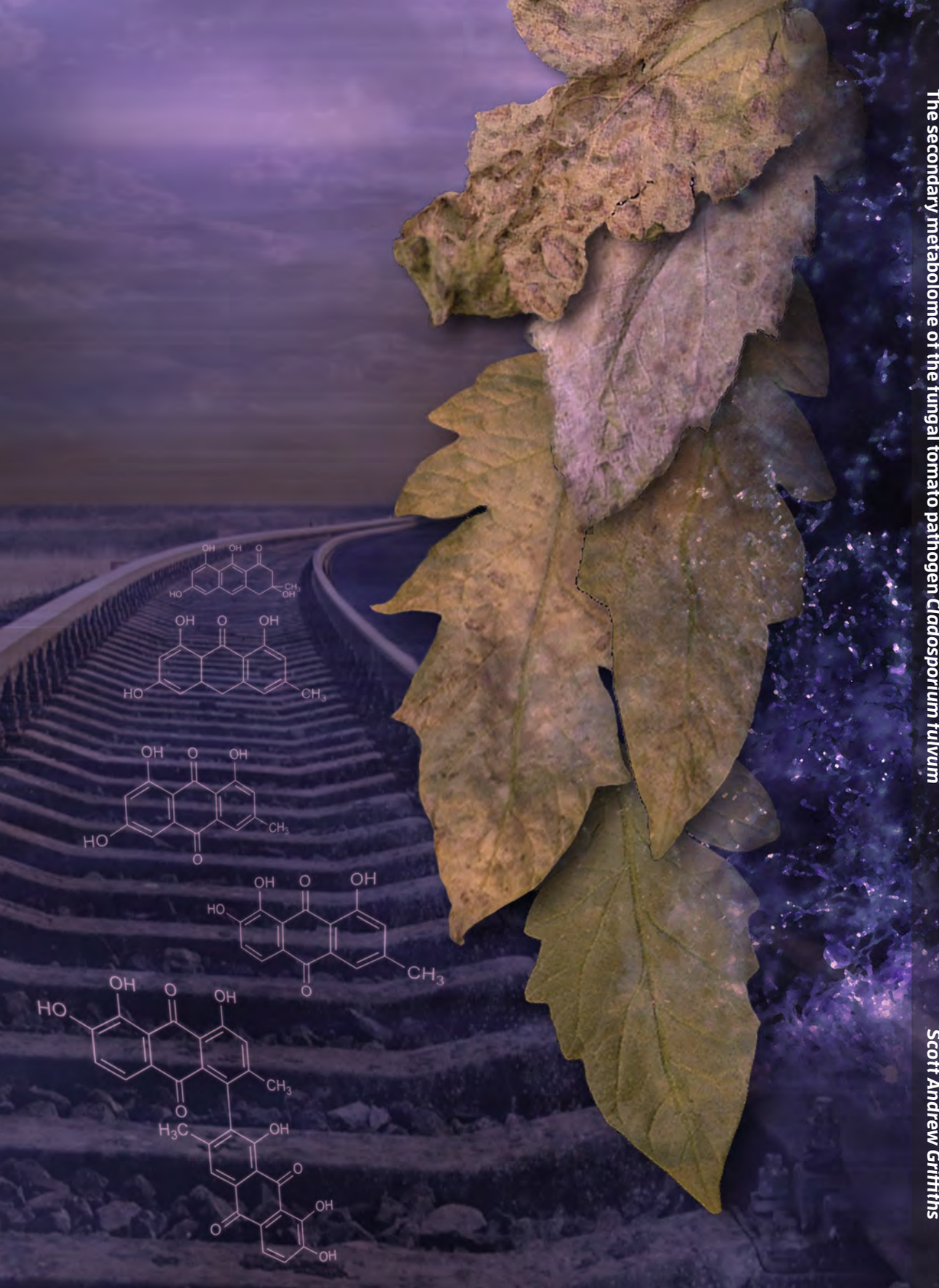

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Thesis committee
Promotors
Prof. Dr P.J.G.M. de Wit
Professor of Phytopathology
Wageningen University

Prof. Dr P.W. Crous
Professor of Evolutionary Phytopathology
Wageningen University

Co-promotor
Dr. Jérôme Collemare
IRHS-INRA, Angers, France

Other members
Prof. Dr R. Hall, Wageningen University
Prof. Dr R.A.L. Bovenberg, University of Groningen
Prof. Dr R.P. de Vries, University of Utrecht
Dr. T.A. van Beek, Wageningen University

