomass was estimated in disease lesions of the experiment shown in Table 5.3, by using a qPCR assay in which copy number of the single-copy *DsPksA* gene from *D. septosporum* was compared to that of the single-copy *CAD* gene of *P. radiata* (method section 2.5.5). This analysis suggested no significant differences in fungal biomass in disease lesions formed from wild-type, *DsVeA* mutant or complemented strains (Figure 5.6 A). Dothistromin levels were also assessed in these

Table 5.3: DsVeA pathogenicity assays.

Strain[a]	% of needles with Dothistroma symptoms	Lesions per infected needle
WT	72.9 ± 23.8	3.0 ± 0.7
KO1	36.8 ± 10.5	1.9 ± 0.1
KO2	86.2 ± 3.4	4.5 ± 1.3
C1	62.4 ± 15.7	3.3 ± 0.6
C2	59.6 ± 24.7	2.8 ± 0.4

[a] There was no significant difference between *ΔDsVeA* mutant (KO1 and KO2) or complemented (C1 and C2) strains compared to the wild-type (WT) for any of the parameters shown ($P > 0.05$). Values are mean ± SD.

lesions but highly variable results were seen between replicates, so we were not able to demonstrate a significant reduction in dothistromin levels in disease lesions infected with *DsVeA* mutants compared to those with the wild-type (Figure 5.6 B). The observed difference between the KO2 and KO1 strain could be attributed to a number of factors: 1) fewer trees inoculated due to difficulties in obtaining sufficient spores; 2) the pathogenicity assay was not well developed at the time when this experiment was done, reflecting the difficulty in getting reproducible infections in laboratory conditions with

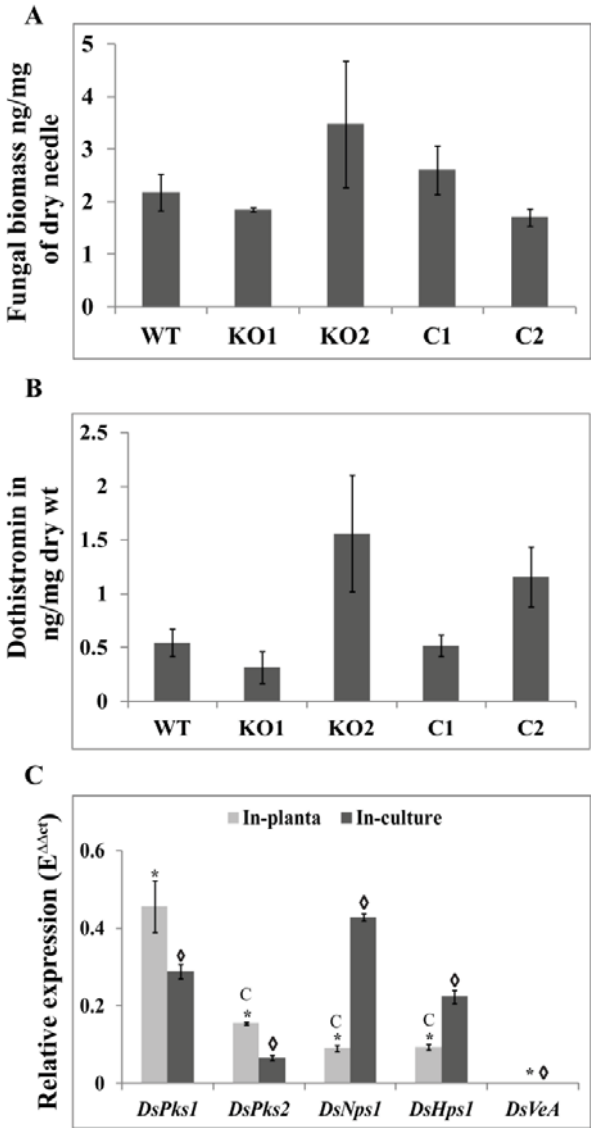


Figure 5.6: Biomass, dothistromin and gene expression of DsVeA mutants *in planta*

(A) Fungal biomass in disease lesions was estimated using a qPCR assay where copy number of the single-copy *DsPksA* gene from *D. septosporum* was compared to that of the single-copy *CAD* gene of *P. radiata.* No significant difference was found between *ΔDsVeA* mutant (KO1 and KO2) or complemented (C1 and C2) strains compared to the wild-type (WT); mean ± SE. ($P > 0.05$).

(B) Dothistromin levels in infected lesions as quantified by HPLC showed no significant difference compared to wild type (WT) mean ± SE. ($P>0.05$).

(C) Expression of secondary metabolite genes *in planta* (light grey) and in culture (dark grey) in *ΔDsVeA* knockout mutant KO1 compared to wild type under the same conditions (WT = 1). The *in planta* data were derived from the same pathogenicity experiment shown in Table 5.2. The *D. septosporum* 18S ribosomal gene was used as a normaliser. Expression is shown for polyketide synthase (*DsPks1*, *DsPks2*), non ribosomal peptide synthase (*DsNps1*) and PKS-NRPS hybrid (*DsHps1*) genes. Values are means ± SE. Significant difference from WT is marked by asterisk for *in-planta* and rhombus for in culture at ($p \leqslant 0.05$) and significant difference between *in planta and in culture* is marked with C.

this pathosystem (Schwelm et al., 2009); 3) the pine seedlings used for the assay were not a clonal population. The difference in host genotype could show varied response to a pathogen. However, *DsVeA* mutants showed severely reduced expression of the dothistromin *DsPksA* gene *in planta*, with less than 10% of wild-type levels.Taken together these results suggest that DsVeA, or the metabolites that are dependent on DsVeA for their expression, are not required for *D. septosporum* to be pathogenic. Expression of four of the other secondary metabolite genes that were down-regulated in DsVeA mutants in culture was assessed *in planta*. Expression of *DsPks2, DsNps1* and *DsHps1* was all significantly different *in planta* compared to in culture (Figure 5.6 C) and expression in *DsVeA* mutants was only 9-15% of wild-type levels (Figure 5.6 C).

5.3 Discussion

In this study, a *D. septosporum* gene, *DsVeA*, was found that is similar in sequence to other characterized *veA* genes. It was demonstrated that *DsVeA* is involved in the regulation of developmental and biosynthetic processes. By use of *DsVeA* deletion and over-expressing strains it was found that sporulation and dothistromin biosynthesis are positively regulated by DsVeA. Furthermore, expression studies of nine other putative secondary metabolite genes in these mutants suggested some involvement of DsVeA in their regulation.

There were differences in morphological phenotypes of *DsVeA* deficient mutants compared to the corresponding *mve1* mutants of *M. graminicola,* a related Dothideomycete (Choi and Goodwin, 2011). Whilst *DsVeA* mutants presented normal hydrophobicity and aerial mycelium, *mve1* mutants showed loss or reduction of both of these traits (Choi and Goodwin, 2011). There was also no evidence for alteration of hyphal or spore morphologies in *DsVeA* mutants similar to those seen with *M.*

graminicola, although different culture conditions were used. A striking reduction in asexual sporulation was observed in *DsVeA* mutants, with less than 10% of wild-type sporulation levels. A similar effect was seen in the AF-producing species *A. parasiticus*, in which sporulation levels dropped to 30% in the light and 46% in the dark in *veA* mutants (Calvo et al., 2004). Sporulation studies done in the *veA* mutants of other fungi showed results that were similar to *D. septosporum* in exhibiting reduced conidial formation in some or increased sporulation in others (Table 5.4). No effect of *veA* deletion was seen on conidiation in *Histoplasma capsulatum* (Laskowski-Peak et al., 2012). In *P. chrysogenum velA* mutants lost the light dependency of sporulation usually seen in wild-type strains (Hoff et al., 2010). Whether deletion of the *veA* ortholog of the Dothideomycete *M. graminicola* (Choi and Goodwin, 2011) affected sporulation levels was not reported.

Table 5.4: Comparison of conidiation changes caused by the *velvet* gene deletion in filamentous fungi.

Species	Conidiation	References
Aspergillus carbonarius		Crespo-Saempere et al., 2013
Aspergillus fumigatus,		Dhingra et al., 2012; Krappmann et al., 2005
Aspergillus parasiticus		Calvo et al., 2004
Fusarium. fujikuroi	Reduced	Wiemann et al., 2010
Fusarium graminearum		Merhej et al., 2012
Magnaporthe oryzae		Kim et al., 2014
Aspergillus. flavus		Duran et al., 2007; Duran et al., 2009
Aspergillus. nidulans		Kato et al., 2003
Botrytis. cinerea		Schumacher et al., 2013
Cochliobolus. heterostrophus	Increased	Wu et al., 2012
Fusarium oxysporum		López-Berges et al., 2013
Neurospora crassa		Bayram et al., 2008

In *A. nidulans*, the function of VeA is affected by light. The VeA protein predominantly accumulates in the nucleus in dark conditions (Stinnett et al., 2007), where it forms complexes with light-sensing and other regulatory proteins to promote

sexual sporulation and secondary metabolism (Purschwitz et al., 2008; Bayram et al., 2008a). Sporulation in *D. septosporum* was shown to be influenced by light, with approximately twice as many spores in dark compared to light conditions. However, this pattern was also seen in *ΔVeA* mutants, suggesting that DsVeA is not essential for light dependent regulation of sporulation in *D. septosporum*. Likewise, reduced conidiation in VeA mutants of *A. parasiticus* and *A. fumigatus* was not light dependent (Calvo et al., 2004; Krappmann et al., 2005). Cellular localization studies are required to determine if the *D. septosporum* VeA protein is located in the nucleus in the dark despite the apparent lack of a nuclear localization signal.

The *nsdD* and *steA* genes are important for regulation of sexual development in *A. nidulans* (Han et al., 2001; Miller et al., 2000). There are no reports of a sexual cycle for *D. septosporum* in laboratory conditions. In our study, expression of *DsNsdD* and *DsSteA* was not affected by deletion of *DsVeA*, although a significant increase in expression of both *DsNsdD* and *DsSteA* in the DsVeA over-expression strain suggests a possible link between these regulatory pathways. Similarly, in *A. nidulans nsdD* and *steA* transcripts were detected in both *veA* mutant and Δ*veA* deletion strains with very slight difference with respect to the wild type (Kato et al., 2003).

Interestingly, another putative morphological regulatory gene, *DsStuA*, showed reduced expression in the absence of *DsVeA*. In *S. nodorum* and *F. graminearum* StuA was shown to be required for asexual sporulation and affect expression of secondary metabolite-encoding genes, sporulation, hydrophobicity and hyphal morphology (Sigl et al., 2011; Lysøe et al., 2011; Ipcho et al., 2010). Until date the effect of velvet knockout affecting expression of a *stuA* gene has not been reported in any fungi. A regulatory link between *veA* and *stuA* might help to explain similarities between *veA* and *stuA* mutant phenotypes in some fungi.

The role of VeA as a secondary metabolite regulator in fungi is well-established (Table 5.1). Elucidating the role of DsVeA in regulating the biosynthesis of dothistromin and other secondary metabolites in *D. septosporum* was a key objective of this study. Loss of the *velvet* gene led to a severe reduction in expression of dothistromin genes, and of dothistromin production in *D. septosporum*, each down to less than 10% of wild-type levels in culture. The regulatory gene *DsAflR* was strongly down-regulated but expression was not abolished. This is in contrast to *A. parasiticus* and *A. flavus*, in which *aflR* expression and aflatoxin production were completely blocked in *veA* mutants (Kato et al., 2003). The DsVeA over-producing strain C2 had approximately two-fold higher levels of *DsAflR* expression, corresponding to the increased expression of DsVeA in this strain. However, a corresponding increase in dothistromin production was not evident. Although significantly more dothistromin was produced in *DsVeA* mutants in the dark compared to light conditions (2-3-fold; Table 5.2), this trend was not seen in the gene expression study. Almost all the dothistromin gene expression data (genes *AflR, AflJ, Ver1 (DotA)*, *PksA*, for both KO1 and KO2) showed equal levels of light-dark expression with just one exception (*DsPksA* with KO2). In our experience, high levels of variability between replicates in dothistromin assays are commonplace, and the gene expression studies are probably a more reliable indicator of the effect of DsVeA in light and dark. Results obtained in this study are consistent with the earlier finding that *DsAflR* is a dothistromin pathway regulatory gene in *D. septosporum* and that DsVeA regulates the expression of *DsAflR*. However, direct effects of DsVeA on expression of *DsPksA* and *DsVer1* pathway genes cannot be ruled out.

Almost all the dothistromin gene expression data (genes *DsAflR, DsAflJ, DsVer1, DsPksA*, for all strains) showed equal levels of expression in light or dark

conditions, with just one exception: *DsPksA* expression in *DsVeA* mutant KO2 (Figure 5.3). This difference could not be confirmed with dothistromin assays. The KO2 mutant also showed lower sporulation than the KO1 mutant (Figure 5.2 A) and small growth-rate differences. In our experience, phenotypic variability is seen even between wild-type clonal isolates of *D. septosporum* (Schwelm et al., 2008), hence the differences seen between KO1 and KO2 are not surprising. It is also possible that small differences in recombination sites during the gene knockout process could affect other regulatory molecules such as overlapping non-coding transcripts that have been shown to affect chromatin structure in some species (Wei et al., 2011).

Velvet proteins have been shown to regulate a wide range of secondary metabolites in fungi. TLC results obtained in this study indicated a decrease in the production of several metabolites in the *ΔVeA* mutants. Expression analysis of the putative secondary metabolite genes showed significant reduction ($p \leq 0.05$) in five (*Pks2, Hps2, Pks1*, *Nps1 & Hps1*) out of nine genes in the *ΔVeA* strain compared to wild type. This result was consistent with its role as a positive regulator of secondary metabolites as seen in other fungi (Table 5.1).

Several putative secondary metabolite genes showed increased expression in the C2 VeA overexpressing strain compared to wild type suggesting VeA protein may be an important rate-limiting step in positive regulation of these genes. Interestingly one of these genes, *DsPks2*, shows homology to the fumonisin pks (*FUM1*) gene from *Gibberella moniliformis*. Although the metabolite made by *DsPks2* is not known, *F. verticillioides* velvet gene product, *FvVE1*, is necessary for the biosynthesis of fumonisin (Myung et al., 2009). *DsPks1* and *DsNps1* showed the same level of expression in the C2 over-expression strain as in C1 and WT. These results suggest that while DsVeA is not rate-limiting and other regulatory factors probably have an

important contribution in the regulation that control expression of these genes. Based on BLAST analyses, the *D. septosporum DsPks1* gene is most closely related to melanin-type *pks* genes and has 79.6% amino acid identity to the putative *M. graminicola* melanin *pks* gene, PID 96592. In *M. graminicola* melanin levels were reduced by 70% in velvet mutants (Choi and Goodwin, 2011) this is similar to the reduction in expression of the *D. septosporum DsPks1* gene (Figure 5.5).

Expression of *DsHps1* was repressed by over-expression of DsVeA. This phenomenon could be due to the requirement of a stoichiometric balance of regulatory proteins in a heteromeric complex as suggested by Calvo (Calvo et al., 2008) or could be due to a feedback-type mechanism in which over-expression of DsVeA protein inhibits the expression of *DsHps1,* a situation comparable to regulation of penicillin production in *A. nidulans* and gliotoxin in *Aspergillus fumigatus* in which deletion as well as over-expression of *veA* caused a decrease in penicillin and gliotoxin levels (Kato et al., 2003; Spröte and Brakhage, 2007; Dhingra et al., 2012). Of the remaining secondary metabolite genes tested, only *DsPks3* DsPks4*, DsNps3* and Ds*Nps2* showed no significant changes or inconsistent expression (*DsPks4*) under the conditions tested indicating DsVeA does not influence their expression.

Pathogenicity was not compromised in *DsVeA* mutants. Kabir et al., 2014 demonstrated the role of dothistromin as a virulence factor. However, the effect of deletion of *DsVeA* on virulence was not adequately assessed; the experiment was done twice but variability was seen due to difficulties with this pathosystem. Similar results were seen in the *M. graminicola mve1* mutants and *Aspergillus fumigatus* (Choi and Goodwin, 2011; Dhingra et al., 2012). Taken together with results showing severe reductions in expression of some secondary metabolite genes in the same mutants, predictions can be made about whether these genes have an important role in the disease

process. Recent transcriptome analysis of a time course of the dothistroma-pine interaction revealed some of the secondary metabolite genes eg (*DsPks2*, *DsNps3*) were up-regulated compared to the beta tubulin gene during early infection stage (Data unpublished). Due to severe down, regulation of *DsPks2* in *ΔDsVeA, DsPks2* is unlikely to be required for pathogenicity however a role in virulence is possible. Other genes showing significantly reduced expression in *DsVeA* mutants, such as *DsHps1, DsHps2* and *DsPks1* may also be dispensable for pathogenicity, although it is not known for any of these cases whether differences in *pks/nps/hps* gene expression are reflected in changes in metabolite levels or whether such metabolites, if they were involved in pathogenicity at all, would be rate-limiting.

Indisputably, VeA protein is conserved in most ascomycetes and is an important regulator of growth, development and metabolism. The *D. septosporum* genome contains candidate genes for interacting protein components of the velvet complex studied in *A. nidulans* (Bayram et al., 2008 a) and their roles need to be characterised. Furthermore, VeA has been shown to interact with LaeA a SAM dependent methyltransferase thought to be involved in be involved in chromatin remodelling and regulation of secondary metabolite in many fungi. The regulatory role of LaeA in *D. septosporum* will be elucidated in the following chapter.

Chapter 6: *Dothistroma septosporum* LaeA regulates secondary metabolism and development.

6.1 Introduction:

In the previous chapter it was demonstrated that the velvet gene product acts as a global regulator in *D. septosporum*. Another gene that is involved in global regulation is *laeA* (''loss of *aflR* expression'') which was predicted to encode a methyltransferase (Bok and Keller, 2004).

Identification and characterisation of *laeA* was an important milestone to better understand global SM gene regulation in filamentous fungi (Bok and Keller, 2004). A crucial function for LaeA in secondary metabolism, development and virulence has been documented for many fungi (Table 6.1).

Table 6.1: LaeA orthologs and their functions in a range of fungal species (Jain and Keller 2013)

Species	Function[a]	References
Aspergillus carbonarius	SM, Dev	Crespo-Sempere et al., 2013
Aspergillus flavus	SM, Dev, Vir	Amaike and Keller, 2009; Kale et al., 2008
Aspergillus fumigatus	SM, Dev, Vir	Bok et al., 2005; Perrin et al., 2007
Aspergillus nidulans	SM, Dev	Bok & Keller 2004
Aspergillus oryzae	SM	Oda et al., 2011
Cochliobolus heterostrophus	SM, Dev, Vir	Wu et al., 2012
Fusarium fujikuroi	SM, Dev, Vir	Wiemann et al., 2010
Fusarium oxysporum	SM, Dev, Vir	Lopez-Berges et al., 2013
Fusarium verticillioides	SM	Butchko et al., 2012
Monascus pilosus	SM, Dev	Lee et al., 2013
Penicillium citrinum	SM	Baba et al.,2012
Penicillium chrysogenum	SM, Dev	Kosalkova et al., 2009
Trichoderma reesei	SM, Dev	Seiboth et al., 2012

[a] SM = secondary metabolism, Dev = fungal development, Vir = virulence.

A transcriptome analysis of the *A. fumigatus ΔlaeA* strain revealed that 13 of the 22 secondary metabolite gene clusters and 9.5 % of all genes in the genome were regulated by LaeA (Perrin et al., 2007).

LaeA contains a conserved S-adenosylmethionine (SAM) binding domain but has no typical transcription factor signature such as a DNA-binding motif (Bok et al., 2006). GFP tagging experiments showed LaeA to be primarily localised in the nucleus in *A. nidulans* (Bok et al., 2004) and to act in a complex with VeA and VelB (Bayram et al., 2008 a). A SAM motif is the signature of histone methyl transferases and the presence of this motif in LaeA suggested that it regulates transcription in a similar mechanism to histone methyltransferases that methylate lysine or arginine residues (Bok and Keller, 2004). Further, its function in controlling a region encompassing 70 kb of the sterigmatocystin cluster in *A. nidulans* (Bok et al., 2006) led to the hypothesis that LaeA may be involved in a chromatin mediated regulatory function by changing the chromatin architecture of a genomic region (Bok et al. 2006). The exact mechanism of how LaeA brings about the global regulation of secondary metabolite biosynthesis is not known. However, recent biochemical studies done to identify the possible substrates of LaeA failed to identify a methyl accepting substrate of LaeA and hence it was suggested that LaeA is not a protein methyltransferase or it requires specific conditions for methylation activity (Patananan et al., 2013). Interestingly LaeA was shown to have automethylation function at a methionine 207 residue located close to the SAM motif (Patananan et al., 2013).

Shwab et al (2007) demonstrated evidence for global regulation of secondary metabolism in which deletion of histone deacetylase (HdaA-HDAC) in *A. nidulans* resulted in a partial bypass of LaeA requirement for ST production. It was subsequently demonstrated that, during exponential growth phase in *A. nidulans* when

sterigmatocystin is not produced, the biosynthetic genes possess high levels of histone H3 lysine 9 trimethylation (H3K9me3) and heterochromatin protein 1 (HepA) marks. These heterochromatic markers decreased in stationary phase with the activation of the sterigmatocystin gene cluster, but failed to be removed in a *ΔlaeA* strain, indicating its role in epigenetic regulation (Reyes-Dominguez et al., 2010). However, in *Trichoderma reseii* Lae1 was not found to affect H3K4 and H3K9 methylation at the CAZyme gene loci (Seiboth et al., 2012).

LaeA has a dynamic role in both fungal morphological and chemical development. To date the importance of LaeA orthologous proteins in regulation/coordination of secondary metabolism and development has not been examined in a forest foliar pathogen. Hence, the key objectives of the investigation described in this chapter were to:

- Identify the LaeA ortholog in *D. septosporum.*
- Functionally characterise the LaeA ortholog by knockout and complementation and by gene expression analysis over a time course of growth *in vitro*.
- Test whether LaeA proteins are functionally conserved between *D. septosporum* and its close relative *C. fulvum*.
- Study the role of chromatin modification during dothistromin biosynthesis.

  Although experiments for the last objective were set up during my PhD studies the ChIP seq data were not available for analysis in time for inclusion in this thesis. Background, methods and experimental design for this objective are in Appendix 8.7.9.

6.2 Results

6.2.1 Identification of the *Dothistroma septosporum* LaeA orthologue.

To identify a LaeA ortholog in *D. septosporum*, functionally verified LaeA proteins from *A. nidulans* (Bok et al., 2005), and *C. fulvum* (Personal communication Prof Pierre de Wit, Wageningen University) were used as queries in a BLASTP analysis of the genome (http://genome.jgi-psf.org/pages/blast.jsf?db=Dotse1). Thirteen hits were obtained with expectation values ranging from $2.22E^{-46}$ to $7.45E^{-6}$. However, when reciprocal blast analyses were performed against *A. nidulans* genome databases, the top three *D. septosporum LaeA* candidates failed to hit the true LaeA ortholog, but the fourth candidate (PID 148869) with an e value of $3.85E^{-20}$ showed a reciprocal match. Initially phylogenetic analysis was done taking all the top hits of putative SAM methyl transferases from sequenced Dothideomycetes fungi belonging to the order capnodiales. A phylogenetic tree revealed that *D. septosporum* methyltransferase (PID 148869) formed a separate clade along with SAM methyltransferases from other capnoidales fungi and was phylogenetically close to characterised LaeA from other fungal species (Appendix 8.7.1) including Llm1 and LlmF LaeA-like methyltransferase in *C. heterostrophus* (Bi et al., 2013) and *A. nidulans* (Palmer et al., 2013). Phylogenetic analysis of only the top LaeA candidate (PID 148869) from *D. septosporum*, along with known LaeA protein sequences from other species (Figure 6.1) produced a tree whose branching was consistent with taxonomic relationships between the various fungi, suggesting orthology of the identified protein sequences. The *D. septosporum* gene (JGI protein ID 148869) was named *DsLaeA*.