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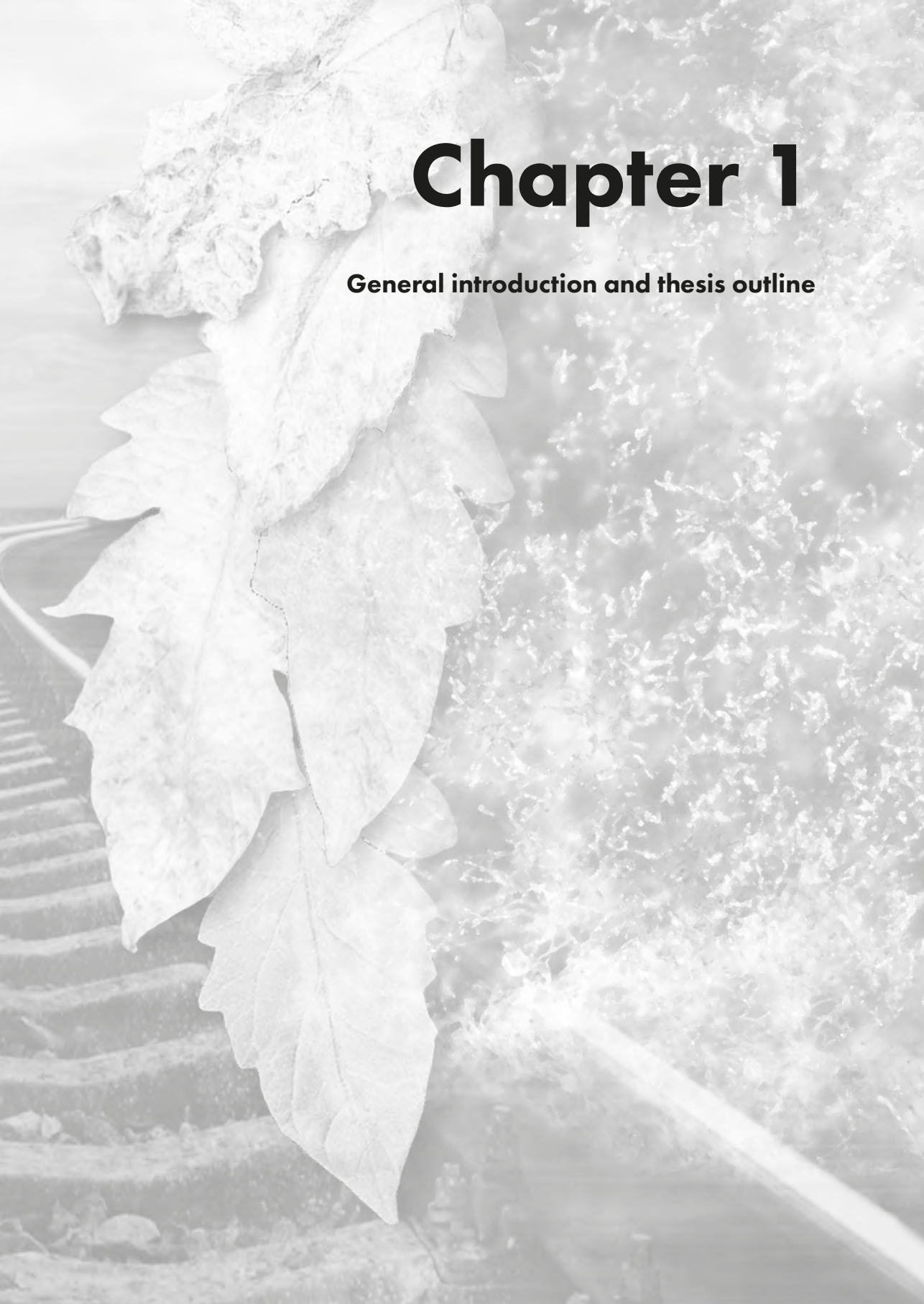

Chapter 1

General introduction and thesis outline

1.1 Fine chemicals, natural products and secondary metabolites

High value organic compounds that are produced in low quantities are termed fine chemicals (FCs), the building blocks of consumer-goods, vital medicines and agrochemicals. FCs were historically produced by using organic chemistry alone, a total synthesis approach that often requires non-renewable precursors and a large amount of energy. The potential repertoire of synthetic molecules at our disposal remains fundamentally limited by our knowledge of organic chemistry. Natural products (NPs) are substances produced by a living organism. Secondary metabolites (SMs) are very bioactive NPs that are predominantly biosynthesised by plants and filamentous microbes for reasons associated with competition, survival, and long-term prosperity within their respective ecological niches. Penicillin is an anti-bacterial fungal SM that not only revolutionised the treatment of infection, but forever altered our perception of natural organic compounds. Since penicillin, many thousands of SMs with diverse biological activities have been isolated from natural sources. SMs which kill or inhibit the growth of living cells have arguably become the most important, as these have provided us with medicinal drugs and agricultural pesticides.

1.1.1 Medicinal secondary metabolites and semi-synthetic derivatives

Medicinal SMs include anti-infective, anti-cancer, anti-cholesterolemic, anti-parasitic, and immunosuppressive drugs (Table 1). Promising SMs are often modified using organic chemistry to yield semi-synthetic derivatives with altered biological activities. Derivatives of natural β-lactam penicillins include the penems, carbapenems, cephems, carboxypenicillins and monobactams, each altered in their spectrum of anti-microbial activity. Daunorubicin is an SM produced by *Streptomyces peucetius* and is the founding member of the anthracycline class of anti-cancer antibiotics (Stutzman-Engwall *et al.*, 1992). Although daunorubicin was used to treat different types of cancer, particularly leukemias and lymphomas, its usage was limited by its side-effects, including cumulative irreversible cardiotoxicity (Bristow *et al.*, 1978). Semi-synthetic derivatives of daunorubicin include doxorubicin, epirubicin and idarubicin, compounds that are reduced in cardiotoxicity and altered in their spectrum of anti-cancer activity.

Illustrating the power of combinatorial biosynthesis, *S. peucetius* was genetically engineered to produce epirubicin (Madduri *et al.,* 1998). Cholesterol lowering statins are the most commonly prescribed and lucrative family of drugs in history, with sales in the USA alone totalling an estimated $18.7 billion in 2005 (Feher *et al.*, 2011). The first commercially available statin was lovastatin, an SM from *Aspergillus terreus*, which became the starting material for the semi-synthesis of simvastatin. These examples illustrate how the costs associated with derivatization of natural SMs are offset by the clinical and commercial benefits.

Table 1. Notable commercial secondary metabolites from bacteria and fungi.[1]

Biological activity	Secondary metabolite	Producer
Anti-bacterial	Abyssomycin	*Verrucosispora sp*
	Chloramphenicol	*Streptomyces venezuelae*
	Erythromycin	*Saccharopolyspora erythraea*
	Gentamycin	*Micromonospora sp*
	Kanamycin	*Streptomyces kanamyceticus*
	Penicillin	*Penicillium chrysogenum*
	Streptomycin	*Streptomyces griseus*
	Tetracycline	*Streptomyces rimosus*
	Rifampicin	*Amycolatopsis mediterranei*
	Vancomycin	*Amycolatopsis orientalis*
Anti-cancer	Actinomycin D	*Streptomyces antibioticus*
	Bleomycin	*Streptomyces verticillus*
	Daunorubicin	*Streptomyces peucetius*
	Mitomycin D	*Streptomyces levendulae*
	Salinosporamide	*Salinosporum sp*
Anti-cholesterolemic	Lovastatin	*Aspergillus terreus*
	Pravastatin	*Streptomyces carbophilus*
Anti-fungal	Amphotericin B	*Streptomyces nodosus*
	Candicidin	*Streptomyces griseus*
	Filipin	*Streptomyces filipinensis*
	Hamycin	*Streptomyces pimprina*
	Natamycin	*Streptomyces natalensis*
	Nystatin	*Streptomyces noursei*
Anti-parasite	Avermectin	*Streptomyces avermitilis*
	Milbemycin	*Streptomyces hygroscopicus*
Growth promoter	Ardacin	*Kibdelosporangium aridum*
	Avoparcin	*Amycolatopsis orientalis*
	Bacitracin	*Bacillus subtilis*
	Bambermycin	*Streptomyces bambergiensis*
	Efrotomycin	*Amycolatopsis lactamdurans*
	Monensin	*Streptomyces cinnamonensis*
	Salinomycin	*Streptomyces albus*
	Spiramycin	*Streptomyces ambofaciens*
	Tylosin	*Streptomyces fradiae*
	Virginiamycin	*Streptomyces virginiae*
Herbicide	Bialophus	*Streptomyces fradiae*
Immunosuppressive	Cyclosporin	*Tolypocladium inflatum*
	Rapamycin	*Streptomyces hygroscopicus*
	Tacrolimus	*Streptomyces sp*

[1]The table was adapted from (Balagurunathan & Radhakrishnan, 2010) and expanded.