The gene model of *DsLaeA* revealed an open reading frame (ORF) of 1035 bp interrupted by three introns with a predicted protein of 345 amino acid residues. DsLaeA shows less than 40% amino acid identity with LaeA proteins from *A. nidulans*,

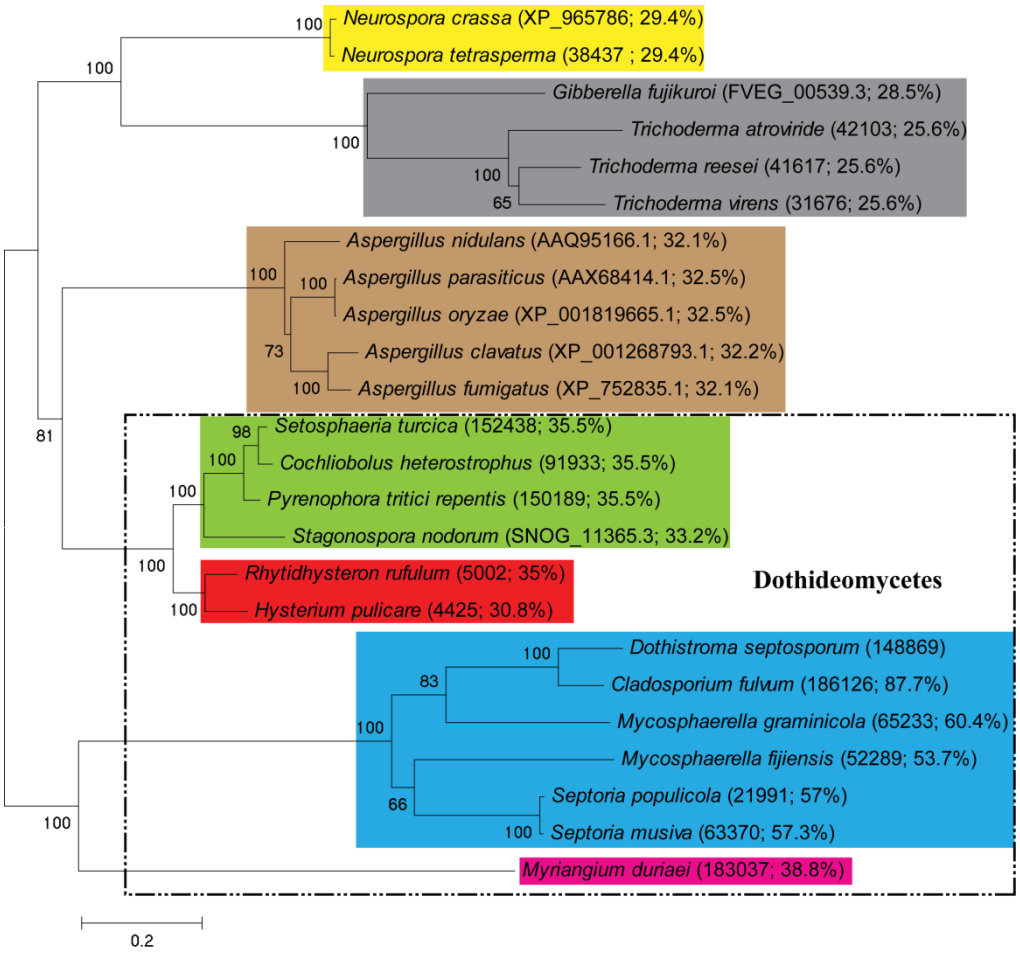


Figure 6.1: Phylogenetic tree of *D. septosporum* LaeA protein and its orthologs in other species. Phylogenetic trees were constructed by neighbor-joining analysis with 1000 bootstrap replicates; the tree was constructed by Neighbor Joining in MEGA 5.0 (Tamura et al., 2011). Numbers on the branches represent the percentage of replicates supporting each branch. Labels on the right indicate the percentage (%) aa identity with *D. septosporum* LaeA (PID 148869) and their corresponding JGI and NCBI gene bank protein ID. The fungi belonging to same order are highlighted and the Dothideomycetes class has been marked with a border. The scale bar represents 20% sequence divergence.

A. parasiticus and *C. heterostrophus,* however it shows 87.7% identity with the functionally characterised LaeA from *C. fulvum* (Appendix 8.7.2). Methionine 207 was shown to automethylate *A. nidulans* LaeA (Patananan et al., 2013) and found to be conserved in many other functionally characterised LaeA proteins; however this feature

is absent in DsLaeA & CfLaeA, as well as in functionally characterised *Trichoderma reesei* LaeA (Patananan et al., 2013).

6.2.2 *D. septosporum* LaeA regulates dothistromin production and gene expression.

To elucidate a possible role of DsLaeA in regulating dothistromin production in *D. septosporum*, *DsLaeA* knockout mutants were generated (methods 2.3.10.2). Analysis of six putative mutants by PCR, using primers that bind outside the 5' and 3' flanking regions of *DsLaeA*, showed products of the expected size for the *DsLaeA* knockout mutants and the wild type control (Figure 6.2 A). To further confirm the *DsLaeA* disruption event all six transformants were analysed by Southern hybridisation using *AvaI* digested genomic DNA using a labelled probe that hybridized to a give 4.1 kb fragment in the replacement mutants but a 2.5 kb fragment in the wild type strain (Figure 6.2 B). Based on these results, two of the six mutants were rejected due to the presence of a wild type band, but four were confirmed as *ΔDsLaeA* strains and named KO1- KO4. Of these, two strains KO1 and KO2 were used for further analysis.

In order to demonstrate that the observed phenotype was due to disruption of *DsLaeA* one of the *ΔDsLaeA* strains KO1 was complemented by co-transformation with a 1:1 ratio of *DsLaeA* coding sequence flanked by 1 kb upstream and downstream sequence (i.e. nucleotides 1802643 to 1806045 of *D. septosporum* scaffold 3), and the vector pBCphleo (pR225) that confers phleomycin resistance. The transformation yielded six *DsLaeA* complemented strains, designated as C1 to C6, which were confirmed by PCR (Figure 6.2 C). To investigate whether LaeA has a conserved function in its close relative *C. fulvum,* a biotroph pathogen of tomato (De Wit et al., 2012), the same *ΔDsLaeA strain* (KO1) was transformed with a functional copy of *CfLaeA* (JGI protein ID 186126). The *CfLaeA* gene, and its native promoter, was ligated

into plasmid pR326 (methods section 2.3.9). Subsequently the *DsLaeA* and *CfLaeA* copy number in these strains was determined by real time PCR (Figure 6.3) (methods section 2.5.5). Of the six *DsLaeA* complemented strains, all were found to carry a single copy of *DsLaeA* and hence strain C1 was selected for further study. The *CfLaeA* transformation yielded three *CfLaeA*-containing strains, confirmed by PCR (Figure 6.2 C; lane 7- 9) that were designated CF1, CF2 & CF3. Copy number estimations by qPCR showed a single copy of *CfLaeA* in CF3, multiple copies in CF2 but variable and unreliable results for CF1 (Figure 6.3).

In *A. parasiticus* and *A. nidulans* nutrient source and light has been shown to affect aflatoxin and sterigmatocystin biosynthesis (Chang et al.,1995; Feng and Leonard, 1998; Calvo et al., 2004). Therefore, the production of dothistromin by *ΔDsLaeA* mutants was assessed in DM (complex) and PMMG (minimal media with pine extract and NH_4^+ as nitrogen source; Appendix 8.1.2). PMMG more closely represents the natural environment in which *D. septosporum* is found (McDougal et al., 2011). The effect of *DsLaeA* deletions on dothistromin production in constant light and constant dark conditions and in these two different media was examined using HPLC (Method section 2.8.5). Dothistromin was produced in both media and under light and dark conditions in either the wild type or *the ΔDsLaeA* strains (Figure 6.4). Quite unexpectedly, both *ΔDsLaeA* (KO1 & KO2) strains produced significantly more (7-10 fold) dothistromin than the WT, in both media and irrespective of light or dark conditions. However, KO2 produced significantly less dothistromin than KO1 in both DM and PMMG media (Figure 6.4 and Appendix 8.7.3). The levels of dothistromin were also significantly higher than the wild type (150%) in the *DsLaeA* complementary strain C1.

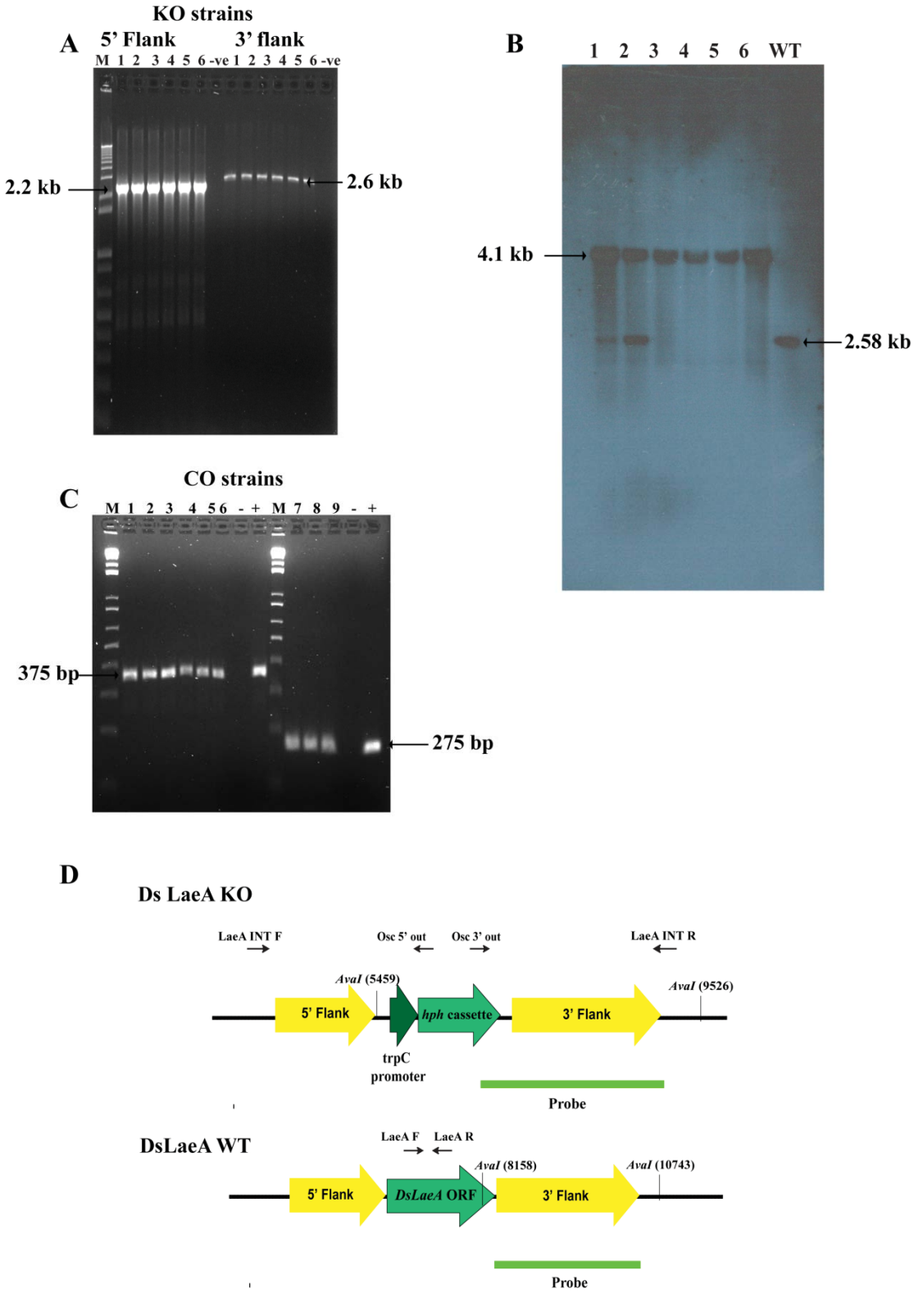

KO strains
A
5' Flank
3' flank
M 1 2 3 4 5 6 -ve 1 2 3 4 5 6 -ve
2.2 kb
2.6 kb
B
1 2 3 4 5 6 WT
4.1 kb
2.58 kb
C
CO strains
M 1 2 3 4 5 6 - + M 7 8 9 - +
375 bp
275 bp
D
Ds LaeA KO
LaeA INT F
Osc 5' out
Osc 3' out
LaeA INT R
AvaI (5459)
AvaI (9526)
5' Flank
hph cassette
3' Flank
trpC promoter
Probe
DsLaeA WT
LaeA F
LaeA R
AvaI (8158)
AvaI (10743)
5' Flank
DsLaeA ORF
3' Flank
Probe

Figure 6.2: Molecular verification of *DsLaeA* gene knockout and complementation.

(A) PCR-based confirmation of *DsLaeA* knockout in six *D. septosporum* transformants (1 lanes 1-6) by amplification of *DsLaeA* 5' flank (left lanes 1-6) using primer combination LaeA INT F & Osc 5' out and 3' flank (right lanes 1-6) using primers LaeA INT R & Osc 3' out, according to the *DsLaeA* KO scheme in (D). All six transformants had PCR products of the expected size (indicated by arrows) for both 5' and 3' flanks, indicating targeted deletion of the *DsLaeA* gene. Lane M is 1 kb plus ladder.

(B) Southern hybridisation of *Ava*I digested genomic DNA of *D. septosporum* wild type (WT), *DsLaeA* knockout strains (lanes 1-6) was performed using a probe complementary to a region of the hygromycin gene as well as to part of the 3' flanking region of *DsLaeA* as shown in (D). The hygromycin cassette replaced a *Ava*I site (8158) present in wild type and resulted in single 4.1 kb fragment in knockout mutant strain while producing a shorter fragment (2.5 kb) in wild type as expected. The transformants in lane 1-2 had an additional hybridizing WT band of ~2.5 kb, indicative of an ectopic integration site of the plasmid; these strains were not used in subsequent analyses. Of the four transformants confirmed by Southern hybridisation (lanes 3-6) were named KO1 – KO4.

(C) PCR-based confirmation *D. septosporum DsLaeA* KO transformant KO1 complemented with *DsLaeA* (C1-C6, lanes 1-6) or *CfLaeA* (CF1-CF3, lanes 7-9) using DsLaeA primers LaeA F and LaeA R (shown in D) or primers CfLaeA F and CfLaeA R (methods Table 2.3) respectively. All transformants had PCR products of the expected size (indicated by arrows) indicating complementation of *DsLaeA* deletion. Lane M is 1 kb plus ladder, lane +ve is control with wildtype *D. septosporum* and *C. fulvum* genomic DNA.

(D) Schematic diagram of *DsLaeA* knockout and wild-type The locations of *AvaI* restriction enzyme recognition sites and annealing sites of primers used to verify gene knockouts and complementation are shown.

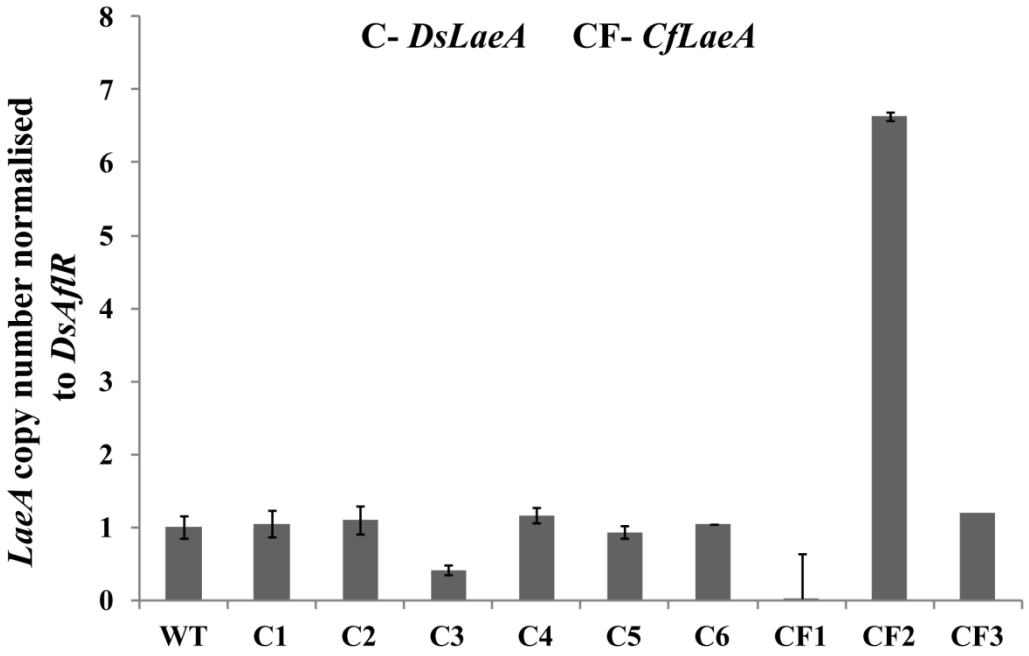


Figure 6.3: Determination of copy number in complementation strains

Quantitative PCR estimation of number of copies of *DsLaeA* (C1-C6) or *CfLaeA* (CF1-CF3) genes in the complementation strains normalised to that of the single copy gene *DsAflR*. Copy number estimates (mean ± SD); n=3 are shown.

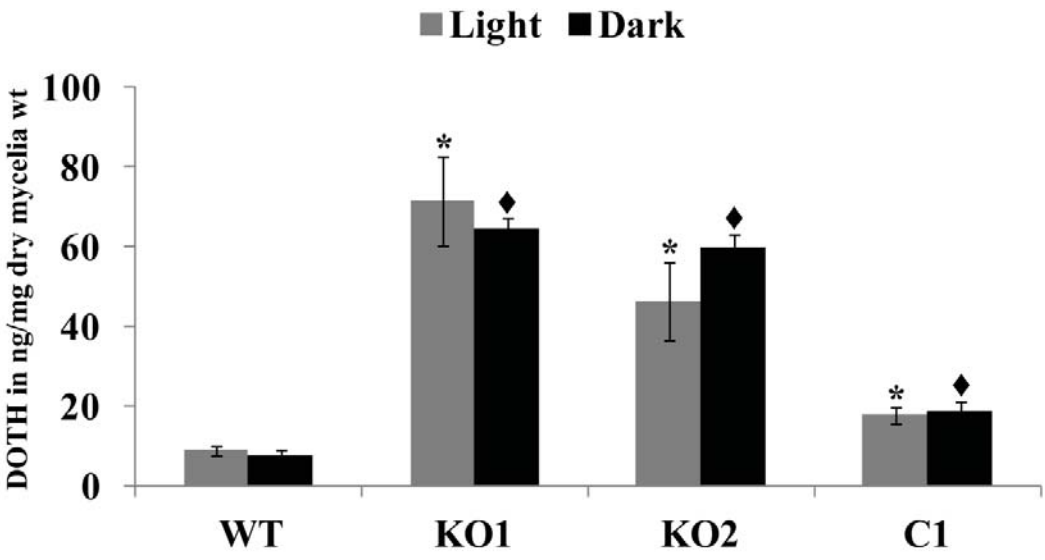


Figure 6.4: Dothistromin production in culture by *DsLaeA* mutants.

Dothistromin production by *DsLaeA* KO strain KO1 & KO2, and *DsLaeA* complemented strain C1, compared to WT strain under light and dark condition. Dothistromin (mean ± SD); n=3 and significant difference to WT ($P<0.05$) in light is indicated by asterisk and in dark by rhombus and there was no significant difference between light and dark levels in any strain.

To further confirm the role of DsLaeA as a regulator of dothistromin production, expression of key dothistromin genes (*DsPksA, DsVer1, DsAflR, DsAflJ* and *DsVbsA*) was examined in WT, *ΔDsLaeA* and *DsLaeA* complemented strains (Figure 6.5). Apart from *DsVbsA,* all dothistromin biosynthesis genes tested were up regulated in the *ΔDsLaeA* mutant in PMMG medium (pine extract minimal medium; showing from 1.6-fold (*DsPksA* & *DsAflR*) to 7.0-fold (*DsAflJ*) increase compared to the wild type (Figure 6.5). This result was in contrast to what was seen in the *laeA* deletion strains of *A. flavus* where expression of the aflatoxin biosynthetic genes (*aflR*, *nor1*, and *aflJ*) was reduced approximately 23-37% compared to that of the wild type strain while that of the late gene *ver1* was less than 0.01% of the wild type (Chang et al., 2012). The higher level of gene expression and dothistromin production seen here may be due to up regulation of the pathway regulator *DsAflR* in the *DsLaeA* mutant, as this is known to enhance dothistromin gene expression (chapter 3).

Dothistromin gene expression was compared between light and dark conditions for *ΔDsLaeA*, complemented and wild type strains. No significant differences were observed between any of the cultures grown in light and dark conditions (Appendix 8.7.4) which was also reflected by similar levels of dothistromin production under light and dark conditions (Figure 6.4 and Appendix 8.7.3). Together these results suggest that functional DsLaeA is a negative regulator of dothistromin production and gene expression, independent of light conditions.

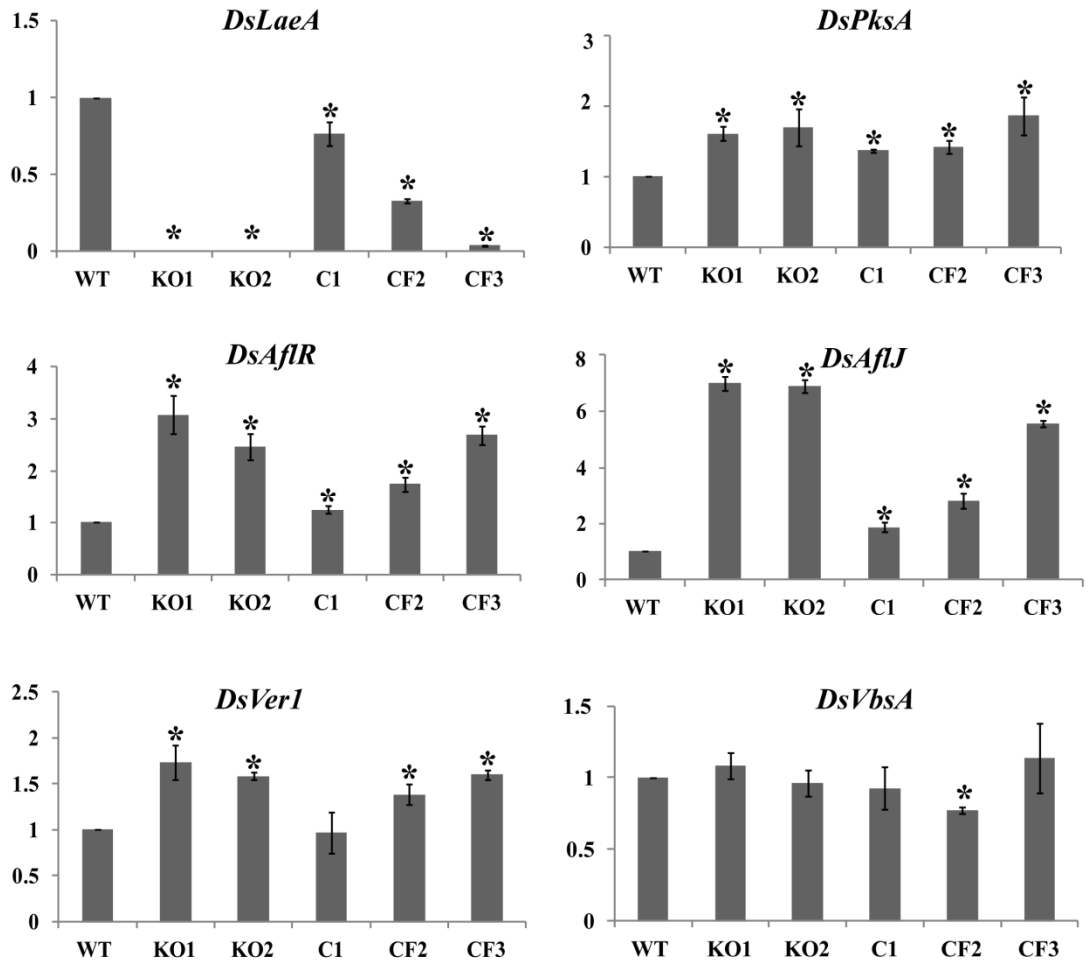


Figure 6.5: Real time PCR analysis of functionally characterised dothistromin genes in *DsLaeA* mutant strains.

Normalized gene expression ratios (mean ± SD; n=3 relative to β Tubulin gene) for *DsLaeA* and dothistromin biosynthetic (*DsPksA, DsVer1*) and regulatory (*DsAflR, DsAflJ, DsVbsA*) genes in *DsLaeA* knockout (KO1 and KO2) and *DsLaeA* complemented (C1) or *CfLaeA* containing (CF2 & CF3) strains grown in light conditions. Significant differences from WT ($p \leq 0.05$) are shown by an asterisk.

To determine if the increased dothistromin production observed in *DsLaeA* knockout mutants could be rescued by *C. fulvum LaeA*, the *CfLaeA* containing strains were grown in PMMG media and toxin quantified as mentioned above. The HPLC result revealed that the single copy *CfLaeA* strain (CF3) did not complement the *DsLaeA* mutation as its dothistromin levels (55±1.9 ng/mg of dry mycelium) were not significantly different from the *DsLaeA* mutant (p=0.177).

However, the multicopy *CfLaeA* strain showed partial complementation as it had a significant reduction in dothistromin level (26 ± 4.3 ng/mg of dry mycelium) compared to the *DsLaeA* mutant (p=0.005) but this was still 3-fold higher than wild type levels.

In *CfLaeA* containing strains the level of expression of dothistromin genes (Figure 6.5) was congruent with the dothistromin levels produced. The promoter of *CfLaeA* was at least partially functional (or fully functional with partially functional protein) in *D. septosporum* as indicated by the expression of *CfLaeA* in the complemented strains CF2 (33% of wild type) and CF3 (1% of wild type) (Figure 6.5). The expression of dothistromin genes *DsPksA DsAflR, DsAflJ* and *DsVer1* showed marked reduction in multi copy complemented strain CF2 compared to the knockout strain but could not revert to wild type level. The levels did not vary significantly in the single copy complemented strain CF3 compared to the knockout strain.

6.2.3 Phenotypic analysis of *DsLaeA* knockout mutant shows defects in sporulation, growth rate, germination and hydrophobicity.

To elucidate the role of DsLaeA in sporulation, *DsLaeA* mutants, WT, and *DsLaeA* complemented strains were grown on PMMG media under constant light or constant dark conditions for 10 days, and the conidia were counted (methods section 2.10.2). The WT and complemented strains produced significantly more conidia in dark compared to light conditions (Figure 6.6 A). However, the *ΔDsLaeA* strains produced significantly fewer spores than the wild type but there was no significant difference in sporulation under light or dark conditions in these mutants.

LaeA is known to regulate sexual development in *A. nidulans* and *A. flavus* (Bayram et al., 2008; Kale et al., 2008). Similarly SteA (Sterile 12 like) and NsdD (never in sexual development) have been shown to regulate sexual sporulation in *A. nidulans* (Vallim et al., 2000, Han et al., 2001). The expression of another putative

regulatory gene, *DsStuA* (PID 45737), was also analysed. StuA (stunted protein) regulates asexual sporulation, mycotoxin and effector gene expression in *S. nodorum* and also *F. graminearum.* The expression of *DsStuA* (stunted protein) was also reduced in the *ΔDsLaeA* strain, concordant with reduced sporulation in this mutant. Expression analysis of *DsNsdD* (PID 68310) *and DsSteA* (PID 72706) by qPCR revealed that DsLaeA is not required for *DsSteA* function, but the expression of *DsNsdD* was reduced significantly compared to wild type in the *ΔDsLaeA* strain (Figure 6.6 B). Another role of LaeA in many fungi is to regulate hydrophobicity (Dagenais et al., 2010; Chang et al., 2012). Hydrophobins reduce the surface tension of the medium on which fungi grow (Fuchs et al., 2004) and are also known to be involved in pathogenicity and virulence determination in many fungi by mediating interactions between the fungus and its host (Bayry et al., 2012, Kim et al., 2005, Dagenais et al., 2010).

The effect of DsLaeA deletion on hydrophobicity in *D. septosporum*, was assessed as described in section 2.10.4. The bromophenol blue solution was maintained as spherical droplets on the colonies of wild type (WT) and complemented strains (C1) while it was gradually absorbed by *ΔDsLaeA* strains, suggesting a marked reduced in hydrophobicity (Figure 6.7 A). To follow up on this observation, the expression of one of the hydrophobin genes *DsHdp1* (PID 75009) was investigated. The *D. septosporum* genome codes for three hydrophobin domain-containing proteins; however, only *DsHdp1* is highly expressed *in planta* (Bradshaw et al, unpublished). qPCR analysis showed that the expression of *DsHdp1* was significantly reduced in the *ΔDsLaeA* strain while it was comparable to wild type level in the complemented strain (Figure 6.7 B).

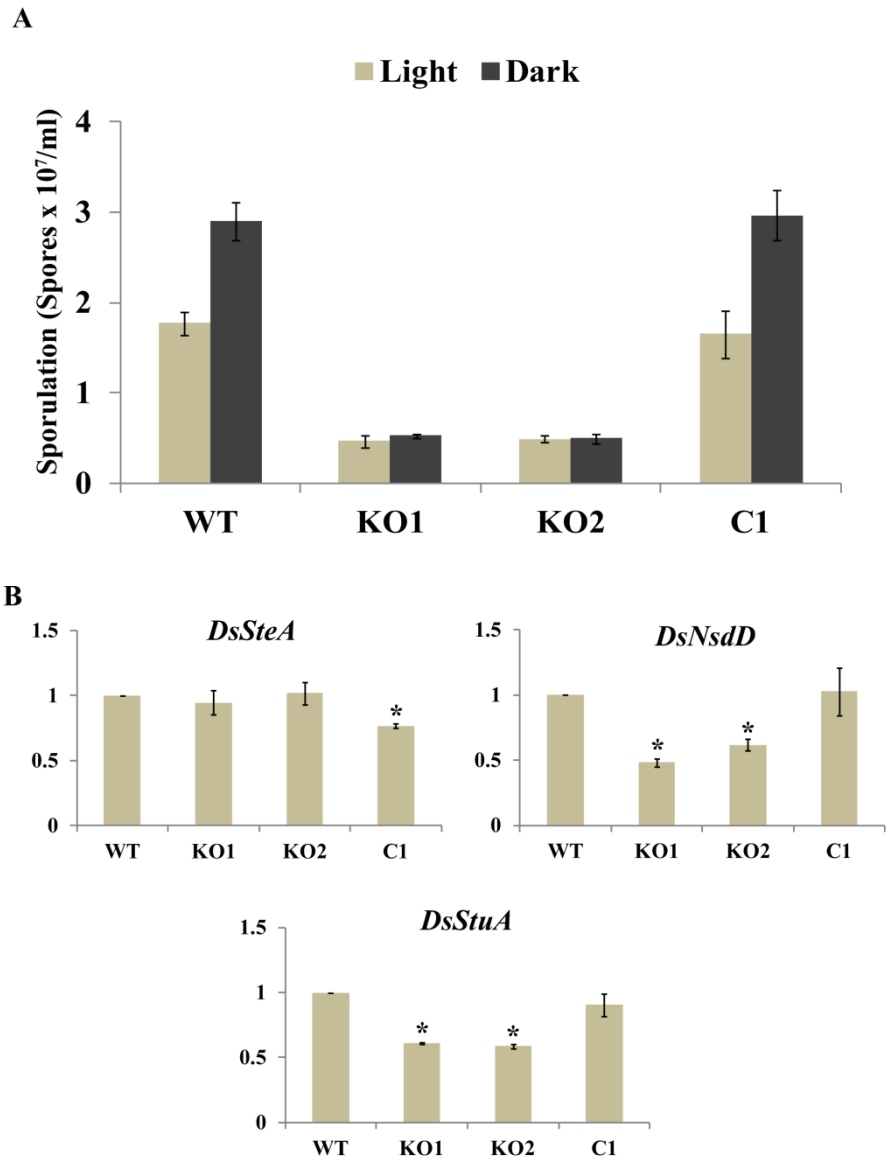
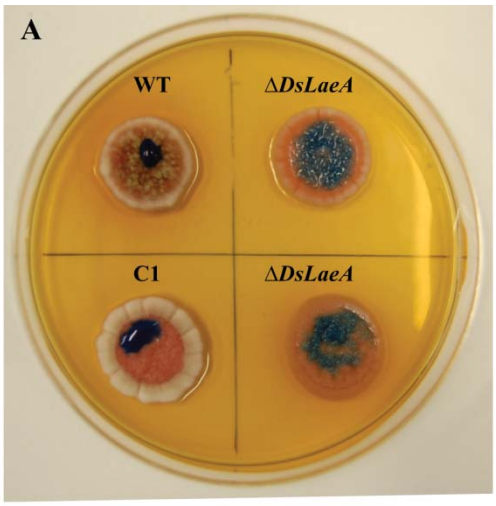


Figure 6.6: Sporulation and expression of developmental regulatory genes in *DsLaeA* mutants.

A) Sporulation of wild type (WT), *DsLaeA* knockout (KO1, KO2) and complemented (C1) strains in *D. septosporum* in light (light bar) and dark (black bar) conditions (mean ± SE). Significant differences ($P < 0.05$) were seen between sporulation of KO and WT strains however there was no significant difference in sporulation in between light and dark conditions in the KO strains.

B) Expression of putative developmental regulatory genes *DsSteA*, *DsNsdD* and *DsStuA* in light conditions. Normalized gene expression ratios for *DsLaeA* knockout (KO1 and KO2) and complemented (C1) strains, relative to wild type (WT); mean ± SD. Significant differences from WT ($P \leq 0.01$) are shown by an asterisk.



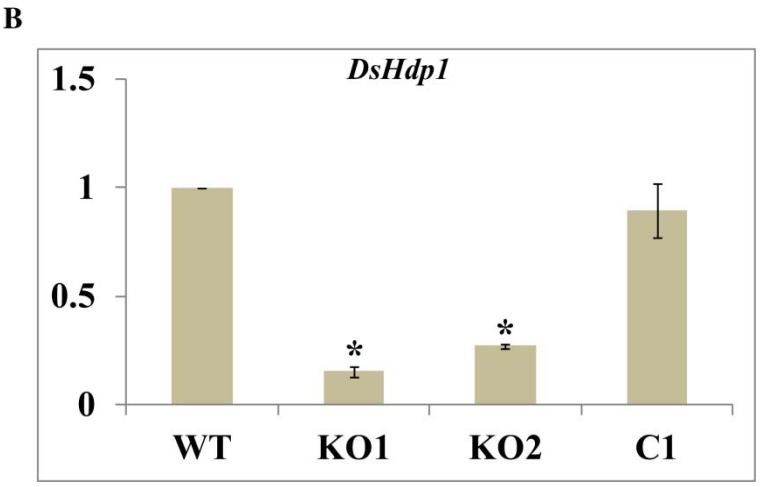


Figure 6.7: Hydrophobicity and hydrophobin gene expresson of the wild type, *ΔDsLaeA* mutants (KO1 & KO2), and the complemented strain (C1).

(A) 30 µl aliquots of bromophenol blue were spotted on colonies, which were photographed after 20 min. Spherical droplets were maintained on the surface of wild type (WT) and complemented strain (C1) but the droplet was readily absorbed by the *ΔDsLaeA* strains. (B) Expression of the *DsHdpI* hydrophobin gene in wild type, *DsLaeA* mutant, and complemented strains as determined by qPCR. Fold changes in mRNA expression relative to the *beta-tubulin* gene (PID 68998) are shown (Methods section 2.5.3). (Mean ± SE); n=3. significant differences from the wild type ($P \leq 0.05$) are marked with asterisks.

Analyses of colony colour and morphology showed some differences in the *ΔDsLaeA* mutants in comparison to wild type and complemented strains on DM agar plates. The *ΔDsLaeA* mutants had darker colonies and, when grown on potato dextrose agar or PMMG showed black exudates on the surface (Figure 6.8 A). The mycelium of *ΔDsLaeA* also looked redder in colour compared to the wild type (not shown), consistent with the elevated levels of dothistromin biosynthesis in the mutants. Growth rates were also estimated under light and dark conditions (methods section 2.10.1). The *ΔDsLaeA* strain grew slower than the wild type strain under light conditions (Figure 6.8 B) but there was no significant difference in growth between *ΔDsLaeA*, WT and complemented strains in the dark. In *A. fumigatus* the growth rate and germination in *ΔlaeA* strain were indistinguishable from wild type (Sugui et al., 2007) while in *A. flavus* an 80% reduction in radial growth rate compared to wild type was reported (Chang et al., 2012).

To compare percentage of germination, conidia were incubated in PMMG medium for up to 48 h (methods 2.10.3; Figure 6.9). The germination of *ΔDsLaeA* conidia was significantly lower than the WT throughout for spores obtained from cultures grown in either light or dark conditions, although over time the *ΔDsLaeA* germination levels became closer to wild type levels. Examination of conidia by light microscopy showed that those of *ΔDsLaeA* were slightly stunted and differed in shape compared to the wild type strain (Appendix 8.7.5). Overall, these results suggested that deletion of *DsLaeA* affects growth in the light and spore development in *D. septosporum.*

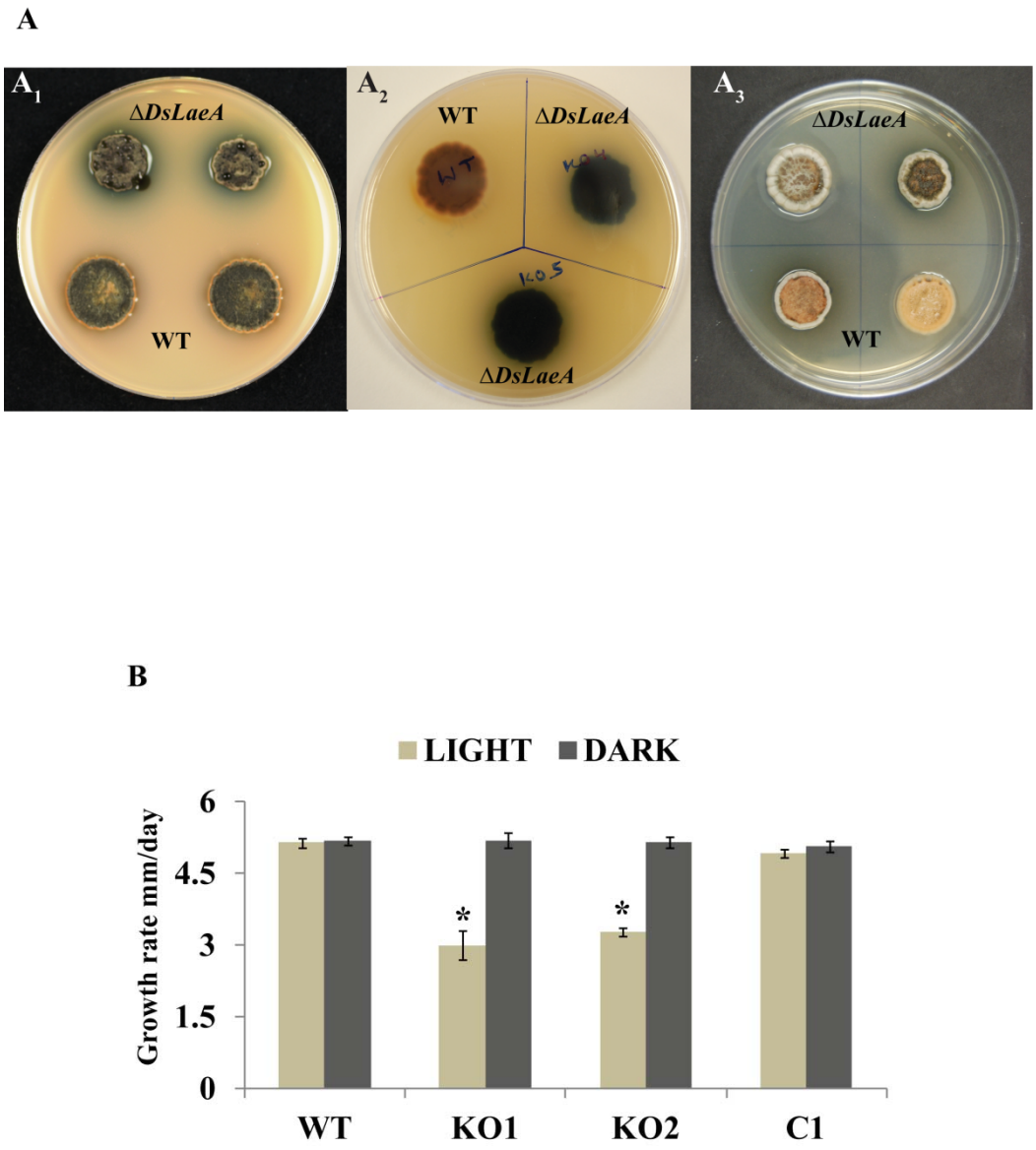


Figure 6.8: Growth characteristics of wild-type (WT), Δ*DsLaeA* mutants (KO1 & KO2) and complemented strain (C1) .

(A) Growth after 7 days of incubation in PDA (A1 & A2 showing rear view) and PMMG (A3). The colony of the mutant shows dark pigmentation and exudates. (B) Average radial colony growth measured for a period of 31 days and average taken. Values are means of three replicates. The error bar indicates standard error. Values significantly different from the WT ($P \leq 0.05$) are marked with an asterisk.

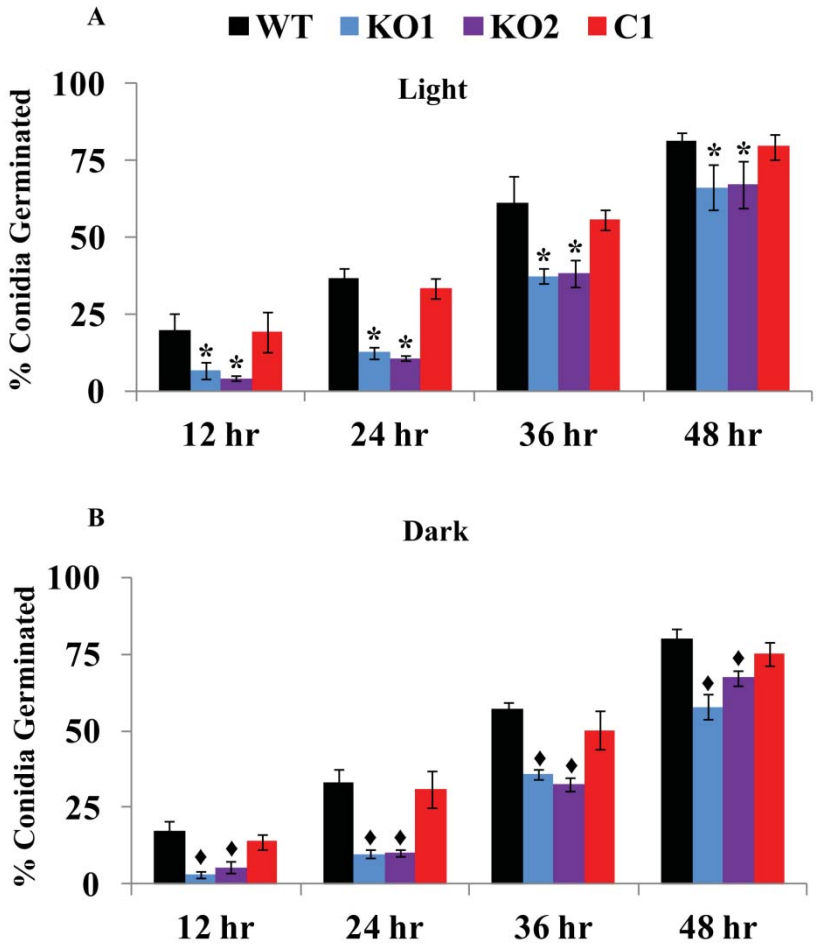


Figure 6.9: Germination % of *DsLaeA* mutants

Δ*DsLaeA* strains (KO1 & KO2) had decreased germination compared to wild type (WT) but wild type levels were restored in the complemented strain (C1). No significant differences were observed in the germination % between spores obtained from cultures grown in light (A) or dark (B) conditions. Three replicates of 100 spores were counted for each strain. Values significantly different from the wildtype (WT) at $P \leq 0.05$ are marked with an asterisk for light and rhombus for dark conditions.

6.2.4. DsLaeA is required for normal expression of other secondary metabolite genes and *DsVeA*

LaeA is known to regulate a diverse range of secondary metabolites in a number of fungi (reviewed by Jain and Keller, 2013). A thin layer chromatograph of an organic solvent extract of a *DsLaeA* KO growth culture showed extra spots compared to wild

type (Figure 6.10 A). As some of these spots were predicted to be due to secondary metabolites, secondary metabolite genes that were highly expressed during the early infection stage of *D. septosporum in planta,* namely *DsPks2, DsHps1 & DsNps3* (Bradshaw et al, unpublished results) were selected for expression analysis in the *DsLaeA* mutant by qPCR The results revealed reduced expression of *DsPks2*, no change in *DsNps3,* but 7-fold increased expression of *DsHps1* in the *DsLaeA* knockout strain compared to the wild type (Figure 6.10 B).

Colonies of *ΔDsLaeA* grown on PDA or PMMG media produced a blackish halo around the colony that was not seen in WT (Figure 6.8 A), indicating that DsLaeA negatively regulates unknown pigmentation in *DsLaeA*. Similar pigmentation was reported in *C. heterostrophus,* a Dothideomycete pathogen in which LaeA negatively regulates melanin synthesis (Wu et al., 2012). The *D. septosporum* genome contains a putative melanin biosynthetic gene *DsPks1* (PID 47338), which has 80% amino acid identity to melanin Pks1 (PID 96592) of *M. graminicola* and 65% identity to Pks18 (PID 30478) of *C. heterostrophus*.

In chapter 5 it was shown that DsVeA is a global regulator of secondary metabolism and sporulation in *D. septosporum.* Comparable results with the *DsLaeA* mutant lead to the question of whether there is a link between these two regulators. Quantative PCR was done to determine the expression of *DsVeA* & *DsLaeA* in wild type (WT) and *ΔDsLaeA* strains at three time points when grown in culture. The results revealed significantly lower expression of *DsVeA* in the *ΔDsLaeA* strain compared to wild type at days 7 and 9 ($P = 1.04E^{-04}$; $5.85E^{-05}$ respectively) but no significant difference at day 4 ($P = 0.27$) (Figure 6.11). Interestingly, expression of *DsLaeA* itself increased from day 4 to day 7, and then appeared to plateau (Figure 6.11).

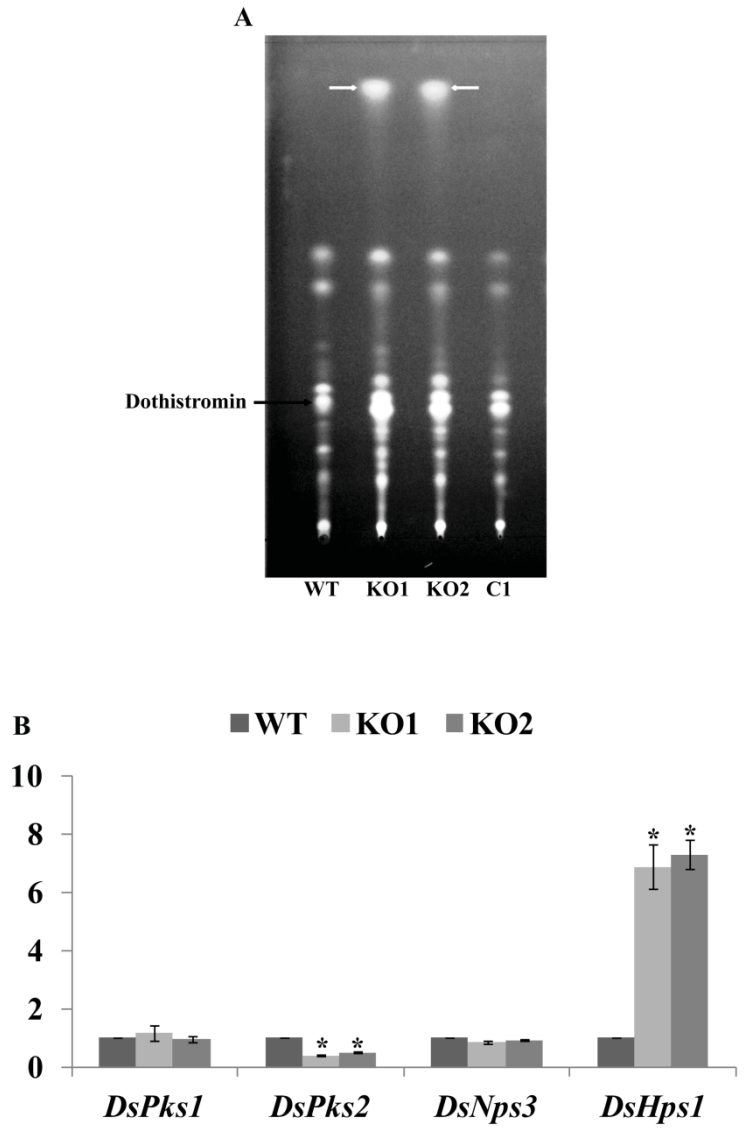


Figure 6.10: Secondary metabolite production and gene expression in *DsLaeA* mutants

A) TLC of extracts from *DsLaeA* knockout (KO1 and KO2), *DsLaeA* complemented (C1) or wild-type (WT) strains. The extra spot in knockout strain KO1 and KO2 has been marked with a white arrow.

B) Gene expression ratios for polyketide synthase *DsPks1*, (similar to melanin Pks1 coding gene in *M. graminicola*) *DsPks2* (similar to fumonisin Pks coding gene in *Fusarium* spp) non-ribosomal peptide synthase *DsNps3* (unknown) and PKS-NRPS hybrid *DsHps1* (similarity to cyclopiazonic acid (CPA) like PKS-NRPS genes in *A. flavus* genes in *DsLaeA* knockout (KO1 and KO2) and complemented (C1) strains, normalised to wild type (WT) levels (WT = 1); mean ± SE. Significant differences from WT ($p \leq 0.05$) are shown by an asterisk.

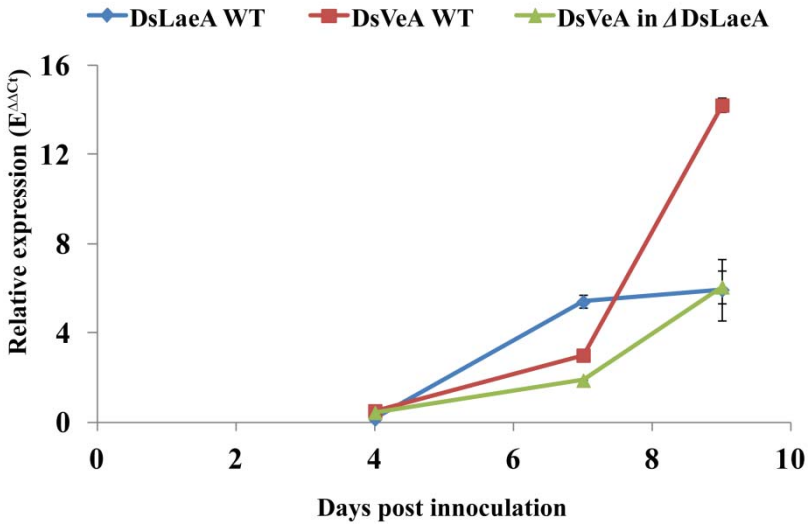


Figure 6.11: Gene expression levels of *DsVeA* and *DsLaeA* relative to *DsTub1* in *D. septosporum* wild type and *ΔDsLaeA* strains.

Each strain was grown in liquid PMMG culture with shaking (200 rpm at 22°C). Total RNA was extracted at Day 4, Day 7 and Day 9 after inoculation. Values are means of three biological replicates and duplicate technical PCR replicates, and bars represent standard deviation.