1.1.2 Secondary metabolites as growth promoters and pesticides

Anti-microbial SMs are extensively used during the rearing of livestock for human consumption, in order to treat infectious diseases and to promote growth (Table 1). Global food production is heavily dependent on the wide-scale use of highly toxic organophosphate and carbamate pesticides. The neonicotinoids are a family of synthetic insecticides that were inspired by the alkaloid nicotine, an SM produced by species of the nightshade (*Solanaceae*) family of plants. Compared to traditional pesticides, neonicotinoids are considerably less toxic towards birds and mammals (Tomizawa & Casida, 2003). Before the recent moratorium on the use of certain neonicotinoids by the European Union and United States regulatory authorities due to their possible role in honey-bee colony collapse disorder (Blacquière *et al.*, 2012), this family of products accounted for 80% of seed treatments and a quarter of global insecticide sales. Similarly, the pyrethroids are a family of successful synthetic and natural insecticides that are inspired and derived from pyrethrins, SMs that are produced by the flowers of pyrethrums (Elliott *et al.*, 1978).

1.2 Biosynthesis of secondary metabolites in fungi

Fungal SMs are classified into different groups based on the enzymes and precursors involved in their biosynthesis. The main groups are polyketides, non-ribosomal peptides, polyketide-peptide hybrids, terpenes and alkaloids, the products of polyketide synthases (PKSs), non-ribosomal peptide synthases (NRPSs), hybrid PKS-NRPSs, terpene cyclases (TCs) and dimethylallyl tryptophan synthetases (DMATs), respectively (Keller *et al.*, 2005). However, these core enzymes only synthesise the first ("raw") compound in their respective biosynthetic pathways. Additional enzymes such as hydrolases, isomerases, oxidases, reductases and transferases are usually involved in transforming raw metabolites into more structurally elaborate SMs. Such enzymes are named decorating, tailoring, or accessory enzymes and they are encoded by discreet genes. Core and decorating genes belonging to the same biosynthetic pathway are co-regulated and often co-localised to form biosynthetic gene clusters (Keller & Hohn, 1997; Keller *et al.*, 2005; Nierman *et al.*, 2005). These clusters may also contain pathway-specific regulators and co-regulators that control only the gene cluster in which they reside. Gene clusters without a pathway-specific activator can also be controlled by global regulators encoded by a gene located elsewhere on the genome. Genes encoding transporters might also be present, such as efflux pumps that facilitate the export of an SM destined for secretion. Fungi produce mainly polyketides and non-ribosomal peptides, which is reflected by the high number of *PKS* and *NRPS* genes encoded in their genomes (Collemare *et al.*, 2008). Genomic analyses have shown that fungi are an untapped reservoir of SMs (Machida *et al.*, 2005; Amaike & Keller, 2011; de Wit *et al.*, 2012). However, even fungi with very large SM gene catalogues do not present an equivalent chemical diversity. The majority of fungal SM biosynthetic genes are silent under standard laboratory conditions.

SM biosynthesis in Ascomycete fungi is tightly controlled by a complex network of regulatory proteins that respond to specific environmental signals. SM production responds to many culture parameters, including carbon source (Espeso & Peñalva, 1992), nitrogen source (Cary *et al.*, 2006), light/dark (Calvo *et al.*, 2004; Blumenstein *et al.*, 2005; Bayram *et al.*, 2008; Atoui *et al.*, 2010) and pH (Ehrlich *et al.*, 1999). SM production is sometimes linked to behavioural responses such as morphological development and tissue differentiation (Bayram & Braus, 2012). Global regulators that link SM production to colony development include chromatin modifying enzymes, such as histone methyltransferases (Bok & Keller, 2004; Connolly *et al.*, 2013) and histone deacetylases (Lee *et al.*, 2009). SMs can also be produced in response to physical or chemical contact with other organisms (Schrader *et al.*, 2000; Kurosawa *et al.*, 2008; König *et al.*, 2013; Chagas *et al.*, 2013; Netzker *et al.*, 2015).

1.2.1 Activating and characterizing biosynthetic pathways

Many strategies have been developed to try and activate silent SM gene clusters (Brakhage & Schroeckh, 2011). In addition to strategies based on culture and co-culture, chemical inhibitors have also been used to alter SM production by inhibiting enzymes involved in the regulation of gene transcription. Examples include the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) that promotes the relaxed form of chromatin through histone hyperacetylation (Trojer *et al.*, 2003), and the nucleoside analogue 5-azacytidine that promotes the loss of methylation marks that normally silence a gene (Williams *et al.*, 2008). The over-expression of local, pathway-specific regulators has been successful in activating the production of SMs in *A. nidulans* (Bergmann *et al.*, 2007; Chiang *et al.*, 2010) and *A. flavus* (Cary *et al.*, 2015). Deletion or over-expression of global regulators has profoundly affected the SM profile and morphological development in many *Ascomycetes* (Yu & Keller, 2005; Jain & Keller, 2013). Biosynthetic genes can also be cloned and expressed heterologously in production hosts such as *Aspergillus oryzae* strain M-2-3 (Pahirulzaman *et al.*, 2012). Although this fungal species contains a high number of SM genes (Machida *et al.*, 2005), strain M-2-3 has a silent SM profile and a proven track record in expressing heterologous SM genes (Halo *et al.*, 2008; Awakawa *et al.*, 2009; Pahirulzaman *et al.*, 2012).

When dealing with an active SM gene cluster that yields a detectable product, gene deletion studies are a powerful method for assigning functions to genes and enzymes. Typically, the manipulation of genes involved in an SM biosynthetic pathway will alter the SM profile of the resulting mutant compared to the parental strain. Loss of an essential activator or early biosynthetic enzyme will abolish production of a given SM entirely, whereas the loss of a later acting biosynthetic enzyme results in the accumulation of intermediate compounds. Enzyme function(s) can be determined by sequentially deleting genes from a biosynthetic gene cluster and identifying the metabolites that accumulate in each mutant. This approach has been successfully applied to several large fungal biosynthetic pathways, including aflatoxin (Yu *et al.*, 2004), sterigmatocystin (Brown *et al.*, 1996) and monodictyphenone (Chiang et al., 2010).

1.3 The natural roles of SM production

SMs are produced for reasons associated with competition, survival and long-term prosperity (Demain & Fang, 2000). When resources are finite, the prosperity of any given species is typically enhanced by the antagonism or death of competitors, grazers and parasites. The enormous diversity of SMs in existence is thought to result from adaptations of the respective producers to different ecological niches (Osbourn, 2010). Assigning biological activities to unknown compounds is difficult and often serendipitous in the absence of high-throughput screening. Understanding the nature of SM production in the ecological niche(s) of the producing organism can guide the determination of these activities.

1.3.1 Defence of an ecological niche from competitors

The majority of our commercial anti-infective and anti-neoplastic chemotherapy drugs originate from *Streptomycetes* (Table 1), an order of free living filamentous bacteria that are ubiquitous in terrestrial soils. Although soils are known to support an enormous quantity of genetically diverse micro-organisms, as growth matrices, most are nutrient poor and subject to discontinuous nutrient availability. Competition for resources is often assumed to have driven the diversification of antibiotics in the soil environment. This interpretation is difficult to prove definitively, but is largely based on the induction of SMs observed during *in vitro* co-culture of *Streptomyces* strains and competitors (Wiener, 1996).

1.3.2 Grazer deterrence

Epichloë festucae, a common symbiont of temperate grasses like *Festuca, Lolium* and *Koeleria spp*. *E. festucae* is known to enhance the survival of host plants through the production of alkaloids with anti-insect (peramine and lolines) and anti-vertebrate (lolitrem B and ergovaline) activity (Schardl, 2001). In another study, *Cladosporium phlei,* pathogen of Timothy grass, was inhibited *in vitro* by p-hydroxybenzaldehyde, a compound produced by an endophyte of Timothy grass, *E. typhina* (Seto *et al.*, 2005). Cyclic peptides, diketopiperadines, were also present in the *E. typhina* culture filtrate. In a clear example of co-evolution, these peptides also stimulated the production of phleichrome in *C. phlei*, a photoactive SM that inhibited the *in vitro* growth of *E. typhina*.

1.3.3 Pathogenicity and virulence factors

Some plant pathogenic fungi deploy SMs as virulence factors during colonization of their respective hosts. Host-specific toxins (HSTs) are structurally diverse pathogen effectors that induce toxicity and promote disease only in the host species expressing a cognate susceptibility gene (Friesen *et al.*, 2008). Examples include the non-ribosomal cyclic tetrapeptide HC toxin that is a determinant of host-specificity and virulence by *Cochliobolus carbonum* during infection of maize (Comstock, 1973). T-toxin is a polyketide and virulence determinant produced by

C. heterostrophus race T, also during infection of maize (Yang, 1996). HSTs determine the host-specificity and virulence of many pathotypes of *Alternaria* species (Nishimura & Kohmoto, 1983). Non host-specific toxins are toxic towards a broader range of hosts without the need for a host susceptibility gene. For example, perylenequinones such as elsinochrome and cercosporin are produced by *Elsinoë* and *Cercospora* species, respectively, during colonisation of their host plants (Lousberg *et al.*, 1969; Gallagher & Hodges, 1972; Daub & Hangarter, 1983). Perylenequinones are photosensitizers that generate reactive oxygen species (ROS) in response to light, damaging host-tissue and promote the infection process. Dihydroxynapthalene (DHN) melanin is an SM produced inside the cell wall of appressoria in *Magnaporthe oryzae* during infection of rice, the loss of which renders the fungus non-pathogenic (Chumley, 1990). Tricyclazole is a commercial pesticide that is used to prevent rice blast by inhibiting 1,3,8-trihydroxynapthalene reductase, an essential enzyme involved in DHN melanin production (Andersson *et al.*, 1996). SMs are clearly important to plant pathogens and can be targeted for disease control.

1.4 A model host-parasite system: *Solanum lycopersicum - Cladosporium fulvum*

It is particularly important to study plant-pathogen systems, as organisms which infect commercial crops and are therefore our direct competitors. For many years, the biotrophic plant pathogen *C. fulvum* has been studied for its rich source of proteinaceous effectors that can facilitate the infection of its host, tomato. However, these effectors are also the molecules that are recognized by disease resistance proteins, rendering the producing fungal strain avirulent on tomato plants that encode them (Stergiopoulos & de Wit, 2009). Thus the effectors have facilitated the discovery of new *Cladosporium fulvum* (*Cf*) disease resistance genes, which has negated the need for fungicides against this pathogen (de Wit *et al.*, 2009).

1.4.1 The high secondary metabolite potential of *C. fulvum*

Far fewer SM genes are encoded in the genomes of biotrophs than necrotrophic and hemi-biotrophic fungi (Collemare *et al.*, 2008). Before the genome of *C. fulvum* was obtained and analysed, its capacity to produce SMs was considered to be negligible. Indeed, the anthraquinone cladofulvin is the sole detectable SM produced by this fungus during growth on artificial media (Agosti *et al.*, 1962). The genome of *C. fulvum* contains 23 predicted core SM genes; 10 PKSs, 10 NRPSs, 2 PKS-NRPS hybrids and one DMATS (de Wit *et al.*, 2012). From these predicted core genes, two were truncated (*Pks4* and *Nps1*) and five were pseudogenized (*Pks9, Hps2, Nps5, Nps7* and *Nps10*), suggesting that *C. fulvum* can produce at least 13 SMs in addition to cladofulvin. Expression of these SM genes was determined by EST and RNA-seq analysis of the fungus grown *in planta* and *in vitro*. Aside from the *PKS6* gene implicated in cladofulvin biosynthesis, no other SM gene was strongly expressed under any tested condition.