Together these results suggest that *DsLaeA* is important in the regulation of biosynthesis of other secondary metabolites in *D. septosporum* and a positive regulator of velvet, which is consistent with its role as a global regulator of secondary metabolism.

6.2.5 *ΔDsLaeA* strain showed early expression of dothistromin genes and dothistromin production similar to wild type strain.

In culture, dothistromin is synthesized during early exponential growth phase in a wild type *D. septosporum* strain (Schwelm et al., 2008). To determine if deletion of *DsLaeA* affects this unique early expression pattern, biomass accumulation, toxin production and gene expression. were analysed out over a time course (Method 2.5.3; 2.7.1; 2.8.1).

As expected from previous results, overproduction of dothistromin was seen in the *ΔDsLaeA* strain, but dothistromin production commenced almost at the same time as wild type strain and there was no significant difference in the level until day 5. However, from day 5 (until day 9) the rate of production of dothistromin (ng DOTH/mg of dry weight mycelium) continued to increase to a significantly higher level ($P= 9.4E^{-9}$) than seen in the WT (Figure 6.12).

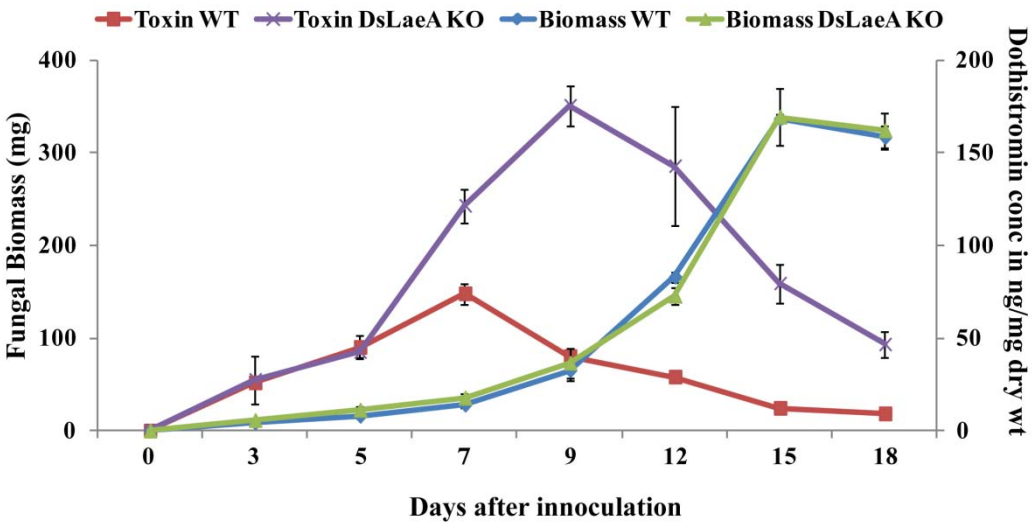


Figure 6.12: Growth and dothistromin production of *Dothistroma septosporum* wild type (WT) and *ΔDsLaeA* strain in liquid media.

Fungal biomass is dry weight of mycelium (mg/25 ml of media) and the rate of dothistromin production estimated as ng dothistromin per mg of dry weight of mycelium for days after inoculation. Values shown are means and standard deviations of three biological replicates.

Eventually the rate of dothistromin production decreased: this occured after day 7 in WT, and day 9 in *ΔDsLaeA*, but both were in early-mid exponential growth stage at this time. However the highest amount of dothistromin accumulated in the media (ng doth/ml media) was seen at day 12 in WT and day 15 in the *ΔDsLaeA* strain (Appendix 8.7.6); this growth stage corresponds to mid to late exponential growth phase. The concentration of dothistromin decreased after this point in both strains.

The expression pattern of selected dothistromin biosynthesis and regulatory genes in the same experiment indicated that genes are switched on during the early stage of growth in culture and gradually peak by day 7 in both wild type and *ΔDsLaeA* (Figure 6.13); These results were concordant with the amount of dothistromin produced (Figure 6.12). The expression of all the dothistromin pathway genes tested showed significant up regulation by 2 to 9 fold in the *ΔDsLaeA* strain over the time course compared to the wild type. Schwelm et al., 2008 earlier demonstrated that dothistromin genes are co-regulated so it was of interest to see whether the deletion of *DsLaeA* affected this co-regulation. To determine this, pairwise comparisons of the relative expression of each dothistromin pathway gene over the time course was done by Pearson correlation. In the *ΔDsLaeA* strain each of the genes showed high correlation (r) in expression levels, varying between 0.7833 to 0.98 (Appendix 8.7.7).

Overall, the results indicated that mutation of *DsLaeA* enhances the production of dothistromin. However, it does not affect the early expression of dothistromin genes and toxin production nor the co-regulation as seen in the wild type strain. Therefore, further investigation needs to be done on the molecular mechanism underlying the early onset of dothistromin production.

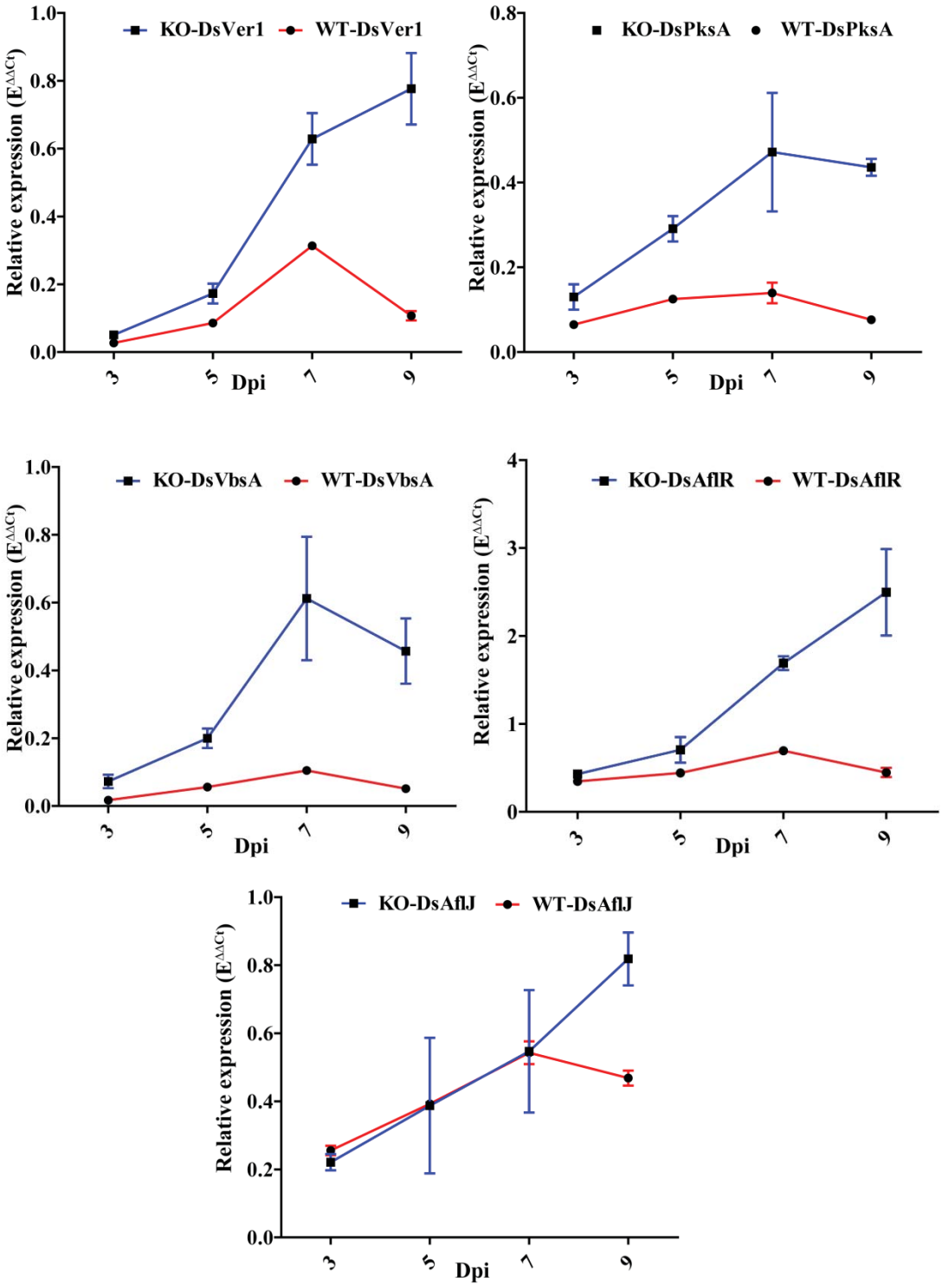


Figure 6.13: Relative expression of dothistromin genes over time in wild type and Δ*DsLaeA* strain

Expression of dothistromin genes *DsVer1 DsPksA, DsVbsA DsAflR* and *DsAflJ* in *D. septosporum* NZE10 (WT – red line) and *DsLaeA* knockout strain (KO - blue line) in low DB medium. Shown are days post inoculation (X axis) and relative gene expression level relative to β*Tubulin* (*DsTub1*) (Y axis). The Δ*DsLaeA* strain showed high expression at an early growth stage similar to WT but an exponential increase after day 5. Each of the points represent (mean ± SD) of triplicate samples.

6.3 Discussion

LaeA is a member of the velvet complex identified in *Aspergillus* spp. It has been characterised in many fungi and regulates secondary metabolism and morphogenetic development (Table: 6.1). In the present study, a predicted *LaeA* gene was identified in *Dothistroma septosporum* using blast and phylogenetic approaches. The *ΔDsLaeA* strain produced significantly more dothistromin compared to wild type in two different media and under light or dark conditions. Similarly, gene expression analysis showed the up regulation of *DsPksA DsVer1, DsAflJ* and *DsAflR* in the mutant. These results completely contrast with what was seen in AF/ST biosynthesis in *Aspergillus* spp. where both toxin production and *AflR* expression was completely abolished (Bok et al., 2004, Chang et al., 2012, Bok et al., 2005). These data suggest that, in *D. septosporum*, DsLaeA negatively regulates dothistromin production. This could be an indirect mechanism in which DsLaeA normally interacts with other toxin biosynthesis regulatory mechanism(s) such as a repressor that is de-repressed by deletion of *DsLaeA*.

In the *DsLaeA* complemented strain (C1) both the expression of *LaeA* and levels of dothistromin indicated incomplete complementation as wild type levels were not restored. A similar phenomenon was reported for velvet complementation in *Trichoderma virens* (Mukherjee et al., 2010). One possible explanation for lower expression in C1 could be due to integration of the complement construct at a non homologous locus causing lower expression due to position effect, as demonstrated in *Aspergillus* spp. (Liang et al., 1997; Chiou et al., 2002). In *D. septosporum* no multiple copy-transformed strains were obtained, leading to the suggestion that LaeA overexpression may be lethal due to indirect effects on other classes of genes which are essential for survival. However, further work needs to be done to test dothistromin production in other *DsLaeA* complemented strains.

LaeA is best known as a positive regulator of secondary metabolism in fungi (Jain and Keller, 2013). The results for dothistromin shown in this chapter contradict this trend but are not unique. In *Fusarium verticillioides* deletion of *laeA* caused increased expression of 3110 out of 14,196 genes (~22 %), of which two were PKS genes; one of these showed co-regulation with flanking genes that indicated a previously uncharacterised SM biosynthetic gene cluster (Butchko et al., 2012).

In this study, it was demonstrated that DsLaeA regulates secondary metabolites other than dothistromin (Figure 6.10 A). In the *ΔDsLaeA* strain, one showed enhanced expression (*DsHps1*) and one reduced (*DsPks2*) compared to wild type. Together these results suggest that DsLaeA acts as a positive as well as a negative regulator of secondary metabolism in *D. septosporum*. It is possible that, in *D. septosporum,* other LaeA-like proteins such as those included in the phylogenetic tree (Appendix 8.7.1) could also regulate secondary metabolism, in the absence of *DsLaeA*. Recently a LaeA-like methyl transferase was identified in *A. nidulans* (Palmer et al., 2013) and *C. heterostrophus* (Bi et al., 2013). The *ΔlaeA-like* mutants showed opposite phenotypes to those exhibited by the *ΔlaeA* strains in both fungi.

LaeA is known to regulate asexual and sexual development in fungi (Bayram et al., 2008). Sporulation in *Dothistroma septosporum* is influenced by light, with more spores produced in dark compared to light conditions (Chettri et al., 2012). In this study, it was shown that sporulation was impaired in *ΔDsLaeA* mutants (Figure 6.6 A) consistent with the role of LaeA in other ascomycetes. Here the most striking result was that sporulation in the *DsLaeA* mutant was independent of light conditions, suggesting DsLaeA is essential for light dependent repression (or dark induction) of sporulation in *D. septosporum.* Similarly, in *Penicillium chrysogenum* deletion of *laeA* also caused light independent reduction of sporulation compared to wild type (Hoff et al., 2010). In

a *ΔlaeA* strain of *Fusarium graminearum* however, although conidiation was less than in wild type it was still light dependent; in this species conidiation is induced rather than repressed by light (Kim et al., 2013).

The *ΔDsLaeA* strain exhibited slightly abnormal conidial morphology (Appendix 8.7.5) similar to that reported in the *A. fumigatus laeA* mutant (Sugui et al., 2007) suggesting a role for DsLaeA in morphogenetic pathway signalling. Expression of two *D. septosporum* putative development related genes *DsNsdD* and *DsStuA* required DsLaeA as their expression was reduced in the *ΔDsLaeA* mutant. A similar result was obtained in the *DsVeA* knock out strain (Chapter 5) which could be explained by its role as a global regulator. The reduced sporulation compared to wild type as seen in the *ΔDsLaeA* strain could be due to an indirect effect of reduced DsStuA. The *D. septosporum* genome does not contain a homolog of brlA (Chettri et al., 2012) which is a key regulator of asexual development in *A. nidulans* (Han and Adams, 2001)

Another abnormality observed in the *ΔDsLaeA* strain was decreased surface hydrophobicity compared to wild type. Fungal cell walls contain various hydrophobic molecules like lipids (Latge, 1999) and adhesins (Tronchin et al., 1997). Reduced hydrophobicity was seen in *laeA* mutants of *A. flavus*, *A. fumigatus*, *P. chrysogenum*, *F. graminearum* and *F. verticillioides* (Chang et al., 2012; Bok et al., 2005; Kosalkova et al., 2009; Jiang, et al., 2011; Kim et al., 2013). To elucidate the regulatory role of DsLaeA on hydrophobicity the effect of *DsLaeA* deletion on *DsHdp1* gene expression was analysed by qPCR (Figure 6.7 B). The result showed that DsLaeA is required for normal expression of the *DsHdp1* gene, which could contribute to the lack of hydrophobicity as seen in the mutant colonies.

In this chapter and chapter 5, it was demonstrated that both DsLaeA and DsVeA play roles in development and secondary metabolism. Further, in this study it was

shown that *DsLaeA* is required for *DsVeA* transcript accumulation. In *A. nidulans* LaeA forms complexes with other proteins as outlined previously (Bayram et al., 2008; Bayram and Braus, 2012). Although analysis of the *D. septosporum* genome has revealed the existence of putative *DsVelB* (PID 139168) and *DsVosA* (PID 71253) genes (with predicted 51 & 31% aa identity with *A. nidulans* VelB and VosA respectively), whether these velvet family candidates interact with each other, and what their roles are, needs to be characterised.

The role of LaeA as a pathogenicity and virulence-determining factor has been demonstrated in a number of fungi. In *A. flavus* deletion of *laeA* reduced host colonisation in peanut (Amaike and Keller, 2009) and *F. oxysporum* and *F. fujikuroi laeA* mutants showed reduced virulence in tomato and rice (Lopez-Berges et al., 2013; Wiemann et al., 2010). In a dothideomycetes maize pathogen *C. heterostrophus*, ChLae1 regulated production of T-toxin, a host-selective pathogenicity factor (Wu et al., 2012). In *D. septosporum* dothistromin is a virulence factor (Kabir et al., 2014). On the basis of results obtained in this study the enhanced dothistromin level, presence of an unknown metabolite band on TLC plates, and higher expression of other secondary metabolite genes in the *ΔDsLaeA* strain compared to wild type, leads to the hypothesis that deletion of *DsLaeA* may enhance virulence in *D. septosporum* by increasing levels of dothistromin and possibly other unknown metabolites. Studies are needed to test this hypothesis. An *in vitro* time course experiment done to study expression and dothistromin production in *ΔDsLaeA* and WT strains revealed early dothistromin production and gene expression in both strains but in *ΔDsLaeA* the rate of dothistromin biosynthesis remained higher for longer than the wild type (Figure 6.12). The time points at which maximum differences between *ΔDsLaeA* and the wild type were seen corresponded to periods when *DsLaeA* was known to be expressed (Figure 6.11),

although *DsLaeA* expression needs to be verified over the complete timecourse. It is possible that *DsLaeA* functions directly by binding to the promoter of *DsAflR* to modulate *DsAflR* expression as reported by (Ehrlich et al., 2011). Alternatively, *DsLaeA* might function indirectly by recruiting an unknown dothistromin repressor. In the *ΔDsLaeA* mutant no repressor would be recruited so toxin production would increase substantially beyond the time point seen in wild type strain. Further work is needed to determine if there is a repressor protein involved and to study the interaction between AflR and LaeA .

The results obtained in this study cast some doubt on the identity of DsLaeA (PID 148869) as a true ortholog of the functionally characterized LaeA proteins from other fungi such as *A. nidulans*, *A. fumigatus*, *A. flavus*, *P. chrysogenum*, *F. fujikuroi* and *C. heterostrophus* (Bok and Keller, 2004; Bok et al., 2005; Amaike and Keller, 2009; Hoff et al., 2010; Wiemann et al., 2010; Wu et al., 2012). The DsLaeA sequence has less than 40% amino acid identity with the above characterised LaeA (Figure 6.1), although it does form a separate clade with LaeA candidates from other capnodiales including CfLaeA (Figure 6.1). Other than reduction in spore production and hydrophobicity which is conserved in many of the aforementioned fungi the marked increase in dothistromin production and increased expression of *DsAflR* gene differ from LaeA mutant phenotypes in *Aspergillus* spp. As mentioned previously the pattern of dothistromin expression and the dothistromin gene arrangement are unique so the aforementioned differences could be due to some different regulatory mechanism of LaeA in dothistromin biosynthesis in *D. septosporum* than in other fungi.

Chapter 7: Summary, Conclusions and Future Directions

The physically separated dothistromin genes and early onset of dothistromin production led to the question how dothistromin biosynthesis is regulated in the pine needle pathogen *D. septosporum.* The aim of these studies was to answer this question by identifying and characterising orthologs of AF/ST pathway specific regulators (AflR and AflJ) and global regulators (VeA and LaeA). DsAflR was shown to be a regulator for the dothistromin pathway. The protein was predicted to contain a conserved Cys_6Zn_2 DNA-binding motif and an AflJ interaction sequence, but in contrast to AF/ST AflR proteins, did not have a recognisable nuclear localisation signal or PEST (protein turnover) motif. Functional characterisation by gene knockout clearly indicated that DsAflR controls the transcriptional activation of most predicted dothistromin genes, although dothistromin production was not completely abolished in the DsAflR mutant. A key conclusion from this study was that secondary metabolite genes do not need to be clustered or sub telomeric to be co-regulated, as is often the case (Palmer and Keller, 2010; Perrin et al., 2007).

Most DsAflR-regulated genes had a predicted AflR-binding sequence (TCG(N_5)CGR) but three did not (*DsEst1, DsDotB* and *DsDotC*) suggesting DsAflR may bind to other sequences and/or another level of regulation operates along with AflR. Further work is needed to experimentally verify the DsAflR binding sites.

The biotrophic tomato pathogen *Cladosporium fulvum* contains the entire set of dothistromin genes but does not produce dothistromin. An attempt to complement the *DsAflR* knockout mutant with the *C. fulvum AflR* was partially successful in restoring dothistromin levels.

The results obtained in this study helped in prediction of genes involved in late stages of the biosynthetic pathway for dothistromin (after versicolorin A) as proposed by Henry and Townsend (2005). Functional analysis of *DsOrdB* and *DsDotB* showed they are required for dothistromin biosynthesis but a third gene, *DsNorB* (the only candidate located on a different chromosome from the dothistromin genes), was not. Further work is required to determine the intermediate compounds involved.

Another important finding was differences in the expression and function of AflJ in *D. septosporum* compared to *Aspergillus* spp. A *D. septosporum ΔDsAflR* mutant showed an eleven-fold increase in *DsAflJ* expression whilst an *A. parasiticus ΔaflR* mutant had decreased levels (Price et al., 2005). In functional studies, *ΔDsAflJ* mutants still produced some dothistromin although dothistromin gene expression was down regulated except for *DsAflR*; this was in contrast to *A. parasiticus /A. flavus* mutants where most genes were expressed at wild type levels but aflatoxin production was abolished (Chang, 2003; Meyers et al., 1998). The fragmented gene cluster and probably another regulatory mechanism may explain these differences in the two fungi.

DsAflJ had other distinct features such as a vacuolar targeting signal near the C terminal and a PEST motif. Cross genera complementation of *ΔDsAflJ* using *AflJ* genes from *A. parasiticus*, *A. nidulans* and *C. fulvum* could not revert dothistromin production to wild type levels, suggesting species-specific function of AflJ. Based on the results obtained with the pathway regulators DsAflR and DsAflJ a model is proposed for their roles in regulation of dothistromin biosythesis (Model 1; Figure 7.1)

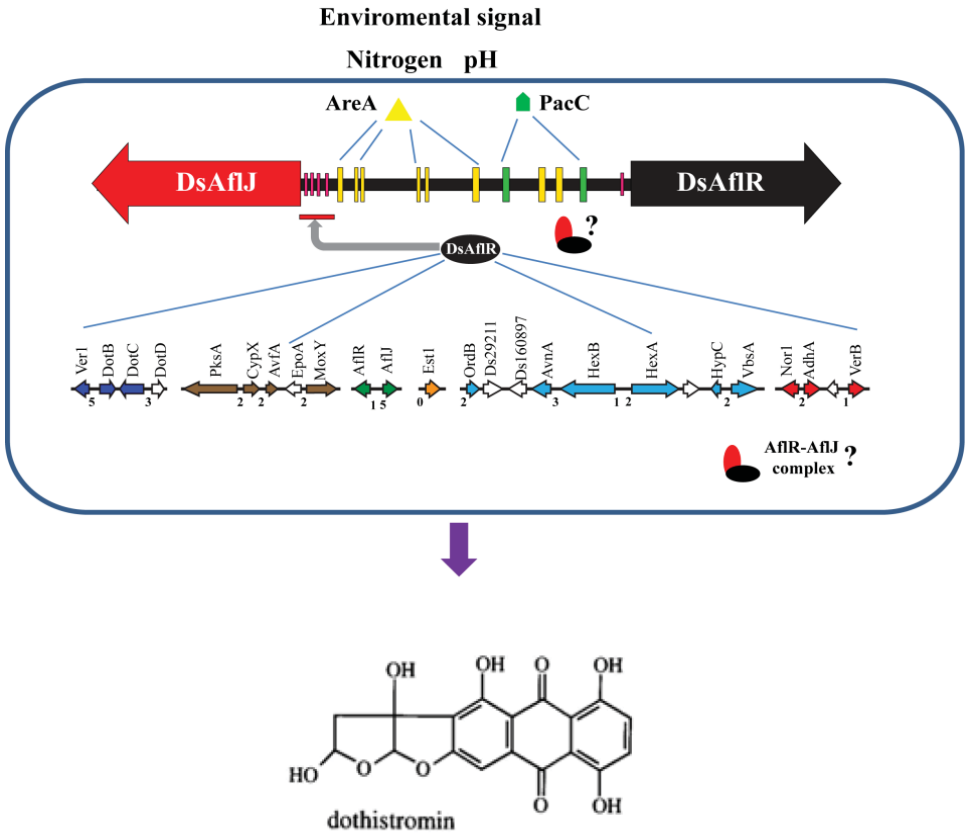


Figure 7.1: Proposed model of dothistromin regulation by DsAflR or DsAflR-DsAflJ

The top section shows divergently transcribed DsAflR and DsAflJ genes with the intergenic region enlarged. Putative binding sites for regulators AreA (yellow bars), PacC (green bars) and AflR (pink bars) are shown on the intergenic region. The lower section shows dothistromin biosynthesis genes. The number below each gene indicates the number of putative AflR binding sites. The model suggests that dothistromin biosynthesis is regulated by DsAflR or a complex of AflR and AflJ. AflR is a negative regulator of DsAflJ and it is proposed that stoichiometric balance between AflR and AflJ is needed for wild type levels of dothistromin production.