1.4.2 The bi-anthraquinone cladofulvin

Anthraquinones form a ubiquitous family of bioactive compounds that are mostly produced by fungi, and plants used in traditional medicine. Anthraquinones contain an aromatic core that serves as a scaffold for the attachment of diverse functional groups. This results in a wide variety of molecules with distinct biological and biochemical characteristics. This diversity is further increased by their assembly into homo- and hetero-dimers, with activities that can be similar or distinct from their respective monomeric components (Zhao *et al.*, 2005; Zheng *et al.*, 2012; Xia *et al.*, 2014; Hussain *et al.*, 2015). Emodin is the most intensively studied anthraquinone due to its ubiquity and diverse biological activities. Emodin has been detected in at least 17 plant families and 94 species (Izhaki, 2002) including the Chinese herb families *Rheum* and *Polygonam* (Srinivas *et al.*, 2007). Emodin has potent anti-cancer (Srinivas *et al.*, 2007), anti-diabetic (Yang *et al.*, 2007), anti-infective (Andersen *et al.*, 1991; Alves *et al.*, 2004; Kong *et al.*, 2009) and anti-inflammatory (Chang *et al.*, 1996) qualities. It is also cathartic (Ali *et al.*, 2004), cardio- (Wu *et al.*, 2007), hepato- (Lin *et al.*, 1996) and neuro- (Liu *et al.*, 2010) protective. Nataloe-emodin is an isomer of emodin that also is active against many human cancer cell-lines, including lung large cell carcinoma, amelanotic melanoma, prostate carcinoma, breast adenocarcinoma, colon adenocarcinoma and chronic myelogenous leukaemia (Diaz *et al.*, 2004; Aponte *et al.*, 2008). As a homodimer of nataloe-emodin, cladofulvin might present similar biological activities.

1.5 Project aims and thesis outline

SM genes are typically deployed by hemi-biotrophic and necrotrophic fungi in order to kill host tissue to facilitate colonization. They do not feature so prominently in the genomes of biotrophic fungi sequenced so far. The genome of *C. fulvum* contains many genes related to SM biosynthesis, a surprising finding given its biotrophic lifestyle. Expression analysis revealed that the majority of core SM genes were poorly expressed or silent *in planta* and *in vivo*, suggesting that strong repression is an alternative to gene reduction for establishing biotrophy. Further analysis of the SM gene catalogue in this fungus was necessary in order to predict the possible SMs that could be produced. The next aim was to increase the chemical diversity of this fungus for the purpose of compound discovery. Genes homologous to Ascomycete-specific global regulators in the *C. fulvum* genome presented an obvious target for manipulation. Anthraquinones are currently enjoying a renaissance period due to their numerous medicinal biological activities and presence in many traditional herbs. Few of their respective biosynthetic pathways are known. Another aim was to elucidate the cladofulvin biosynthetic pathway and discover the enzyme(s) involved in the dimerization of nataloe-emodin. Identifying such an enzyme should accelerate the discovery of additional enzymes with different substrate specificities. Finally, we aimed to address the relevance of cladofulvin to the infection of tomato and to the maintenance of biotrophy.

Thesis outline

Chapter 2: In this chapter, the SM genes identified during the analysis of the *Cladosporium fulvum* genome were analysed in further detail using a combined phylogenetic and comparative genomic approach. Each locus containing a core SM gene was inspected for other genes linked to SM production, such as decorating enzymes or regulators. Products of these SM genes or gene clusters were predicted by their similarity to homologs that have been functionally characterized in other fungi. The expression of each core SM gene was determined during growth *in planta* and under several conditions *in vitro*. Based on the strength of their expression, the relevance of each core SM gene or respective biosynthetic cluster to the lifestyle of the fungus was discussed.

Chapter 3: In this chapter, an attempt to increase the chemical diversity of *C. fulvum* using a combination of targeted genetic mutations and altered culture conditions is detailed. Previously identified global regulators of SM production were deleted or over-expressed. Each strain was grown on a variety of carbons sources and subjected to metabolic profiling by ultra violet-high performance liquid chromatography (UV-HPLC). The interplay between global regulators, SM production and fungal development is discussed.

Chapter 4: In this chapter the core PKS responsible for cladofulvin production was confirmed. The cladofulvin biosynthetic pathway was elucidated using a combination of gene deletion and heterologous expression of early cladofulvin biosynthetic genes in *Aspergillus oryzae* M-2-3. The cytotoxicity of cladofulvin and metabolic precursors was tested against a catalogue of immortalized human cell-lines. The effect of dimerization on the biological activity of anthraquinones is discussed.

Chapter 5: In this chapter the relevance of cladofulvin to parasitism and to the success of the fungus *ex-planta* was explored. Deletion mutants unable to produce cladofulvin were tested for their resilience to environmental stresses and their ability to grow on tomato. The local regulator inside the cladofulvin gene cluster was manipulated to activate cladofulvin biosynthesis during the ordinarily biotrophic phase of growth. The importance of SM down-regulation to biotrophic parasites is discussed.

Chapter 6: In this chapter the most important findings of this thesis are considered and directions for future research are suggested.

References

Agosti G, Birkinshaw JH, Chaplen P. 1962. Studies in the biochemistry of micro-organisms. 112. Anthraquinone pigments of strains of Cladosporium fulvum Cooke. *The Biochemical Journal* **85**: 528-30.

Ali S, Watson MS, Osborne RH. 2004. The stimulant cathartic, emodin, contracts the rat isolated ileum by triggering release of endogenous acetylcholine. *Autonomic and Autacoid Pharmacology* **24**: 103-5.

Alves DS, Pérez-Fons L, Estepa A, Micol V. 2004. Membrane-related effects underlying the biological activity of the anthraquinones emodin and barbaloin. *Biochemical Pharmacology* **68**: 549-61

Amaike S, Keller NP. 2011. Aspergillus flavus. *Annual Review of Phytopathology* **49**: 107-33.

Andersen DO, Weber ND, Wood SG, Hughes BG, Murray BK, North JA. 1991. In vitro virucidal activity of selected anthraquinones and anthraquinone derivatives. *Antiviral Research* **16**: 185-96.

Andersson A, Jordan D, Schneider G, Lindqvist Y. 1996. Crystal structure of the ternary complex of 1,3,8-trihydroxynaphthalene reductase from Magnaporthe grisea with NADPH and an active-site inhibitor. *Structure* **4**: 1161-70.

Aponte JC, Vaisberg AJ, Rojas R, Caviedes L, Lewis WH, Lamas G, Sarasara C, Gilman RH, Hammond GB. 2008. Isolation of Cytotoxic Metabolites from Targeted Peruvian Amazonian Medicinal Plants. *Journal of Natural Products* **71**: 102-105.

Atoui A, Kastner C, Larey CM, Thokala R, Etxebeste O, Espeso EA, Fischer R, Calvo AM. 2010. Cross-talk between light and glucose regulation controls toxin production and morphogenesis in Aspergillus nidulans. *Fungal Genetics and Biology* **47**: 962-72.

Awakawa T, Yokota K, Funa N, Doi F, Mori N, Watanabe H, Horinouchi S. 2009. Physically discrete beta-lactamase-type thioesterase catalyzes product release in atrochrysone synthesis by iterative type I polyketide synthase. *Chemistry & Biology* **16**: 613-23.

Balagurunathan R, Radhakrishnan M. 2010. *Industrial Exploitation of Microorganisms*. IK Publisher Co., New Dehli.

Bayram O, Braus GH. 2012. Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiology Reviews* **36**: 1-24.

Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, Braus-Stromeyer S, Kwon N, Keller NP, Yu J, *et al.* 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* **320**: 1504-6.

Bergmann S, Schümann J, Scherlach K, Lange C, Brakhage AA, Hertweck C. 2007. Genomics-driven discovery of PKS-NRPS hybrid metabolites from Aspergillus nidulans. *Nature Chemical Biology* **3**: 213-7.

Blacquière T, Smagghe G, van Gestel CAM, Mommaerts V. 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21**: 973-92.

Blumenstein A, Vienken K, Tasler R, Purschwitz J, Veith D, Frankenberg-Dinkel N, Fischer R. 2005. The Aspergillus nidulans phytochrome FphA represses sexual development in red light. *Current Biology* **15**: 1833-8.

Bok JW, Keller NP. 2004. LaeA, a regulator of secondary metabolism in Aspergillus spp. *Eukaryotic Cell* **3**: 527-35.

Brakhage AA, Schroeckh V. 2011. Fungal secondary metabolites - strategies to activate silent gene clusters. *Fungal Genetics and Biology* **48**: 15-22.

Bristow MR, Thompson PD, Martin RP, Mason JW, Billingham ME, Harrison DC. 1978. Early anthracycline cardiotoxicity. *The American Journal of Medicine* **65**: 823-32.

Brown DW, Yu JH, Kelkar HS, Fernandes M, Nesbitt TC, Keller NP, Adams TH, Leonard TJ. 1996. Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in Aspergillus nidulans. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 1418-22.

Calvo AM, Bok J, Brooks W, Keller NP. 2004. veA is required for toxin and sclerotial production in Aspergillus parasiticus. *Applied and Environmental Microbiology* **70**: 4733-9.

Cary JW, Ehrlich KC, Kale SP, Calvo AM, Bhatnagar D, Cleveland TE. 2006. Regulatory elements in aflatoxin biosynthesis. *Mycotoxin Research* **22**: 105-9.

Cary JW, Uka V, Han Z, Buyst D, Harris-Coward PY, Ehrlich KC, Wei Q, Bhatnagar D, Dowd PF, Martens SL, *et al.* 2015. An Aspergillus flavus secondary metabolic gene cluster containing a hybrid PKS–NRPS is necessary for synthesis of the 2-pyridones, leporins. *Fungal Genetics and Biology* **81**: 88-97.

Chagas FO, Dias LG, Pupo MT. 2013. A mixed culture of endophytic fungi increases production of antifungal polyketides. *Journal of Chemical Ecology* **39**: 1335-42.

Chang CH, Lin CC, Yang JJ, Namba T, Hattori M. 1996. Anti-inflammatory effects of emodin from ventilago leiocarpa. *The American Journal of Chinese Medicine* **24**: 139-42.

Chiang Y, Szewczyk E, Davidson AD, Entwistle R, Keller NP, Wang CCC, Oakley BR. 2010. Characterization of the Aspergillus nidulans monodictyphenone gene cluster. *Applied and Environmental Microbiology* **76**: 2067-74.

Chumley FG. 1990. Genetic Analysis of Melanin-Deficient, Nonpathogenic Mutants of Magnaporthe grisea. *Molecular Plant-Microbe Interactions* **3**: 135-143.

Collemare J, Billard A, Böhnert HU, Lebrun M. 2008. Biosynthesis of secondary metabolites in the rice blast fungus Magnaporthe grisea: the role of hybrid PKS-NRPS in pathogenicity. *Mycological Research* **112**: 207-15.

Comstock JC. 1973. Role of Host-Selective Toxin in Colonization of Corn Leaves by Helminthosporium carbonum. *Phytopathology* **63**: 24-29.

Connolly LR, Smith KM, Freitag M. 2013. The Fusarium graminearum histone H3 K27 methyltransferase KMT6 regulates development and expression of secondary metabolite gene clusters. *PLoS Genetics* **9**: e1003916.

Daub ME, Hangarter RP. 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. *Plant Physiology* ***73***, 855-7.

Demain AL, Fang A. 2000. The natural functions of secondary metabolites. *Advances in Biochemical Engineering/ Biotechnology* **69**: 1-39.

Diaz F, Chai H, Mi Q, Su B, Vigo JS, Graham JG, Cabieses F, Farnsworth NR, Cordell GA, Pezzuto JM, *et al.* 2004. Anthrone and oxanthrone C-glycosides from Picramnia latifolia collected in Peru. *Journal of Natural Products* **67**: 352-6.

Ehrlich KC, Cary JW, Montalbano BG. 1999. Characterization of the promoter for the gene encoding the aflatoxin biosynthetic pathway regulatory protein AFLR. *Biochimica et Biophysica Acta* **1444**: 412-7.

Elliott M, Janes NF, Potter C. 1978. The future of pyrethroids in insect control. *Annual Review of Entomology* **23**: 443-469.

Espeso EA, Peñalva MA. 1992. Carbon catabolite repression can account for the temporal pattern of expression of a penicillin biosynthetic gene in Aspergillus nidulans. *Molecular Microbiology* **6**: 1457-65.

Feher A, Pusch G, Koltai K, Tibold A, Gasztonyi B, Szapary L, Feher G. 2011. Statin therapy in the primary and the secondary prevention of ischaemic cerebrovascular diseases. *International Journal of Cardiology* **148**: 131-8.