A coactivator model (Figure 7.1) is proposed to explain dothistromin pathway regulation by a DsAflR-DsAflJ complex. The model suggests that DsAflJ acts as a coactivator of DsAflR as both proteins are required for normal levels of dothistromin biosynthesis. The model further suggests that the ratio between AflR/AflJ is important for production of dothistromin at wild type levels. The deletion of either *DsAflR* or

DsAflJ would lead to disruption of cellular AflR-AflJ protein balance leading to significantly less toxin production than wild type. When *DsAflJ* is over expressed the balance is disturbed and there is no corresponding increase in dothistromin production above wild type levels (Figure 4.5). However, one caveat for this model is that when *DsAflR* is overexpressed (C2 in Figure 3.4) there is elevated dothistromin production. In this scenario it is possible that very high levels of DsAflR by-pass the need for the DsAflJ coactivator. In this model DsAflR has a dual function: it interacts with DsAflJ for activation of dothistromin production and is also a negative regulator of *DsAflJ* expression, as evidenced by the high level of *DsAflJ* seen in the *ΔDsAflR* mutant. The presence of the PEST motif in DsAflJ is an indicator of the short half life of DsAflJ, further suggesting the importance of cellular balance with AflR. In future mutational analysis of the promoter region of *DsAflJ*, along with ChIP using a DsAflJ specific antibody would help to determine if DsAflJ colocalises with DsAflR to dothistromin gene promoters.

The role of AflJ in anthraquinone synthesis in other ascomycetes is still a mystery and very limited work has been done in *Aspergillus* spp. The presence of AflJ in *D. septosporum* may help to better understand this regulatory protein. The regulation of dothistromin by AflJ presented in this study identifies several knowledge gaps and generates questions that still need to be answered. A recent study in *A. parasiticus* demonstrated the requirement of AflJ for transport and activation of AflR (Ehrlich et al., 2012). A similar role is possible in *D. septosporum* becasue of the predicted membrane-spanning domain and vacuolar location signal in DsAflJ. Rigorous work in cellular localisation studies needs to be done to study DsAflR and DsAflJ functions in dothistromin biosynthesis.

The hypothesis that orthologs of *Aspergillus* spp. global regulators VeA and LaeA are present in *D. septosporum* and regulate development and secondary metabolism was supported in this study. The knockout study of *DsVeA* showed that *D. septosporum* VeA did not appear to regulate growth rate, hyphal morphology or hydrophobicity but indicated it is a positive regulator of sporulation and dothistromin production independent of light. The finding suggested that another regulatory protein might be involved in regulating light repression of sporulation as seen in the wild type strain. This study also demonstrated the dependency of *DsStuA* on VeA, a novel finding connecting velvet with another developmental regulatory network. Expression analysis of nine putative secondary metabolite genes in the *ΔDsVeA* mutant revealed five of them to be positively regulated, suggesting a conserved role in regulation of secondary metabolism as previously reported (Duran et al., 2007, Bayram and Braus, 2012).

The study of *D. septosporum* LaeA revealed contrasting results. DsLaeA regulated conidial morphology, hydrophobicity, pigmentation and expression of *DsVeA*, as well as expression of several secondary metabolism genes, revealing its function as a global regulator. It was also a positive regulator of sporulation and appeared to be required for light repression of sporulation. The most interesting outcome of these studies was the overproduction of dothistromin and increased expressed of dothistromin genes (including *DsAflR*) by *ΔDsLaeA* knockout mutants. This was in stark contrast to the situation in AF/ST biosynthesis for which *laeA* mutants are named due to "Loss of AflR Expression" (Bok and Keller, 2004). A time course experiment revealed that dothistromin production is extended at a higher rate for longer in a *ΔDsLaeA* mutant compared to the wild type and this was consistent with gene expression, further suggesting a role for DsLaeA in repression of dothistromin biosynthesis.

Based on these results a model is proposed to explain the role of the global regulators LaeA and VeA in regulation of dothistromin and other secondary metabolites in *D. septosporum* (Figure 7.2, Model 2). Dothistromin production is negatively regulated by DsLaeA as evidenced from *ΔDsLaeA* mutants. We hypothesize that LaeA may recruit a repressor protein (Rep) that functions by binding in the AflR-AflJ intergenic region, and/or may recruit a chromatin modifying enzyme

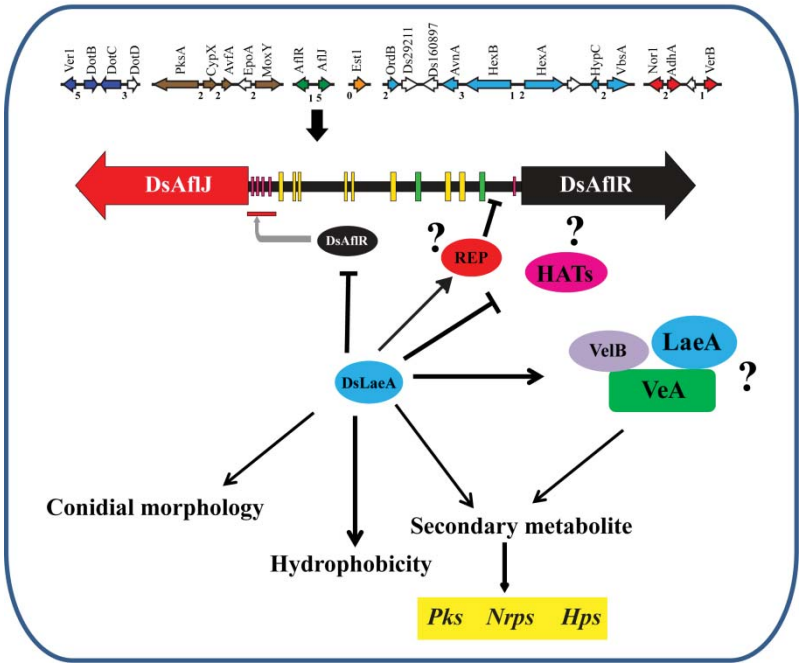


Figure 7.2: Secondary metabolite and developmental regulation by DsLaeA

DsLaeA acts as a negative regulator of DsAflR and regulates conidial morphology, hydrophobicity and production of other secondary metabolites. Interaction with members of the velvet family has not been demonstrated. Rep: unknown repressor protein; *Pks*, *Nrps*, *Hps*: genes coding for polyketide synthases, nonribosomal peptide synthetases and Pks-Nrps hybrids. HATs: chromatin modifier. All functions proposed but not validated by genetic analysis are marked with question marks (?). Repressing functions are marked with blocked arrows (T). Other arrows indicate influence of DsLaeA on development.

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In the *ΔDsLaeA* mutant, *AflR* and *AflJ* were up-regulated 3 & 7 fold compared to WT. A more direct effect of DsLaeA is also possible: Ehrlich et al (2012) reported direct interaction between AflR and LaeA of *Aspergillus* spp. so the possibility of an AflR-LaeA complex binding the DsAflR promoter cannot be overruled. In future, more protein - protein interaction studies are recommended to validate this hypothesis and support the model.

Model 2 also suggests a regulatory link between DsVeA and DsLaeA by the formation of a trimeric complex as reported in *A. nidulans* (Bayram et al., 2008) that brings about coordination of secondary metabolism and development. In future, interactions between these proteins need to be studied and purification of protein complexes might help to identify the interactive network of proteins that regulate secondary metabolism and development in *D. septosporum*.

LaeA is known to counteract repressive heterochromatin marks at the AflR promoter of the ST cluster during stationary growth when ST biosynthesis occurs in *A. nidulans* **(**Reyes-Dominguez et al., 2010) and has more regulatory control on genes that are sub telomerically located (Perrin et al., 2007). We speculate that, in *D. septosporum*, DsLaeA may recruit chromatin modifiers by an unknown mechanism and can repress dothistromin gene expression during exponential growth phase as shown in Figure 6.12, where dothistromin is being produced by the WT but produced at a higher level by the *ΔDsLaeA* strain. The fact that there is any expression of dothistromin genes in WT during exponential phase might be due to the position of *DsAflR* away from the telomeric region where the effects of LaeA are not so strong. In future chromatin immunoprecipitation using specific histone antibodies for different histone modification signatures in dothistromin gene promoters in both wild type and *DsLaeA* knockout strains and *DsLaeA* expression over time in *D. septosporum* wild type strain needs to be

done to validate the model. Some of this work is in progress (Appendix 8.7.8) but could not be completed in time for this thesis.

Expression of *DsVeA* was decreased in a *DsLaeA* knockout strain, but whether LaeA influences DsVeA or other predicted members of the velvet family at the protein level is not known. It would also be of interest to determine if velvet proteins function with similar mechanisms to those seen in other fungi. To address these question identifications and characterization of velvet family of proteins needs to be done.

DsVeA contains a PEST motif that suggests a potential target for ubiquitination. Creation of VeA-GFP mutants of *D. septosporum* could provide an opportunity to apply fluorescence microscopy or flow cytometry to analyse protein degradation. ChIP assays could give more insight into the nature of other posttranslational modifications of DsVeA like phosphorylation.

The study revealed that although fragmented, the dothistromin biosynthesis pathway is regulated by pathway specific transcription factor DsAflR. More importantly, the location of *AflR* half way from the telomeric ends in *D. septosporum* is distinct from AF/ST biosynthesis cluster. This implies that *DsAflR* is away from the influence of telomeric silencing effect, thus may not be so tightly regulated as in the AF/ST pathway. The early onset of dothistromin production *in vitro* could also partly be due to the fragmented nature of the cluster where it escapes from the regulation by telomere silencing and suppressive chromatin modification. The cluster has the advantage to switch on early in the growth stage.

Secondary metabolite clusters are usually located in sub-telomeric regions that undergo extensive changes and such changes can in turn can affect secondary metabolite production that can affect niche adaptation of an organism (Wong and Wolfe, 2005). A recent model on the evolution of

aflatoxin/stergimatocystin/dothistromin (ASD) like gene clusters (Bradshaw et al., 2013) suggests that the current fragmented arrangement of dothistromin genes evolved from a tighter cluster of ASD genes, suggesting a selective advantage of the fragmentation. Movement of some dothistromin genes away from the telomeres, including the pathway regulators *AflR* and *AflJ,* could be a evolutionary adaptive feature for early onset of dothistromin production. As dothistromin is virulence factor involved in lesion formation during rapid biomass growth this adaptation would help *D. septosprorum* to be successful as a hemibiotroph pine pathogen in the ecosystem.

These studies provide a step towards the understanding the fundamental differences in regulation of clustered and fragmented secondary metabolite genes and may shed light on understanding adaptations involved in development of biological roles of fungal secondary metabolites.

Chapter: 8 Appendix

8.1 Media

All media used in this study were prepared in milli Q water and autoclaved at 121°C for 15 minutes and allowed to cool by standing in a water bath maintained at 50°C before adding any antibiotics.

8.1.1 *E. coli* growth media

Luria B broth (LB)

LB Lennox L broth base (Invitrogen) 20 g/L.

Luria B agar (LA)

LB Lennox L broth base (Invitrogen) 20 g/L, Bacteriological agar (Oxoid) 17 g/L.

For selective media

Compound	Stock concentration mg/ml	Final concentration µg/ml
Kanamycin	100	100
Ampicillin	50	50
Spectinomycin	100	100
IPTG	23.83	23.83
X-gal	20	40

8.1.2 Fungal growth media

***D. septosporum* Media (DM)**

Bacteriological agar (Oxoid) 15 g/L, Malt extract (Oxoid) 50 g/L, Nutrient broth (Oxoid) 23 g/L.

***D. septosporum* Broth (DB)**

Malt extract (Oxoid) 50 g/L, Nutrient broth (Oxoid) 23 g/L.

Low *D. septosporum* Broth (Low DB)

Malt extract (Oxoid) 25 g/L, Nutrient broth (Oxoid) 20 g/L.

***D. septosporum* Sporulating Media (DSM)**

Malt extract (Oxoid) 15 g/L, Yeast Extract (BD) 15 g/L

Potato Dextrose Agar (PDA)

Potato dextrose agar (Merck) 39 g/L.

Pine Needle Minimal Media with glucose (PMMG)

Minimal Mineral (MM)	**g/l**
Magnesium sulphate heptahydrate (Merk, Darmstadt, Germany)	0.2
Di potassium hydrogen orthophosphate (BDH, Poole, England)	0.9
Potassium chloride (Sigma, Louis, Germany)	0.2
Ammonium nitrate (Sigma Aldrich, Steinheim, Germany)	1.0
Iron sulphate (APS Chem.Ltd. NSW, Australia)	0.002
Zinc Sulphate heptahydrate (BDH, Poole, England)	0.002
Manganese chloride (BDH, Poole, England)	0.002
Aspargine (Sigma Life Science, St.Louis, USA)	2.0
Glucose (APS Chem.Ltd. NSW, Australia)	3.0
Agar (Neogen corporation, Michigan, USA)	20.0

Water for this media was prepared by soaking fresh pine needles ([10% w/v]) in 1 l Milli Q water for 24 hhours at room temperature based on Carsolio et al. (1994) and Ph adjusted to 6.2

8.1.3 *Dothistroma septosporum* media for transformation

Regeneration Media (RG)

Bacteriological agar (Oxoid) 15 g/L, Malt extract (Oxoid) 50 g/L, Nutrient broth

(Oxoid) 23 g/L, Sucrose (BDH) 273.8 g/L.

0.8% Regeneration Media overlay

Bacteriological agar (Oxoid) 8 g/L, Malt extract (Oxoid) 50 g/L, Nutrient broth (Oxoid) 23 g/L, Sucrose (BDH) 273.8 g/L.

For selection media:

Hygromycin B 50 mg/ml (Roche): Used at 70µg/ml

Phleomycin 21mg/ml (Invivogen): Used at 7 µg/ml

8.2 Buffers and Solutions

8.2.1 General buffers and solutions

All buffers and solutions were prepared using milli-Q water and autoclaved at 121°C for 15 min and were autoclaved at 121°C for 15 min and allowed to cool before use unless otherwise stated

TE buffer ($T_{10}E_1$)

10 mM Tris (Carl Roth), 1mM EDTA (Sigma), pH 8.0.

TBE buffer (10 X)

Tris (Carl Roth) 108.0 g/L, Boric acid (Ajax) 55 g/L, EDTA (Sigma) 7.44 g/L, dissolve in approx. 750 mL milli-Q water. Volume made up with Milli-Q water to make up 1 litre, and adjust pH to 8.2 with 10 M HCl (BDH).

Proteinase K

Dissolve at the concentration of 20 mg/ml in sterile 50mM Tris (pH 8.0), 1.5 mM calcium acetate.

RNAse A (DNAse free)

10 mg of RNAse (Sigma) was dissolved in 0.01 M Sodium acetate (pH 5.2) and kept in a boiling bath for 15 minutes and then 0.1 volume of TE buffer was added to it making stock of 10 mg/ml.Working concentration is $1/10^{th}$ the volume.

Loading dye composition (6x)

0.25% bromophenol blue (Sigma) and 40% sucrose (BDH) dissolved in water and filter sterilized using 0.2 μM syringe filter.

Ethidium bromide gel staining solution

For stock preparation 10 mg/mL ethidium bromide (BDH) was dissolved in milli-Q water and 1μl per 10 ml of water was used for staining agarose gels.

Acid and Bases

11.1 M HCl (37.1 g/L) (BDH) and 10 M NaOH (Scientific Supplies Ltd) (39.9 g/L)

8.2.2 Buffers for DNA extraction

CTAB 2% (w/v), 100mM Tris HCl (pH 8), 1.4 M NaCl and 20 mM EDTA volume made up to 100 ml by sterile water. The solution was autoclaved and 1 ml β mercapto ethanol PVP 100 mg was added to make the volume 100 ml.

8.2.3 *Dothistroma septosporum* transformation reagents

OM buffer

1.4 M $MgSO_4$ (Ajax), 10 mM Na_2HPO_4 (BDH), dissolved in 100 mL Milli-Q water, adjust pH with 100 mM $NaH_2PO_4.2H_2O$ (BDH) to pH 5.8. Add Milli-Q water to a final volume of 300 mL.

ST buffer

0.6 M sorbitol (Sigma), 100 mM Tris (Carl Roth) pH 8.0

STC buffer

1 M sorbitol (Sigma), 50 mM Tris pH 8.0 (Carl Roth), 50 mM $CaCl_2$ (Merck).

40% Polyethylene Glycol (PEG) solution

40% (w/v) PEG 4000 (BDH), 50 mM $CaCl_2$ (Merck), 50 mM Tris pH 8.0 (Carl Roth), 1M sorbitol (Sigma)

Glucanex (Novozymes)

10 mg /ml in OM buffer

8.2.4 Buffer and reagents for Southern Hybridisation

0.25 M HCl acid treatment:

Take 21ml HCl and add it to 979 ml of water.

Denaturation buffer

0.5 M NaOH (BDH), 0.5 M NaCl (Pure Science Ltd.)

Neutralization buffer

0.5 M Tris, pH 7.4 (Carl Roth), 2.0 M NaCl (Pure Science Ltd.).

SSC (20 X)

3.0 M NaCl (Pure Science Ltd.), 0.3 M Trisodium citrate (Scientific Supplies Ltd.).

Buffer I (Southern blot, hybridisation)

100 mM Tris, pH 7.5 (Carl Roth), 150 mM NaCl (Pure Science Ltd.).

Buffer II (Southern blot, hybridisation)

100 mM Tris, pH 7.5 (Carl Roth), 150 mM NaCl (Pure Science Ltd.), 1% (w/v)

blocking reagent (Roche)

Buffer III (Southern blot, hybridisation)

100 mM Tris, pH 9.5 (Carl Roth), 100 mM NaCl (Pure Science Ltd.).

Antibody solution

AntiDig AP antibody (Roche) dissolved diluted 1:10000 in buffer II

Wash Solution I

2 x SSC, 0.1% SDS

Wash Solution II

0.1 x SSC, 0.1% SDS

Stripping Buffer

0.2 M NaOH, 0.1% SDS (w/v)

8.2.5 Buffers and reagents for RNA extraction

10x Formaldehyde Agarose Gel Buffer

200 mM Na-MOPS, 50 mM NaOAc and 10 mM EDTA

Electrophoresis Buffer

Dilute 10x Formaldehyde Agarose Gel Buffer to 1x using DEPC treated water

RNA loading dye 5x

4µl of saturated aqueous bromophenol blue solution, 20 µl of 500 mM EDTA pH 8, 180 µl 37% formaldehyde, 500 µl 100% glycerol, 771 µl of formamide and volume made up to 2.5 ml with 10x Formaldehyde Agarose Gel Buffer.

8.2.6 Buffer and reagents for Chromatin immunoprecipitation

Sonication buffer

50 mM HEPES–KOH pH 7.5, 140 mM

NaCl (Pure Science Ltd), 1 mM EDTA (Sigma), 1% Triton X-100 (Sigma), 0.1% Na-deoxycholate (1× Fungal protease inhibitor (Promega).

ChIP dilution buffer

1% Triton X-100(Sigma), 1.2 mM EDTA (Sigma),16.7 mM Tris-HCl pH 8 (Carl Roth), 167 mM NaCl (Pure Science Ltd), 1× fungal protease inhibitor (Promega).

Low salt wash buffer

150 mM NaCl (Pure Science Ltd), 0.2% SDS (BDH limited), 0.5% TritonX-100 (Sigma), 2 mM EDTA(Sigma), 20 mM Tris-HCl pH 8 (Carl Roth).

High salt wash buffer

500 mM NaCl(Pure Science Ltd), 0.2% SDS (BDH limited), 0.5% TritonX-100 (Sigma), 2 mM EDTA (Sigma), 20 mM Tris-HCl pH 8 (Carl Roth).

LiCl salt wash buffer

0.25 mM LiCl (Prolabo), 0.5% Na-deoxycholate,1 mM EDTA (Sigma), 10 mM Tris-HCl pH 8 (Carl Roth), 0.5% Ig epal CA 630 (Sigma)

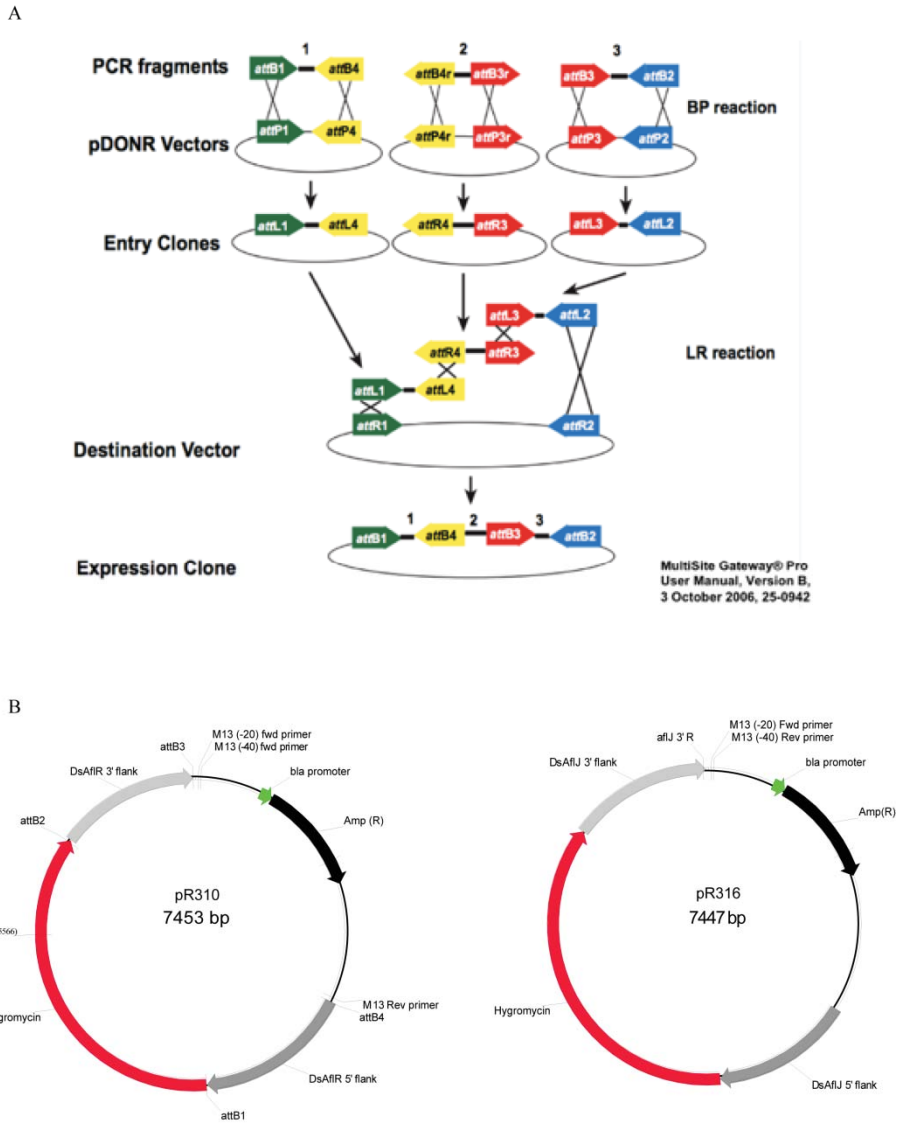
TE buffer

1 mM EDTA (Sigma), 10 mM Tris-HCl pH 8 (Carl Roth).

Resuspension buffer

SDS 1% (BDH limited), $NaHCO_3$ 0.1 M. (BDH limited)

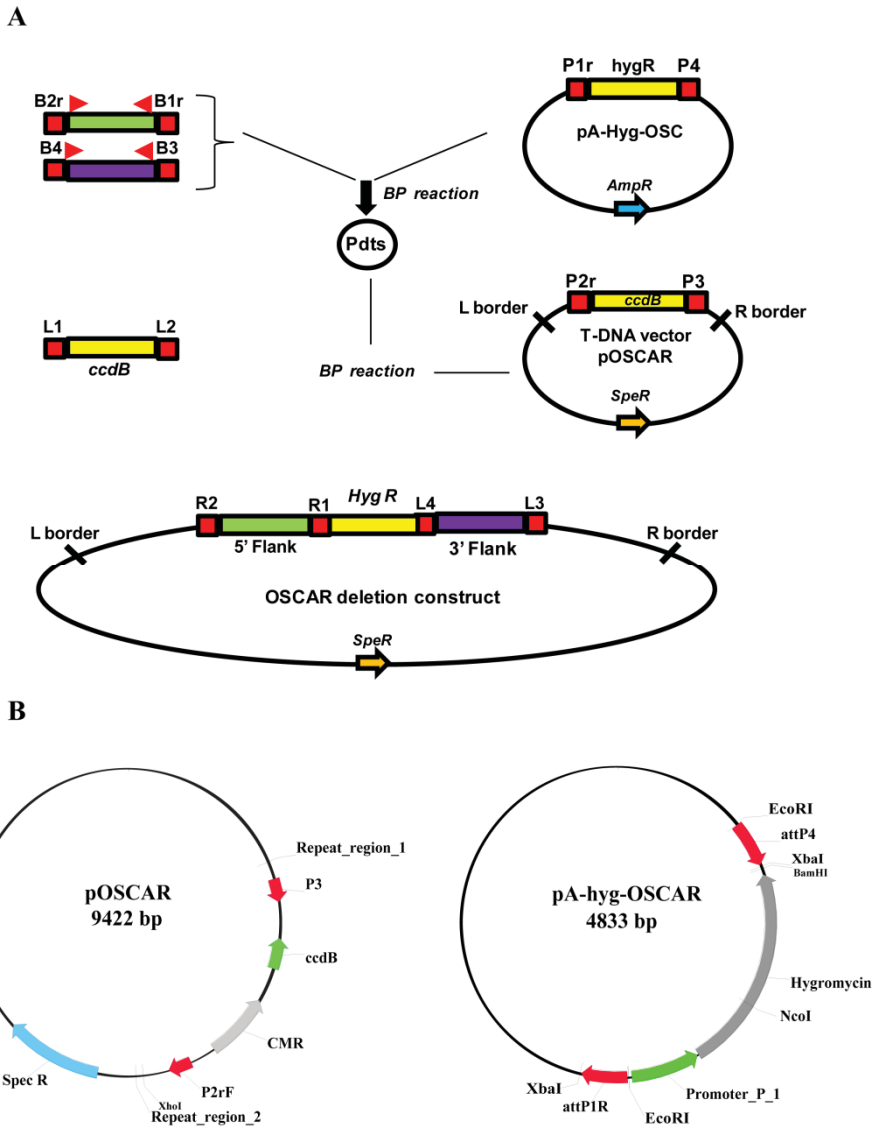
Appendix 8.3 Vectors and constructs



Appendix 8.3.1: MultiSite Gateway® 3 Fragment recombination reaction based vector construction

A) Three PCR products (5', 3' flanking sequence of gene of interest and the fragment of interest) flanked by specific attB or attBr sites and three MultiSite Gateway® Donor vectors are used to create three independent entry clones by BP reaction. Finally entry constructs are recombined with destination vector pDEST™ R4-R3 to get the destination construct.