Friesen TL, Faris JD, Solomon PS, Oliver RP. 2008. Host-specific toxins: effectors of necrotrophic pathogenicity. *Cellular Microbiology* **10**: 1421-8.

Gallagher R, Hodges R. 1972. The Chemistry of Dothistromin, a Difuronanthraquinone From Dothistroma pini. *Australian Journal of Chemistry* **25**: 2399-2407.

Halo LM, Marshall JW, Yakasai AA, Song Z, Butts CP, Crump MP, Heneghan M, Bailey AM, Simpson TJ, Lazarus CM, *et al.* 2008. Authentic heterologous expression of the tenellin iterative polyketide synthase nonribosomal peptide synthetase requires coexpression with an enoyl reductase. *ChemBioChem* **9**: 585-94.

Hussain H, Al-Harrasi A, Green IR, Hassan Z, Ahmed I. 2015. *Recent Advances in the Chemistry and Biology of Natural Dimeric Quinones*. *Studies in Natural Products Chemistry* **46**: 1-567.

Izhaki I. 2002. Emodin - a secondary metabolite with multiple ecological functions in higher plants. *New Phytologist* **155**: 205-217.

Jain S, Keller N. 2013. Insights to fungal biology through LaeA sleuthing. *Fungal Biology Reviews* **27**: 51-59.

Keller NP, Hohn TM. 1997. Metabolic Pathway Gene Clusters in Filamentous Fungi. *Fungal Genetics and Biology* **21**: 17-29.

Keller NP, Turner G, Bennett JW. 2005. Fungal secondary metabolism - from biochemistry to genomics. *Nature Reviews Microbiology* **3**: 937-47.

Kong WJ, Wang JB, Jin C, Zhao YL, Dai CM, Xiao XH, Li ZL. 2009. Effect of emodin on Candida albicans growth investigated by microcalorimetry combined with chemometric analysis. *Applied Microbiology and Biotechnology* **83**: 1183-90.

Kurosawa K, Ghiviriga I, Sambandan TG, Lessard PA, Barbara JE, Rha C, Sinskey AJ. 2008. Rhodostreptomycins, antibiotics biosynthesized following horizontal gene transfer from Streptomyces padanus to Rhodococcus fascians. *Journal of the American Chemical Society* **130**: 1126-7.

König CC, Scherlach K, Schroeckh V, Horn F, Nietzsche S, Brakhage AA, Hertweck C. 2013. Bacterium induces cryptic meroterpenoid pathway in the pathogenic fungus Aspergillus fumigatus. *ChemBioChem* **14**: 938-42.

Lee I, Oh J, Shwab EK, Dagenais TRT, Andes D, Keller NP. 2009. HdaA, a class 2 histone deacetylase of Aspergillus fumigatus, affects germination and secondary metabolite production. *Fungal Genetics and Biology* **46**: 782-90.

Lin CC, Chang CH, Yang JJ, Namba T, Hattori M. 1996. Hepatoprotective effects of emodin from Ventilago leiocarpa. *Journal of Ethnopharmacology* **52**: 107-11.

Liu T, Jin H, Sun Q, Xu J, Hu H. 2010. Neuroprotective effects of emodin in rat cortical neurons against beta-amyloid-induced neurotoxicity. *Brain Research* **1347**: 149-60.

Lousberg RJJC, Salemink CA, Weiss U, Batterham TJ. 1969. Pigments of Elsinoe species. Part II. Structure of elsinochromes A, B, and C. *Journal* of the *Chemical Society*: 1219-1227.

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, *et al.* 2005. Genome sequencing and analysis of Aspergillus oryzae. *Nature* **438**: 1157-61.

Madduri K, Kennedy J, Rivola G, Inventi-Solari A, Filippini S, Zanuso G, Colombo AL, Gewain KM, Occi JL, MacNeil DJ, *et al.* 1998. Production of the antitumor drug epirubicin (4'-epidoxorubicin) and its precursor by a genetically engineered strain of Streptomyces peucetius. *Nature Biotechnology* **16**: 69-74.

Netzker T, Fischer J, Weber J, Mattern DJ, König CC, Valiante V, Schroeckh V, Brakhage AA. 2015. Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Frontiers in Microbiology* **6**: 299.

Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, *et al.* 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. *Nature* **438**: 1151-6.

Nishimura S, Kohmoto K. 1983. Host-specific toxins and chemical structures from alternaria species. *Annual Review of Phytopathology* **21**: 87-116.

Osbourn A. 2010. Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. *Trends in Genetics* **26**: 449-57.

Pahirulzaman KAK, Williams K, Lazarus CM. 2012. A toolkit for heterologous expression of metabolic pathways in Aspergillus oryzae. *Methods in Enzymology* **517**: 241-60.

Schardl CL. 2001. Epichloë festucae and related mutualistic symbionts of grasses. *Fungal Genetics and Biology* **33**: 69-82.

Schrader K, Dayan F, Allen S, de Regt M, Tucker C, Paul Jr, C. 2000. 9,10-Anthraquinone Reduces the Photosynthetic Efficiency of Oscillatoria perornata and Modifies Cellular Inclusions. *International Journal of Plant Sciences* **161**: 265-270.

Seto Y, Kogami Y, Shimanuki T, Takahashi K, Matsuura H, Yoshihara T. 2005. Production of phleichrome by Cladosporium phlei as stimulated by diketopiperadines of Epichloe typhina. *Bioscience, Biotechnology, and Biochemistry* **69**: 1515-9.

Srinivas G, Babykutty S, Sathiadevan PP, Srinivas P. 2007. Molecular mechanism of emodin action: transition from laxative ingredient to an antitumor agent. *Medicinal Research Reviews* **27**: 591-608.

Stergiopoulos I, de Wit PJGM. 2009. Fungal effector proteins. *Annual Review of Phytopathology* **47**: 233-63.

Stutzman-Engwall KJ, Otten SL, Hutchinson CR. 1992. Regulation of secondary metabolism in Streptomyces spp. and overproduction of daunorubicin in Streptomyces peucetius. *Journal of Bacteriology* **174**: 144-54.

Tomizawa M, Casida JE. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annual Review of Entomology* **48**: 339-64.