B) Examples of Destination construct pR310 and pR316 made by Gateway three fragment recombination reaction for deleting *DsAflR* and *DsAflJ* in *D. septosporum.*

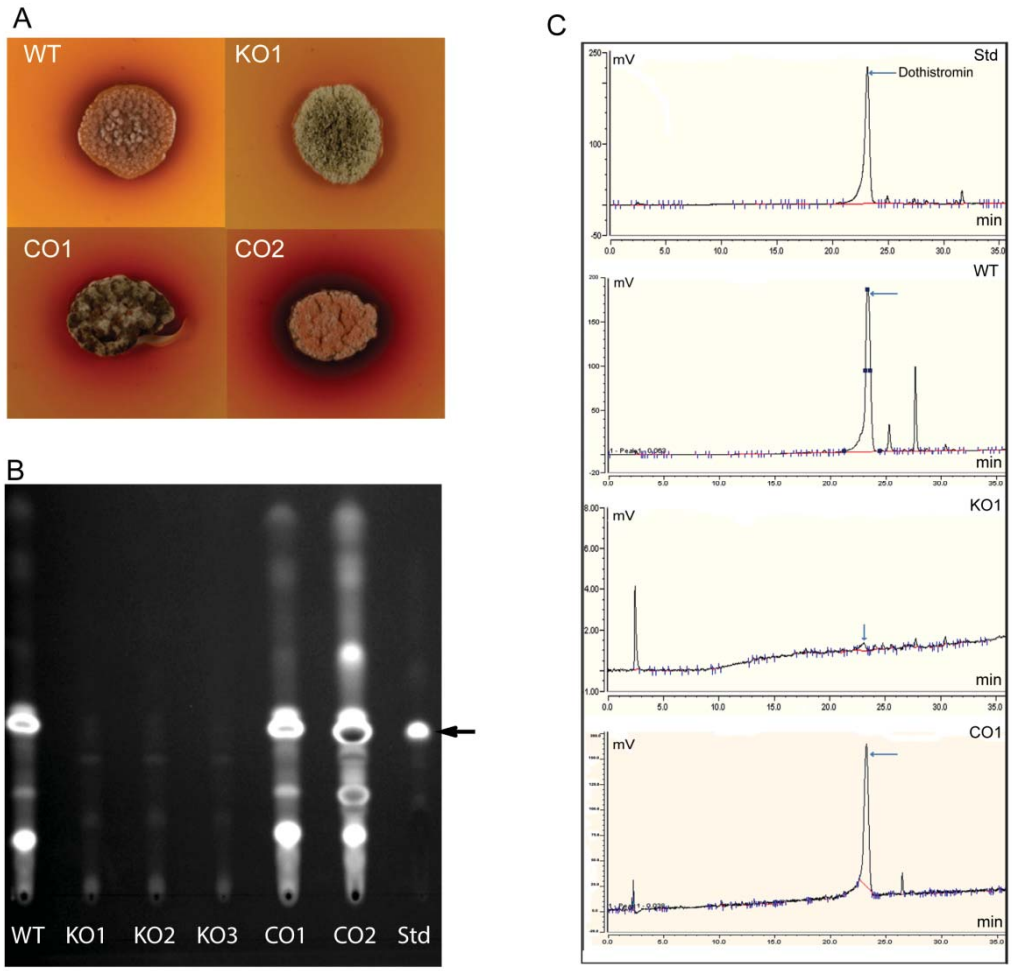


Appendix 8.3.2: OSCAR deletion vector construction methodology.

A) 5' and 3' flanks of the gene to be deleted are PCR amplified. This is followed by a BP clonase recombination reaction of flank products and the binary (pOSCAR) and selection marker (hygromycin) plasmids. B1r & B2r from 5' flank PCR, B3 & B4 from 3' flank PCR, P1r & P4 from pA-Hyg-OSCAR vector and P2r &P3 from pOSCAR vector. The final product is verified by PCR.

B) Examples of vector pOSCAR and pA-hyg-OSCAR used for creating pOSCAR deletion construct

8.4 Appendices for chapter 3

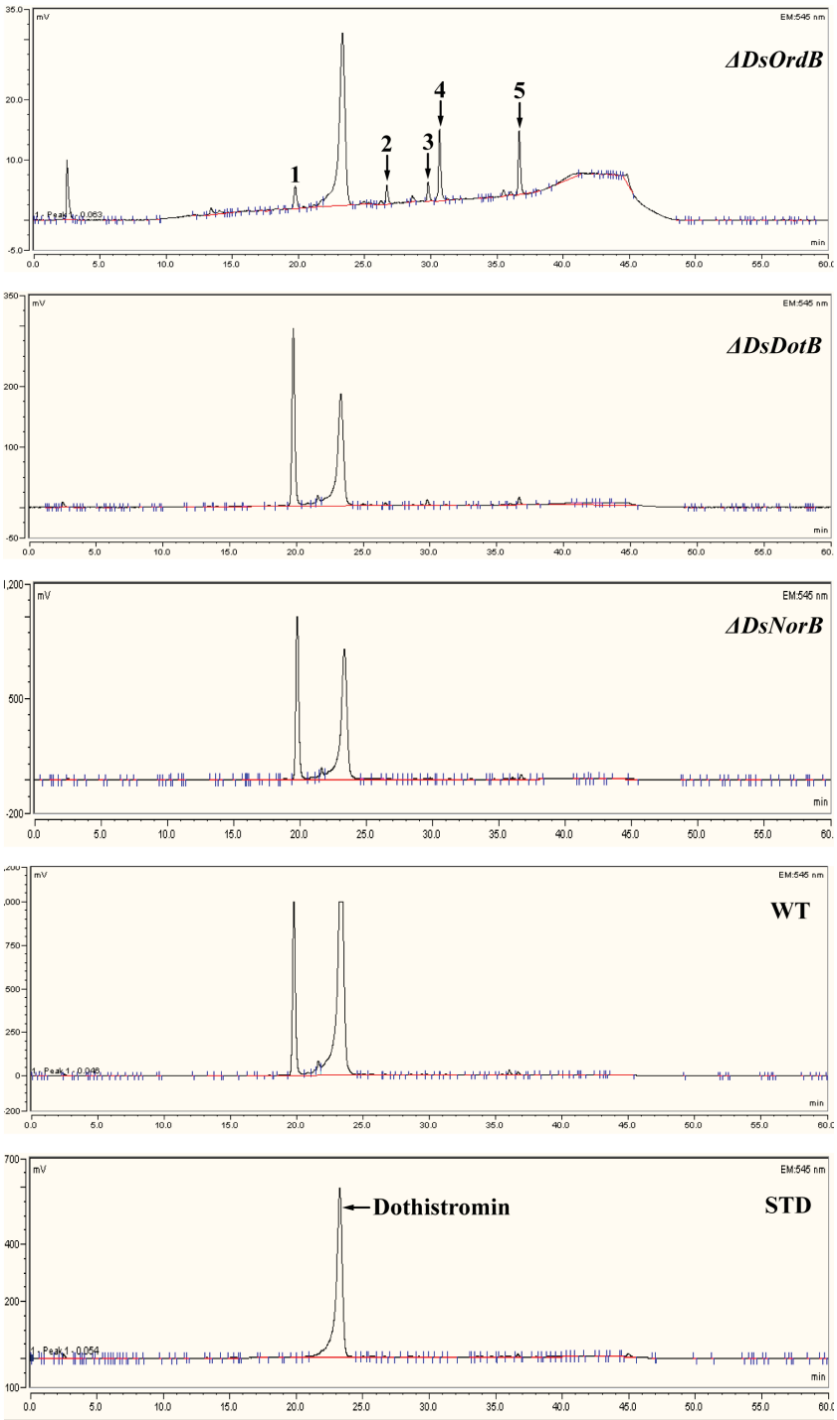


Appendix 8.4.1: *D. septosporum ΔDsAflR* mutants are severely impaired in dothistromin production.

(A) Wild type (WT), *ΔDsAflR* mutant (KO1), and complemented strains with one (CO1) or two (CO2) copies of *DsAflR* grown on DM agar plates. The red-brown compound secreted into the agar is dothistromin.

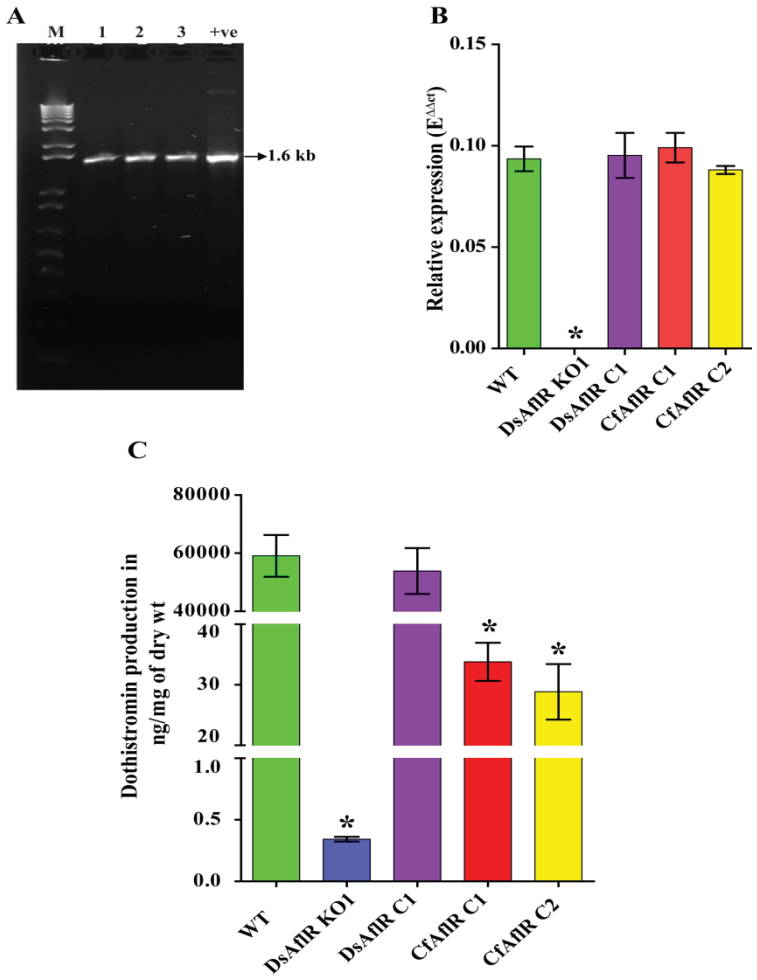
(B) Extracts from growth media of the same strains as in (A), along with two other independent *ΔDsAflR* mutants (KO2, KO3), separated by thin layer chromatography, along with a dothistromin standard (Std; indicated by arrow).

(C) HPLC chromatograms of dothistromin standard (Std), wild type (WT), *DsAflR* knockout (KO1) and complemented (CO1) strains of *D. septosporum* showing dothistromin (arrowed) at retention time of 23.1±0.05 minutes.



Appendix 8.4.2: HPLC chromatograms of dothistromin assay

Standard (Std), wild type (WT), *DsOrdB* knockout, *DsDotB* knockout and *DsNorB* knockout strains of *D. septosporum* showing dothistromin (arrowed) at retention time of 23.1±0.05 minutes. Note the bands marked 2, 3, 4, 5 were prominent only in *ΔDsOrdB* strain. The peak marked 1 at retention time of 19.5 minutes is present only in culture grown in pine minimal broth (PMMG). The area of each unknown peak corresponding to standards concentration were determined and calculated as shown in figure 3.8.



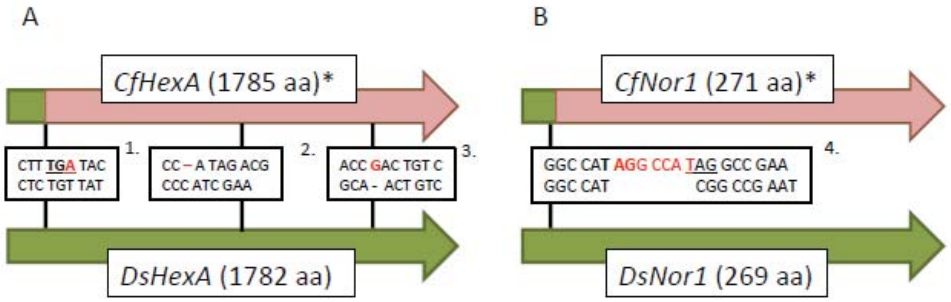
Appendix 8.4.3: Analysis of *CfAflR* complemented strain of *DsAflR* gene replacement mutant (KO1).

(A) PCR confirmation of *CfAflR* complemented strain *CfAflR* C1, *CfAflR* C2, *CfAflR* C3 (Lane 1, 2 and 3) and WT *C. fulvum* genomic DNA as positive control (Lane 4) using primers that bind to *CfAflR* coding sequence and the 3' flank (Cf AflJ ApaI F and CfAflr expR ; method table 2.3)

(B) *CfAflR* gene expression in complemented strains was evaluated by quantitative real-time PCR using RNA isolated from mycelium grown in Dothistroma broth (DM) rich media. The expression values shown in the *DsAflR* knockout mutant *DsAflR* KO1, complementation mutant *DsAflR* C1 (purple bar), *CfAflR* complementation mutant C1 (red bar) and *CfAflR* complementation mutant C2 (yellow bar) relative to expression in the wild type (green bar).

(C) Dothistromin production by the above (B) strains as quantified by HPLC.

In B and C, the significant difference from the wild type (P≤0.05) has been marked with asterisk.



Appendix: **8.4.4. A comparison of *HexA* and *Nor1* transcripts from *C. fulvum* and *D. septosporum*.**

Green corresponds to the functional portion of each transcript, pink denotes the pseudogenized regions. *Denotes size of polypeptide if all stop codons were ignored. (A) *CfHexA* has (1) a premature in frame stop codon at nucleotide position 276 (counting from A of start codon), (2) 1 nt deletion at position 3138 and (3) 1 nt insertion at position 4148. (B) *CfNor1* has (4) a 7 nt insertion at position 36 resulting in introduction of a stop codon.

* Note this work was done at The Laboratory of Phytopathology (Wageningen University)

Appendix 8.4.5: Predicted conserved domain of Ds PID29211 a putative transcription factor on chromosome 12 of *D. septosporum*.

Fungal transcription factor domain; This domain is present in the large family of fungal zinc cluster transcription factors that contain an N-terminal GAL4-like C6 zinc binuclear cluster DNA-binding domain. Examples of members of this large fungal group are the *Saccharomyces cerevisiae* transcription factors, GAL4, STB5, DAL81, CAT8, RDR1, HAL9, PUT3, PPR1, ASG1, RSF2, PIP2, as well as the C-terminal domain of the Cep3, a subunit of the yeast centromere-binding factor 3. It has been suggested that this region plays a regulatory role (Bauer et al 2009).

	Expression relative to WT in PMMG[a]				WT/KO	WT/KO	R
Gene	**WT**	**KO1[b]**	**C1**	**C2**	**P-VALUE[c]**	**Fold diff.**	
Ver1	1	0.084±0.002	0.996±0.079	7.851±0.063	4.30E-06	11.90	
DotB	1	0.229±0.029	1.102±0.011	NC	1.46E-03	4.37	
DotC	1	0.028±0.001	0.994±0.005	0.516±0.003	1.17E-06	35.71	
DotD	1	0.509±0.045	1.032±0.052	NC	8.48E-03	1.96	
PksA	1	0.609±0.015	1.036±0.044	3.436±0.021	1.48E-03	1.64	
CypX	1	0.306±0.002	0.996±0.002	1.459±0.033	5.59E-06	3.27	
AvfA	1	0.339±0.0135	0.881±0.018	0.483±0.005	3.88E-04	2.95	
EpoA	1	0.995±0.047	0.888±0.046	NC	9.29E-01	1.01	
MoxY	1	0.127±0.007	0.977±0.018	1.618±0.053	6.91E-05	7.87	
Ds-31	1	0.366±0.003	0.892±0.051	NC	2.20E-05	2.73	
AflR	1	0	1.001±0.006	2.17±0.083	0.00E+00	0	
AflJ	**1**	**7.747±0.107**	**1.076±0.052**	**0.415±0.011**	**2.51E-04**	**7.75**	
Est1	1	0.093±0.002	1.008±0.018	0.859±0.001	3.01E-06	10.75	
OrdB	1	0.124±0.002	0.848±0.003	1.981±0.042	9.36E-06	8.06	
Ds29211	1	0.963±0.013	0.939±0.044	NC	1.07E-01	1.04	
Ds160897	1	0.368±0.015	0.975±0.015	NC	6.30E-04	2.72	
AvnA	1	0.011±0.0002	0.837±0.0293	1.964±0.104	6.22E-08	90.91	
HexB	1	0.114±0.002	0.848±0.041	1.496±0.013	3.15E-06	8.77	
HexA	1	0.418±0.008	0.986±0.014	0.585±0.023	1.97E-04	2.39	
HypC	1	0.582±0.011	0.962±0.117	NC	6.91E-04	1.72	
VbsA	1	0.146±0.009	0.961±0.117	2.367±0.081	1.21E-04	6.85	
Nor1	1	0.139±0.003	0.857±0.02	0.664±0.028	1.78E-05	7.19	
AdhA	1	0.025±0.002	1.044±0.031	2.332±0.03	4.46E-06	40.00	
VerB	1	0.012±0.0003	0.823±0.006	2.743±0.017	1.17E-07	83.33	
NorB	1	0.042±0.0006	0.953±0.024	0.174±0.011	4.16E-07	23.81	

Appendix 8.4.6 (a) Expression of dothistromin cluster-associated genes in *D. septosporum* wild-type (WT), AflR and C2 in PMMG (minimal media).

[a]PMMG (minimal salts medium with pine needle extract)
[b]KO1 values shown in black suggest significance difference between WT and *DsAflR* KO; values shown in red show no
qRT-PCR gene expression normalised to wild-type; mean and SD of 3 biological and 2 technical replicates.
[c]P Value: probability of no significant difference between KO1 and WT
Increased expression of *AflJ* is shown in bold and highlighted.
Raw values show mean expression relative to *β tubulin*.

	Expression relative to WT in DM[a]				WT/KO	WT/KO	R
Gene	**WT**	**KO1[b]**	**C1**	**C2**	**P-VALUE[c]**	**Fold diff.**	
Ver1	1	0.096±0.006	0.844±0.022	48.573±4.25	4.49E-05	10.42	
DotB	1	0.85668±0.02237	1.102±0.049	1.1988±0.032	5.35E-02	NC	
DotC	1	0.0192±0.001	1.039±0.019	0.93±0.096	1.35E-06	52.08	
DotD	1	0.049±0.003	0.975±0.011	0.855±0.039	1.66E-05	20.41	
PksA	1	1.425±0.068	0.949±0.011	68.257±0.447	1.62E-01	NC	
CypX	1	1.198±0.041	0.977±0.022	38.899±0.066	7.95E-02	NC	
AvfA	1	0.858±0.072	0.917±0.061	7.932±0.489	1.83E-01	NC	
EpoA	1	0.856±0.034	1.091±0.037	102.691±8.075	5.06E-01	NC	
MoxY	1	0.4811±0.046	1.021±0.061	64.517±2.884	7.85E-03	2.08	
Ds-31	1	0.329±0.002	1.009±0.007	x	1.54E-05	3.04	
AflR	1	0	0.951±0.048	4.079±0.107	0.00E+00	0.00	
AflJ	**1**	**83.511±3.704**	**1.243±0.026**	**6.356±0.355**	**2.01E-03**	**83.51**	
Est1	**1**	**10.65±0.926**	**0.966±0.099**	**2.144±0.248**	**9.08E-03**	**10.65**	
OrdB	1	0.556±0.053	1.036±0.013	33.516±1.547	1.43E-02	1.80	
Ds29211	1	1.085±0.035	0.956±0.059	x	1.36E-01	NC	
Ds160897	1	0.448±0.015	0.935±0.01	x	7.69E-04	2.23	
AvnA	1	0.135±0.011	1.167±0.084	197.298±7.014	1.71E-04	7.41	
HexB	1	0.324±0.0281	0.922±0.045	39.922±1.269	1.72E-03	3.09	
HexA	**1**	**2.175±0.063**	**0.982±0.046**	**6.561±0.216**	**2.93E-03**	**2.18**	
HypC	1	0.607±0.014	0.954±0.054	x	4.13E-02	1.70	
VbsA	1	0.298±0.019	0.893±0.04	144.956±2.243	7.88E-04	3.36	
Nor1	1	1.055±0.025	1.19±0.015	49.811±1.223	1.58E-01	NC	
AdhA	1	0.302±0.006	1.338±0.103	59.318±6.377	9.35E-05	3.31	
VerB	1	0.118±0.025	0.908±0.095	143.522±20.202	8.21E-04	8.47	
NorB	1	0.136±0.019	1.201±0.024	1.051±0.031	5.05E-04	7.35	

Appendix 8.4.6 (b) Expression of dothistromin cluster-associated genes in *D. septosporum* wild-type (WT), AflR and C2 in DM (rich media).

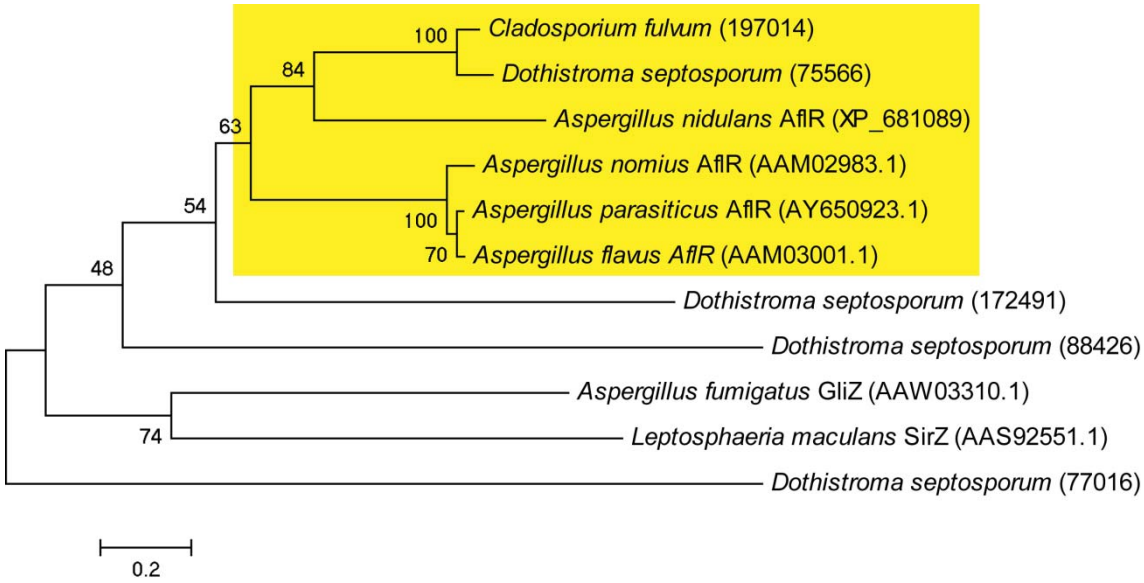
[a]DM *Dothistroma septosporum* specific media (rich media)

[b]KO1 values shown in black suggest significance difference between WT and *DsAflR* KO strain; values shown in red sh
Values are qRT-PCR gene expression normalised to wild-type; mean and SD of 3 biological and 2 technical replicates.

[c]P Value: probability of no significant difference between KO1 and WT

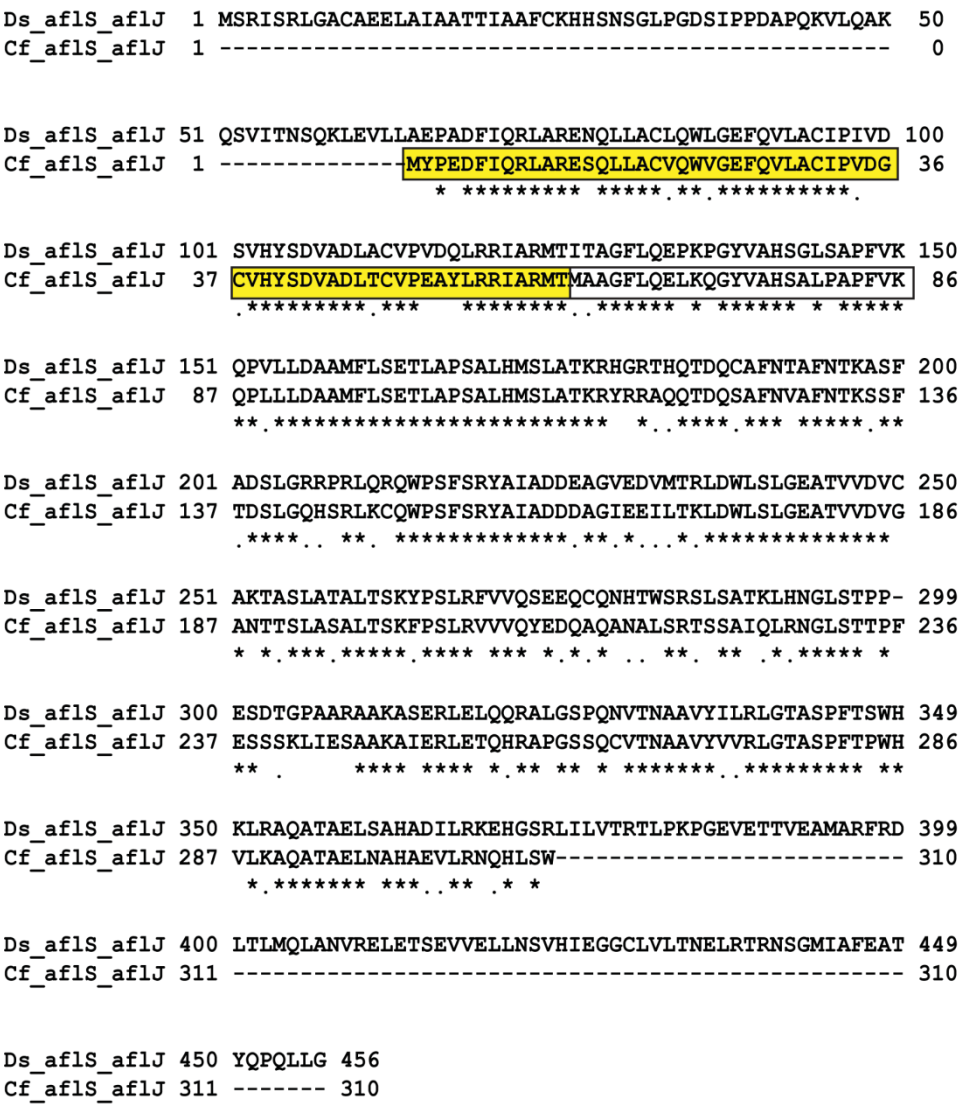
Increased expression of *AflJ*, *Est1* and *HexA* are shown in bold and highlighted.

Raw values show mean expression relative to *β tubulin*.



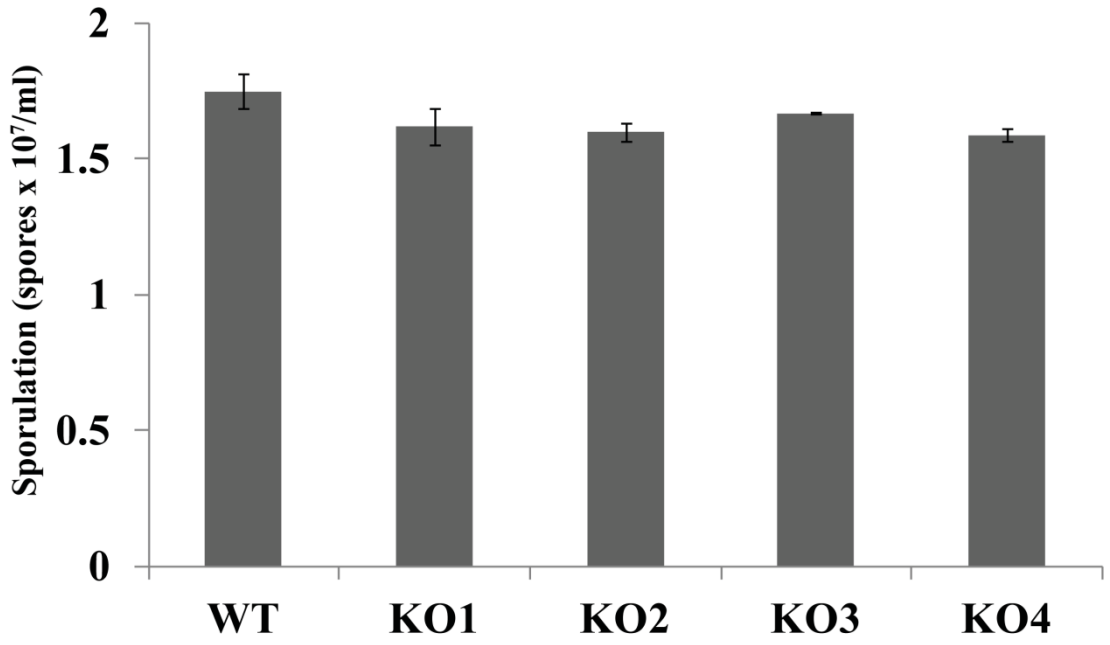
Appendix 8.4.7 A phylogenetic tree was constructed using the top hits of putative AflR like protein sequence from *D. septosporum* genome along with other characterised AflR from *Aspergillus spp* and . C_6Zn_2 containg transcription factor SirZ (Sirodesmin regulator in *L. maculans*) and GliZ (Gliotoxin regulator in *A. fumigatus*). The amino acid sequences were obtained by BLASTP using *A. nidulans* and *A. parasiticus* functionally characterised AflR protein sequences to the JGI database (http://genome.jgi-psf.org/pages/blast.jsf?db=dothideomycetes). The tree was constructed by Neighbor Joining in MEGA 5.0 (Tamura et al., 2011) with 1000 bootstrap replicates (coefficients are indicated below the respective nodes).

8.5 Appendix for Chapter 4



Appendix 8.5.1: Alignment of DsAflJ and Cf AflJ predicted amino acid sequences

The JGI gene model of CfAflJ (PID 197013) has a shorter N and C terminal than shown here. About 60 amino acids are absent from the gene model and have been added after manual annotation. The starting aa methionine as predicted by JGI model is marked with a black rectangle and the sequence added after manual annotation is highlighted in yellow.



Appendix 8.5.2: Sporulation of *DsAflJ* knockout mutants.

Sporulation of wild type (WT), *DsAflJ* knockout strains (KO1, KO2, KO3 & KO4) (mean ± SE). Significant differences ($P < 0.05$). There was no significant difference in sporulation between the knockout strains. KO1 was selected for further work

Appendix 8.5.3 DsAflJ TMpred analysis

Type	Position Range	Centre	Score
Inside to outside helices	7-25	15	99
	84-101	92	571
	157-173	165	152
Outside to inside helices	7-25	17	254
	84-102	92	66
	152-174	162	335
	329-348	339	299

Possible transmembrane helices within DsAflJ of *Dothistroma septosporum*. Only scores above 500 are considered significant.

Suggested models for transmembrane topology

These suggestions are purely speculative and should be used with **extreme caution** since they are based on the assumption that all transmembrane helices have been found.

2 possible models considered, only significant TM-segments used

***** the models differ in the number of TM-helices ! *****

> STRONGLY preferred model: N-terminus inside

1 strong transmembrane helices, total score : 517

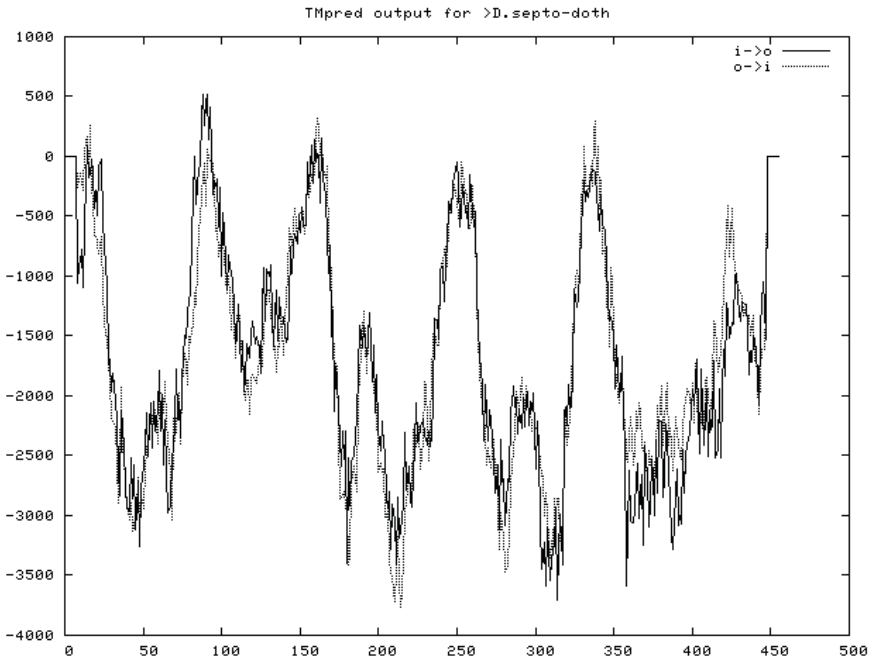
from to length score orientation

1 84 101 (18) 517 i-o

> **Alternative model**

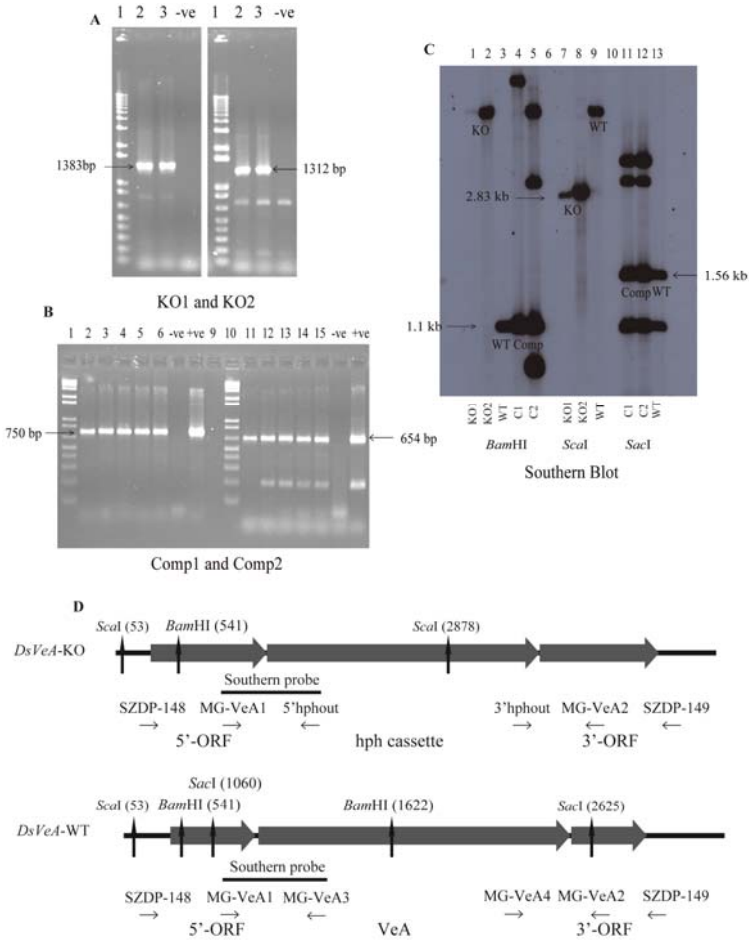
0 strong transmembrane helices, total score : 0

from to length score orientation



Appendix 8.5.3: The pattern of orientation showing transmembrane helices within AflJ of *D. septosporum*.

8.6: Appendix for chapter 5

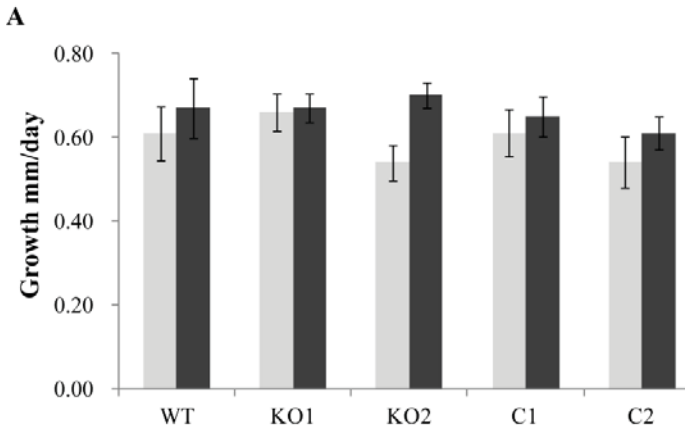


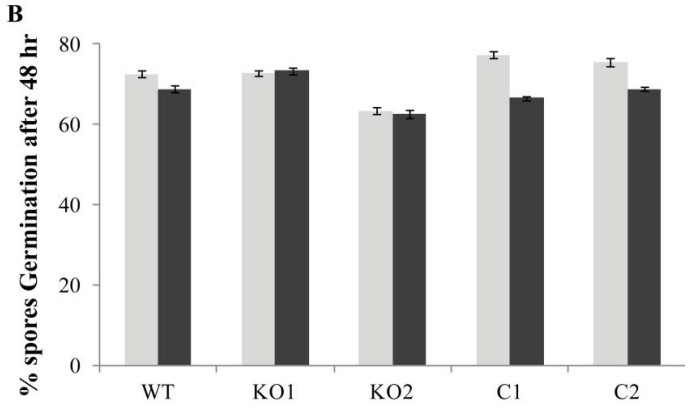
Appendix 8.6.1: Molecular verification of *DsVeA* gene replacement and complementation.

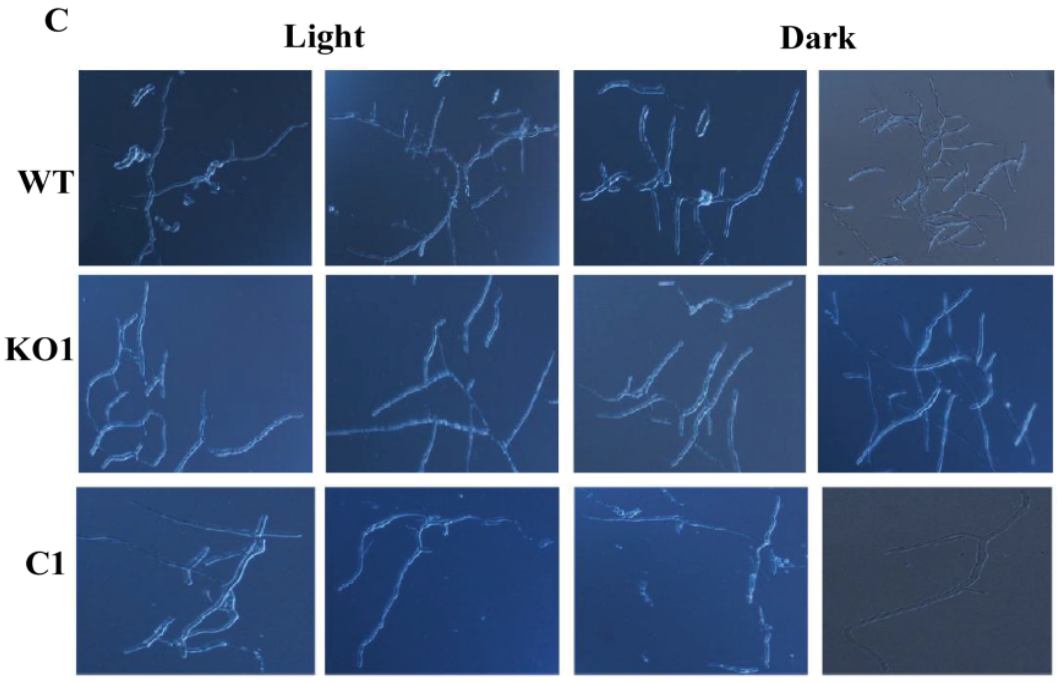
A) PCR results for 5' (left; primers SZDP148, 5'hphout) and 3' (right, primers SZDP149, 3'hphout) knockout of *DsVeA* coding region by hygromycin cassette in *D. septosporum*. Transformants KO1 and KO2 (lanes 2 and 3) had correct sized PCR products, indicating knockout of the *DsVeA* gene. Lane 1 is 1 kb plus ladder

B) PCR results for 5' (left; primers MG-VeA1, MG-VeA3) and 3' (right, primers MG-VeA2, MG-VeA4) complementation of *DsVeA* coding region in KO1. Five transformants C1 to C5 (lanes 2-6 and 11-15) had correct sized PCR products, indicating complementation of *DsVeA* mutation. Lane 1 is 1 kb plus ladder, lane +ve is control with *D. septosporum* gDNA template.

C) Southern hybridisation and (D) schematic diagram of *DsVeA* knockout and complemented plasmids. *BamHI* and *ScaI* digested genomic DNA of *D. septosporum* WT, KO1 and KO2 were hybridized with *VeA* plus hygromycin cassette probe. The hygromycin cassette introduced a ScaI (2878) site which replaced the BamHI (1622) site in the WT strain and resulted in a 2.83 kb fragment in knockout strains. *BamHI* and *SacI* digested genomic DNA of WT, C1 and C2 were hybridized with a *DsVeA* probe. Complemented strains had the same sized *BamHI* (1.1 kb) and *SacI* (1.56 kb) fragments as WT.


A
Growth mm/day
0.80
0.60
0.40
0.20
0.00
WT
KO1
KO2
C1
C2


B
% spores Germination after 48 hr
80
60
40
20
0
WT
KO1
KO2
C1
C2


C
Light
Dark
WT
KO1
C1

Appendix 8.6.2. Phenotypic analyses of *DsVeA* knockout, complemented and WT strains under light and dark conditions.

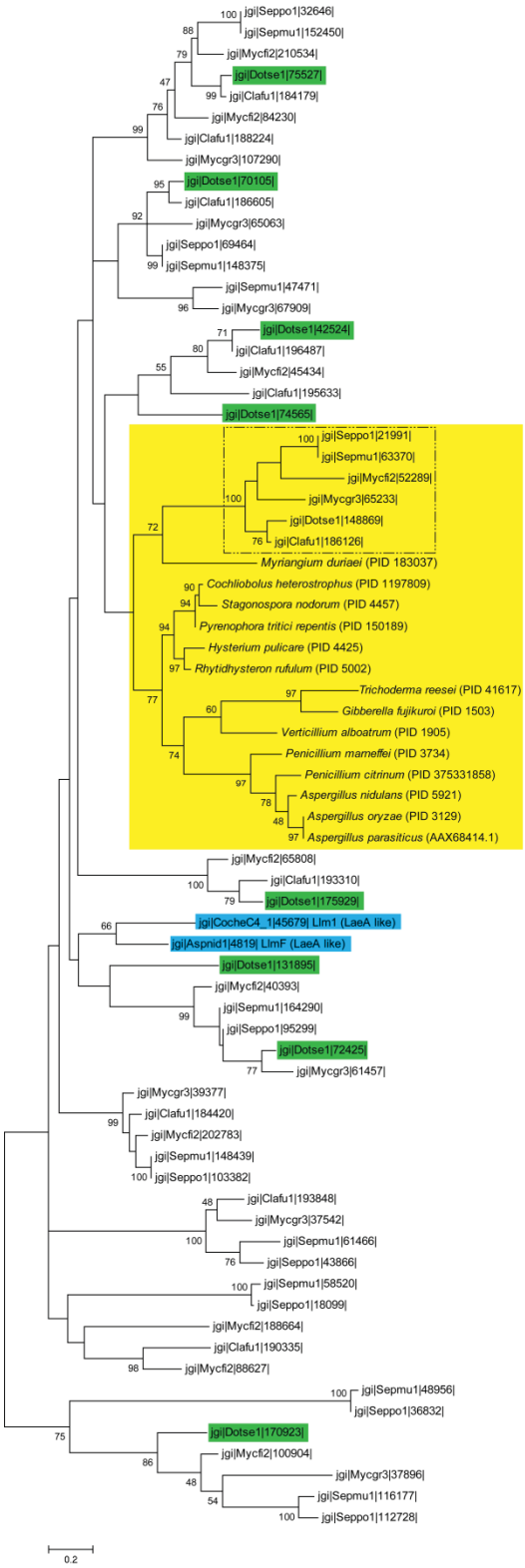
(A) Shows radial growth rates measured at seven day intervals for 21 days in DM (rich) media under light (light grey bar) and dark (black bar) conditions. Experiment was done in triplicate. All strains grew slightly faster in the dark than the light, but this difference was only significant ($P < 0.05$) for *ΔDsVeA* strain KO2.

(B) Percentage (%) of germinated spores after 48 hrs of incubation in DM broth under light and dark condition. No significant difference was observed in germination % for *ΔDsVeA* strains KO1 & KO2 or complemented strains C1 & C2 compared to wild type (WT) under either light and dark condition.

(C) Microscopic analysis of ΔDs*VeA* mutants grown on agar plates. No difference was observed in hyphal morphologies nor in their ability to produce aerial hyphae between *DsVeA* KO strains (KO1 & KO2), complemented strains (C1 & C2) compared to wild type (WT) in both light and dark conditions tested.

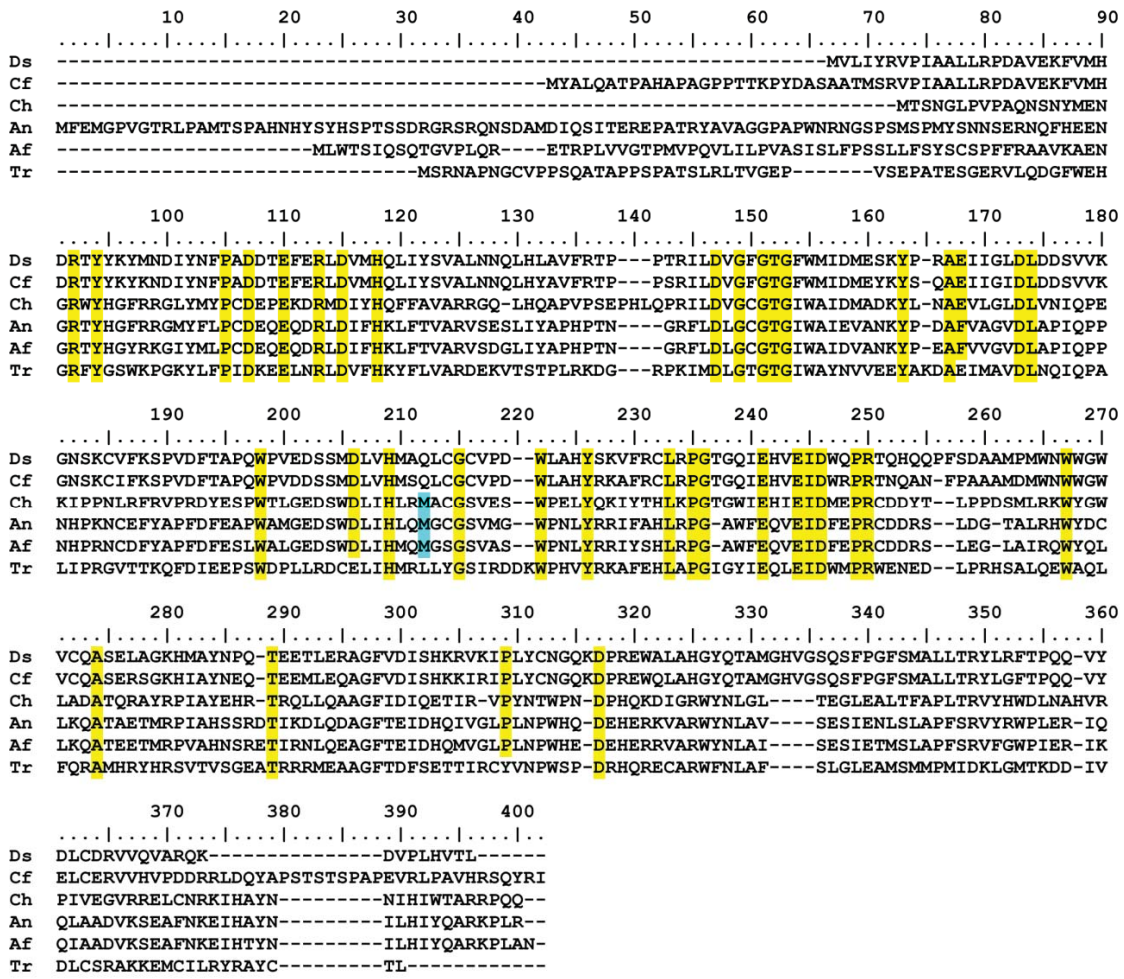
(* This phenotypic characterisation was done by Yanan Guo)

8.7 Appendix for Chapter 6



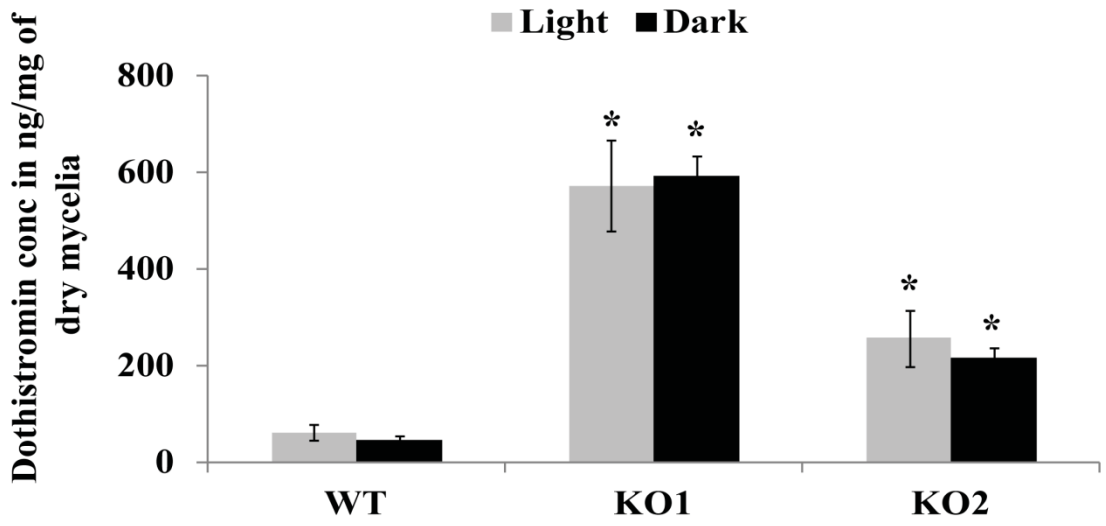
Appendix 8.7.1: Phylogenetic analysis of putative LaeA protein sequences.

A phylogenetic tree was constructed using the top hits of putative SAM dependent methyl transferase from order capnodiales within the Dothideomycetes class, along with other characterised LaeA from Eurotiomycetes, Dothideomycetes and Sordariomycetes. The amino acid sequence were obtained by BLASTP using *Aspergillus nidulans* functionally characterised LaeA protein sequence to the JGI database (http://genome.jgi-psf.org/pages/blast.jsf?db=dothideomycetes). The tree was constructed by Neighbor Joining in MEGA 5.0 (Tamura et al., 2011) with 1000 bootstrap replicates (coefficients are indicated below the respective nodes). Boot strap values below 40 are not shown in the figure. The predicted LaeA in order capnodiales is marked with a dash line. *D. septosporum* top nine hits having LaeA like candidates are highlighted in small green boxes and LlmF (LaeA like of *A. nidulans*) and Llm1 (LaeA like of *C. heterostrophus*) is highlighted with blue.



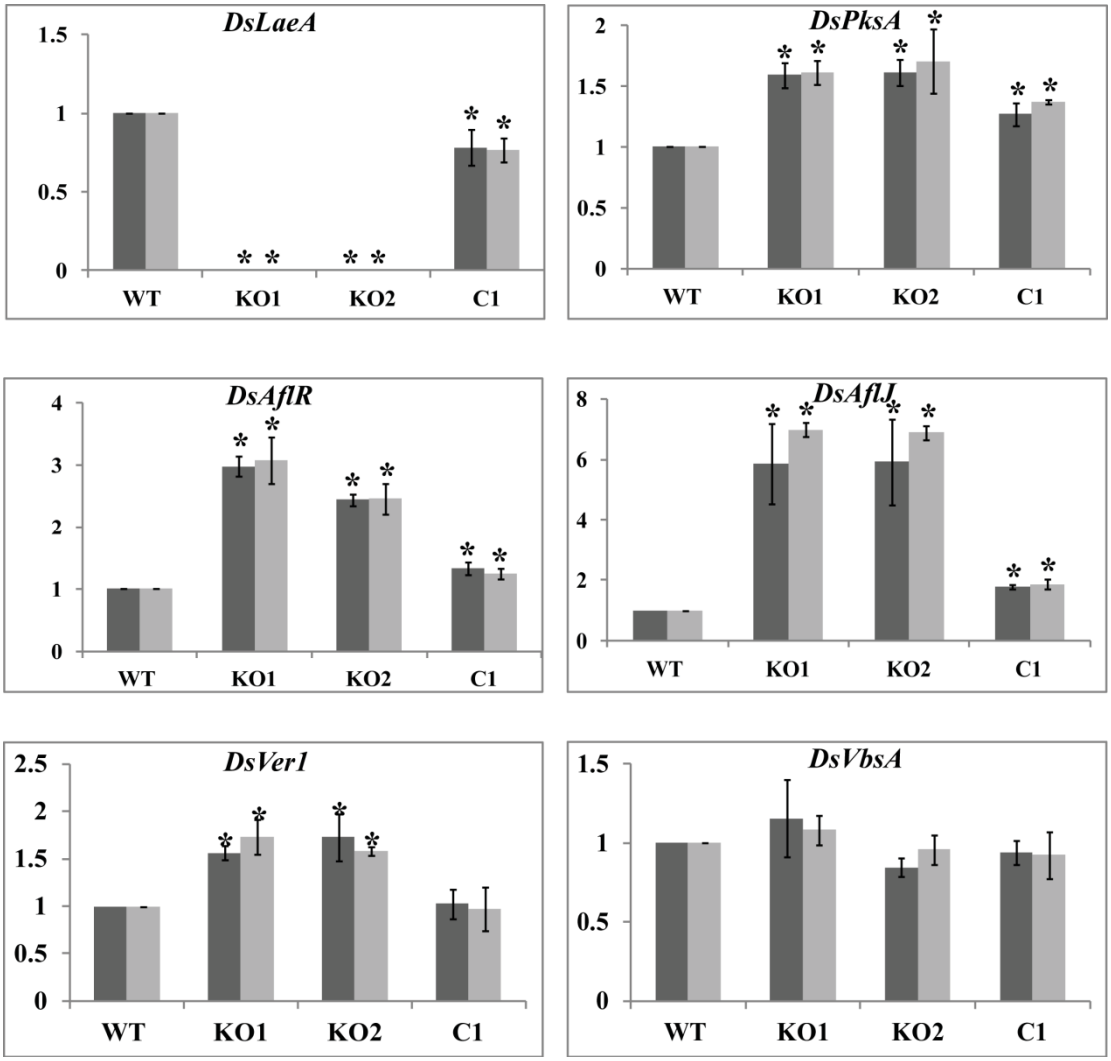
Appendix 8.7.2: Alignment of LaeA sequences

Sequence alignment of LaeA from *Dothistroma septosporum* (Ds) with its orthologs from other fungal species *Aspergillus nidulans* (An), *Cladosporium fulvum* (Cf), *Cochliobolus heterostrophus (Ch), Aspergillus flavus (Af) and Trichoderma reesei (Tr).* Conserved regions are highlighted in yellow. Methionine 207 conserved in functionally characterised LaeA is highlighted in blue.



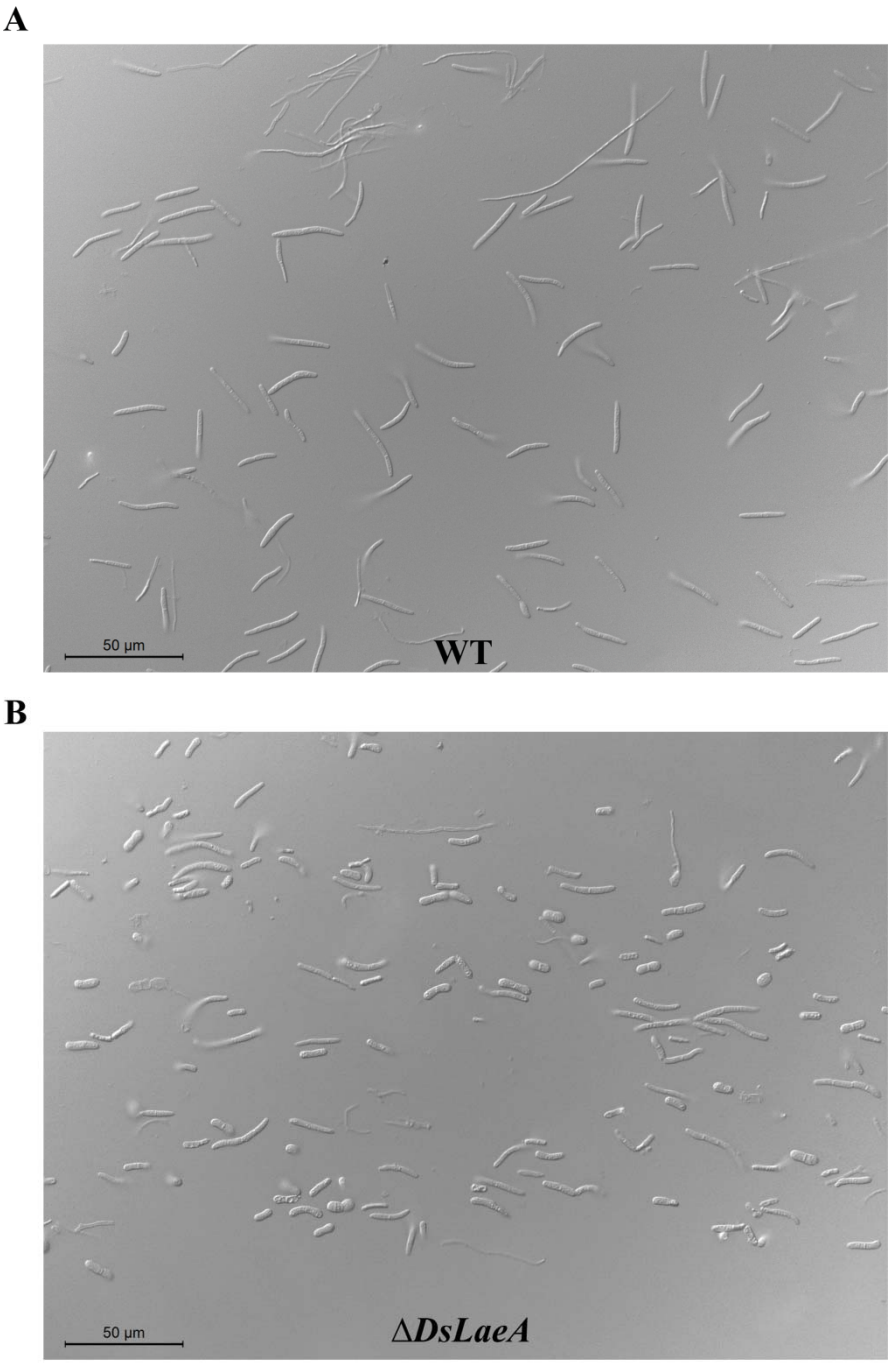
Appendix 8.7.3: Dothistromin production by *ΔDsLaeA* strain in DM medium.

Dothistromin production as estimated by HPLC by *DsLaeA* knockout strain (KO1 & KO2) compared to wild type under light (light bar) and dark (dark bar) conditions. The values are means and SD (n=3). Values significantly different to wildtype ($P \leq 0.05$) are marked with an asterisk.



Appendix 8.7.4: Expression of dothistromin biosynthesis pathway genes in *DsLaeA* mutants.

Normalized gene expression ratios for *DsLaeA* knockout (KO1 and KO2) and complemented (C1) strains, relative to wild type (WT); mean ± SE (n=3). Significant differences from WT ($p \leq 0.05$) are shown by an asterisk. Expression of *DsLaeA* and dothistromin genes *DsPksA*, *DsAflR, DsAflJ, DsVer1* and *DsVbsA* in cultures grown either in light (light bars) or dark (dark bars) conditions. No significant difference was observed between expression in light and dark conditions for any of the strains or gene shown.



Appendix 8.7.5: Light microscope image of Δ*DsLaeA* and wild type strain conidia

Conidiospores were obtained from *D. septosporum* wildtype (WT) and *ΔDsLaeA* strain (KO1) mycelium plated on PMMG media and incubated at 22^{o}C for ten days in light conditions.

Days after inoculation	Dothistromin conc per ml[a]	
	WT	***DsLaeA* KO**
3	4.44 ± 0.82	11.98 ± 4.57
5	28.49 ± 1.11	38.47 ± 9.06
7	82.49 ± 5.63	174.66 ± 3.47
9	105.06 ± 9.85	518.86 ± 9.732
12	193.27 ± 7.57	826.54 ± 169.38
15	165.33 ± 26.73	1087.19 ± 230.56
18	116.95 ± 8.48	602.37 ± 66.22

Appendix 8.7.6 Dothistromin production (ng/ml) in culture by wild type and *DsLaeA* KO strain.

[a] Dothistromin (mean ± SD); n =3.

GENES	DS29211	*DsAflJ*	*DsAflR*	*DsVbsA*	*DsPksA*	*DsVer1*
	0.407					
DsVer1		0.951	0.98	0.91	0.926	
	0.64					
DsPksA		0.856	0.983	**0.785**		
	0.452					
DsVbsA		**0.78**	0.852			
	0.379					
DsAflR		0.9826				
	0.51					
DsAflJ						
Ds29211						

Appendix 8.7.7: Pair wise comparison of gene expression in *ΔDsLaeA* strain Pearson correlation

Pearson product moment correlation coefficients between functionally characterised dothistromin biosynthesis or regulatory genes (*DsDotA, DsPksA DsVbsA DsAflR and DsAflJ*) are compared along with another gene (PID: 29211) not involved in dothistromin biosynthesis. Grey shading indicates statistically significant positive correlations ($p \leq 0.1$). Figures highlighted in yellow indicate p value slightly more than 0.1. Ds29211 did show positive correlations with other genes but none were statistically significant ($p > 0.3$).

Pearson correlation analysis was done using an online program at (http://department.obg.cuhk.edu.hk/researchsupport/PearsonCorrelation.asp).

8.7.8 Chromatin immuniоprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is a method for studying interactions between specific proteins and a genomic DNA region and can be used to determine the specific location of various histone modifications. The methodology of ChIP involves fixing of the cells/tissue with formaldehyde followed by shearing protein associated chromatin into smaller fragments then immunoprecipitating the DNA–protein complex using protein-specific antibody. After washing and centrifugation the isolated DNA–protein complexes are reverse crosslinked by heat treatment and the specifically enriched DNA segment is purified. The final product is analysed by a PCR, microarray or sequencing based method.

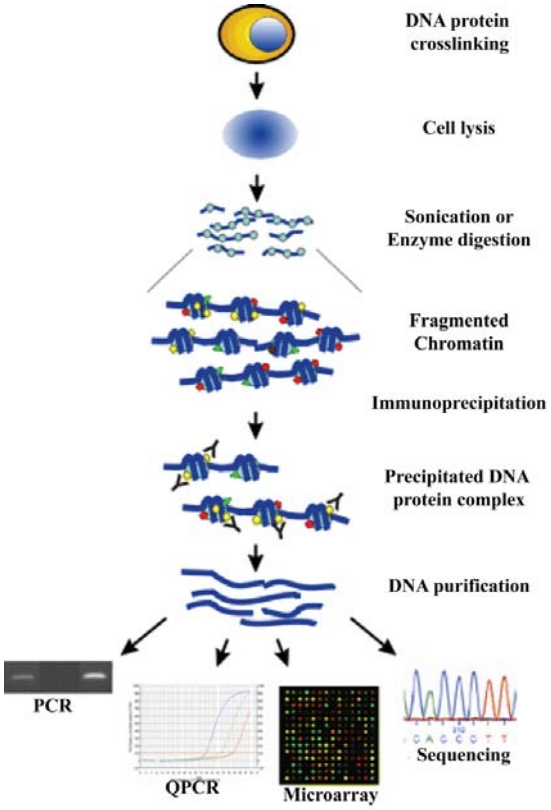


Figure 8.7.8. The chromatin immunoprecipitation (ChIP). (Adapted from Collas, 2010)
The steps involved in chromatin immunoprecipitation reaction and important alternatives used in final analysis of the immunoprecipitated DNA.

Appendix 8.7.9 ChIP analysis

To investigate whether early induction of dothistromin gene expression *in vitro* is associated with changes of the histone signature, chromatin immunoprecipitation followed by high throughput sequencing was performed on *D. septosporum* NZE10 that had been grown in low DB medium. Due to initial problems with standardisation of the ChIP protocol for *D. septosporum* and time constraints for thesis submission, sequencing results were not available so data are not presented here but the experimental procedure is included here for completion.

8.7.9.1 Chromatin Immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed following protocol given by Boedi et al (2012).

8.7.9.2 Cross-linking and chromatin preparation

Conidia (10^5 per ml) of D. septosporum NZE10 wildtype were inoculated in low DB medium at 22°C and mycelium harvested at day 4 (early) and day 9 (late). The time points for sampling were decided on the basis of the growth curve in chapter 6 (figure 6.12). At day 4 the culture is in the early exponential growth phase where the dothistromin starts to be synthesized and day 9 at early-mid exponential but in wildtype the rate of dothistromin production has strated to decline. This was reflected in gene expression as well. Mycelia were collected by filtration through a nappy liner, washed with PBS (Invitrogen) and suspended in 10 ml of PBS in a 125 ml flask. For cross linking of the chromatin 270 µl of 37% formaldehyde (final concentration of 1%) was added to the above flask which was kept in a shaker at 100 rpm for 15 min at room temperature. The cross linking was quenched by the addition of 500 µl 2.5 M glycine (USB chemicals, Cleveland, USA) in water and mycelia were collected by filtration followed by a wash with PBS and the frozen at -80°C

Frozen mycelium (0.1g x 2) was ground in liquid nitrogen with a mortar and pestle and then powdered mycelia suspended in 1 ml of pre chilled Chip DNA extraction buffer (Appendix: 8.2.6) containing Complete protease inhibitor (1 tablet per 10 ml extraction buffer) (Roche) and 5 mM of sodium butyrate per 10 ml. Shearing of chromatin was performed by sonication using a (Misonix Sonicator® S-4000 (Misonix Inc.) with micro tip in a alcohol bath at (amplitude 35%, 25 times 20s followed by cooling for 1 mins after each cycle. The sheared chromatin was centrifuged for 15 min at 15,685 x g at 4°C and supernatant transferred to a new tube and kept in an ice bath.

8.7.9.3 Chromatin pre clearing and immunoprecipitation

To pre-clear the samples, 20 µl of protein A agarose (Thermo fisher) was added and held on a rotary shaker for 1 hr at 4°C. The samples were then centrifuged at for 15,685 x g for 30 sec and supernatant was transferred to a fresh microcentrifuge tube. The concentration of the protein was determined by a Lowry assay (Lowry et al., 1950), and the sample was diluted to a concentration of 200 µg protein/mL in a final volume of 1.2 ml. A 200 µl aliquot was saved for an input control at -20°C. For immunoprecipitation of the target protein about 2 µg of each H3K9 (me3), H3K9(Ac), H4K12 (Ac) antibody (Epigentek,NY, USA) (Reyes-Dominguez et al., 2010; 2011; Nutzmann et al., 2011; Roze et al., 2007) with Pre immune rabbit serum (Igg) and H3c terminal antibody as control (Berger et al., 2008; Bok et al., 2009) was diluted at 1:100 ratio and added to each tube and incubated overnight at 4°C on a rotary shaker.

8.7.9.4 IP washes and elution

The following day after overnight incubation at 4°C 20 µl of protein A/G agarose beads **(**Thermo Fisher**)** was added and incubate for 2 hours at 4ºC on a rotator to allow antibody binding followed by centrifugation at 5000 rpm for 1 min to pellet beads and

the supernatant was discarded. About 1 ml of low salt buffer (Appendix 8.2.6) was added to each tube and incubated for 5 min at 4 degree on a rotating platform followed by centrifugation at 5000 rpm and discarding the supernatant. This step was repeated twice. Next wash was done using 1 ml high salt buffer (Appendix: 8.2.6) followed by centrifugation carried out as in the previous step. Two other washes and centrifugation with LiCl Wash buffer (Appendix: 8.2.6) and then TE buffer as done in the previous step. Chromatin was resuspended in 50 µl of resuspension buffer (Appendix: 8.2.6) and incubated at 65°C for 15 minutes and centrifuged at 13,000 rpm for 2 min. This step was repeated yielding 100 µl final volume of chromatin. For reverse cross linking of the chromatin 4 µl of NaCl (5M) was added to 100 µl chromatin and incubated at overnight at 65°C. 100 µl of previously saved input DNA was taken in a separate tube and similarly treated. To 100 µL samples of the resuspended chromatin, 1 µl of RNAse was added and incubated at 65°C for 30 mins, followed by addition of 2 µL EDTA (0.5 M), 4 µl 1 M Tris-HCl pH 6.5, and 2 µl Proteinase K (1 mg/ml, Sigma) and another incubation for 2 hrs at 45°C. DNA was purified from the samples phenol/chloroform extraction, precipitated by isopropyl alcohol, followed by 70% ethanol wash and finally dissolved in 50 µl. TE buffer.

Antibody	Day- 4 Rep	Day- 9 Rep	Reason for use	Reference
H3K9 Me3	R-1 R-2	R-1 R-2	Determine H3K9Me3 silent histone mark at dothistromin gene locus.	Smith et al., 2011; Chujo & Scott, 2014
H3K9 Ac	R-1 R-2	R-1 R-2	Active histone mark	Nutzmann et al., 2011
H4K12 Ac	R-1 R-2	R-1 R-2	Active histone mark	Roze et al., 2007; Belden et al., 2007
H3 c terminal	R-1 R-2	R-1 R-2	Help to quantify modifi cations of the histone relative to histone presence	Reyes-Dominguez etal., 2010; Berger et al., 2008
Rabbit Igg	R-1 R-2	R-1 R-2	negative control	Roze et al., 2007
Input DNA	R-1 R-2	R-1 R-2	negative control	Ahmed et al., 2013

Table 8.7.9.4 : List of ChIP purified DNA submitted for sequencing

ChIP was performed with three antibodies and three controls for two time points with duplicate samples (R-1, R2).

8.7.9 5 ChIP seq library preparation and run

ChIP Seq library preparation and sequencing was done at NZGL, Massey University, New Zealand. 2x75 base pair end read, Illuminia TruSeq ChIP Seq library of 24 samples was prepared and 2x Illumina MiSeq was run.

In general, histone acetylation and methylation are associated with transcriptional activation and silencing respectively in fungi (Boedi et al., 2012). In the present study ChIP seq was adopted to understand the mechanism that triggers dothistromin synthesis during early growth phase. A whole genome sequencing approach was taken to enable analysis of other genes across chromosome 12, providing a chromosomal level view of chromatin level regulation. This will allow us to see if other secondary metabolite genes show enrichment in the ChIP-seq result that may be useful to study the silent clusters which in turn may help to understand the organism better at the molecular level to develop a control strategy.

Appendix 8.8: Publication and conference presentation

Chettri, P., Calvo, A.M., Cary, J.W., Dhingra, S., Guo, Y., McDougal, R.L., Bradshaw, R.E., 2012. The *veA* gene of the pine needle pathogen *Dothistroma septosporum* regulates sporulation and secondary metabolism. Fungal Genetics and Biology 49, 141–151.

Chettri, P., Ehrlich, K.C., Cary, J.W., Collemare, J., Cox, M.P., Griffiths, S.A., Olson, M.A., de Wit, P.J.G.M., Bradshaw, R.E., 2013. Dothistromin genes at multiple separate loci are regulated by AflR. Fungal Genetics and Biology 51, 12–20.

De Wit, P.J.G.M., van der Burgt, A., Ökmen, B., Stergiopoulos, I., Abd-Elsalam, K.A., Aerts, A.L., Bahkali, A.H., Beenen, H.G., **Chettri, P**., Cox, M.P., Datema, E., de Vries, R.P., Dhillon, B., Ganley, A.R., Griffiths, S.A., Guo, Y., Hamelin, R.C., Henrissat, B., Kabir, M.S., Jashni, M.K., Kema, G., Klaubauf, S., Lapidus, A., Levasseur, A., Lindquist, E., Mehrabi, R., Ohm, R.A., Owen, T.J., Salamov, A., Schwelm, A., Schijlen, E., Sun, H., van den Burg, H.A., van Ham, R.C.H.J., Zhang, S., Goodwin, S.B., Grigoriev, I. V, Collemare, J., Bradshaw, R.E., 2012. The genomes of the fungal plant pathogens *Cladosporium fulvum* and *Dothistroma septosporum* reveal adaptation to different hosts and lifestyles but also signatures of common ancestry. PLoS genetics 8, e1003088.

Bradshaw, R.E., Slot, J.C., Moore, G.G., **Chettri, P.,** de Wit, P.J.G.M., Ehrlich, K.C., Ganley, A.R.D., Olson, M.A., Rokas, A., Carbone, I., Cox, M.P., 2013. Fragmentation of an aflatoxin-like gene cluster in a forest pathogen. The New phytologist 198, 525–35.

In preparation

Chettri, P., Ehrlich, K.C., Bradshaw, R.E., 2014. *Dothistroma septosporum* AflJ regulates biosynthesis of the aflatoxin-like toxin dothistromin

Conferences attended

Chettri, P., Ehrlich, KC., Cary, JW., Collemare, J., Cox, MP., Griffiths, SA.,Bradshaw, RE. 2013. The Fragmented Dothistromin Gene Cluster and its Regulation by AflR. 10th international congress of plant pathology, Beijing, China [Conference poster]

Pranav Chettri, Ana M. Calvo, Jeffrey W. Cary, Sourabh Dhingra, Yanan Guo, Rebecca L. McDougal, Rosie E. Bradshaw, 2011. Functional characterisation of the velvet gene in the pine needle pathogen *Dothistroma septosporum*. NZMS 2011 Annual Conference Palmerston North. [Conference Oral Presentation]

Pranav Chettri, Austen Ganley, Murray Cox and Rosie E. Bradshaw (2010). Dothistroma septosporum genome analysis. Poster presentation. Symposium on Fungal and Yeast Cell Biology: November 29-30, Massey University, Auckland, New Zealand. [Conference Poster]

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