Trojer P, Brandtner EM, Brosch G, Loidl P, Galehr J, Linzmaier R, Haas H, Mair K, Tribus M, Graessle S. 2003. Histone deacetylases in fungi: novel members, new facts. *Nucleic Acids Research* **31**: 3971-81.

Wiener P. 1996. Experimental studies on the ecological role of antibiotic production in bacteria. *Evolutionary Ecology* **10**: 405-421.

Williams RB, Henrikson JC, Hoover AR, Lee AE, Cichewicz RH. 2008. Epigenetic remodeling of the fungal secondary metabolome. *Organic Biomolecular Chemistry.* **6**: 1895-7.

Wu Y, Tu X, Lin G, Xia H, Huang H, Wan J, Cheng Z, Liu M, Chen G, Zhang H, *et al.* 2007. Emodin-mediated protection from acute myocardial infarction via inhibition of inflammation and apoptosis in local ischemic myocardium. *Life Sciences* **81**: 1332-8.

Xia G, Li J, Li H, Long Y, Lin S, Lu Y, He L, Lin Y, Liu L, She Z. **2014**. Alterporriol-type dimers from the mangrove endophytic fungus, Alternaria sp. (SK11), and their MptpB inhibitions. *Marine Drugs* **12**: 2953-69.

Yang G. **1996**. A Polyketide Synthase Is Required for Fungal Virulence and Production of the Polyketide T-Toxin. *The Plant Cell* **8**: 2139-2150.

Yang Y, Shang W, Zhou L, Jiang B, Jin H, Chen M. **2007**. Emodin with PPARgamma ligand-binding activity promotes adipocyte differentiation and increases glucose uptake in 3T3-Ll cells. *Biochemical and Biophysical Research Communications* **353**: 225-30.

Yu J, Keller N. **2005**. Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology* **43**: 437-58.

Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW. **2004**. Clustered Pathway Genes in Aflatoxin Biosynthesis. *Applied and Environmental Microbiology* **70**: 1253-62.

Zheng C, Shao C, Guo Z, Chen J, Deng D, Yang K, Chen Y, Fu X, She Z, Lin Y, *et al.* 2012. Bioactive hydroanthraquinones and anthraquinone dimers from a soft coral-derived Alternaria sp. fungus. *Journal of Natural Products* **75**: 189-97.

Zhao B, Guengerich FP, Bellamine A, Lamb DC, Izumikawa M, Lei L, Podust LM, Sundaramoorthy M, Kalaitzis JA, Reddy LM, *et al.* 2005. Binding of two flaviolin substrate molecules, oxidative coupling, and crystal structure of Streptomyces coelicolor A3(2) cytochrome P450 158A2. *Journal of Biological Chemistry* **280**: 11599-607.

de Wit PJGM, Mehrabi R, van den Burg HA, Stergiopoulos I. **2009**. Fungal effector proteins: past, present and future. *Molecular Plant Pathology* **10**: 735-47.

de Wit PJGM, van der Burgt A, Ökmen B, Stergiopoulos I, Abd-Elsalam KA, Aerts AL, Bahkali AH, Beenen HG, Chettri P, Cox MP, *et al.* 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.

Chapter 2

Secondary metabolism and biotrophic lifestyle in the tomato pathogen *Cladosporium fulvum*

Jérôme Collemare
Scott Griffiths
Yuichiro Iida
Mansoor Karimi Jashni
Evy Battaglia
Russell Cox
Pierre J.G.M. de Wit

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Abstract

Cladosporium fulvum is a biotrophic fungal pathogen that causes leaf mould of tomato. Analysis of its genome suggested a high potential for production of secondary metabolites (SM), which might be harmful to plants and animals. Here, we have analysed in detail the predicted SM gene clusters of *C. fulvum* employing phylogenetic and comparative genomic approaches. Expression of the SM core genes was measured by RT-qrtPCR and produced SMs were determined by LC-MS and NMR analyses. The genome of *C. fulvum* contains six gene clusters that are conserved in other fungal species, which have undergone rearrangements and gene losses associated with the presence of transposable elements. Although being a biotroph, *C. fulvum* has the potential to produce elsinochrome and cercosporin toxins. However, the corresponding core genes are not expressed during infection of tomato. Only two core genes, *PKS6* and *NPS9*, show high expression *in planta*, but both are significantly down regulated during colonization of the mesophyll tissue. *In vitro* SM profiling detected only one major compound that was identified as cladofulvin. *PKS6* is likely involved in the production of this pigment because it is the only core gene significantly expressed under these conditions. Cladofulvin does not cause necrosis on *Solanaceae* plants and does not show any antimicrobial activity. In contrast to other biotrophic fungi that have a reduced SM production capacity, our studies on *C. fulvum* suggest that down-regulation of SM biosynthetic pathways might represent another mechanism associated with a biotrophic lifestyle.

Key words: *Cladosporium fulvum*, secondary metabolome, biotrophy, phylogeny, comparative genomics, polyketide, non-ribosomal peptide, cladofulvin.

2.1 Introduction

Fungi are a major source of natural compounds, also known as secondary metabolites (SMs), with diverse biological activities. Fungal SMs include important pharmaceuticals such as penicillin and lovastatin, but also harmful food and feed contaminants known as mycotoxins including aflatoxins and trichothecenes. They can also serve as pathogenicity factors such as host-specific and non-specific toxins produced by many fungal plant pathogens [1,2]. Fungal SMs are classified into four main groups based on core enzymes and precursors involved in their biosynthesis: polyketides, non-ribosomal peptides, terpenes and alkaloids [3]. In contrast to plants, fungi produce mainly polyketides and non-ribosomal peptides, and accordingly contain a higher number of core genes encoding polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) in their genomes [4]. SM biosynthetic pathways often require several enzymes that are encoded by co-regulated genes located at the same locus in the genome, which defines a gene cluster organization [5].

The genomics era has provided new tools to study fungal SMs and their biosynthesis at the whole genome scale. The core enzymes typically responsible for the synthesis of the first intermediate in biosynthetic pathways, *i.e.* PKSs, NRPSs, hybrid PKS-NRPSs, terpene cyclases (TCs) and dimethylallyl tryptophan synthase (DMATSs), have highly conserved domains which allows efficient identification of their encoding genes. From the inventory of these genes in a given genome, the SM production capacity of a fungal species can be assessed. This genome-wide approach already showed that Pezizomycotina have a greater potential for SM production than Saccharomycotina, Taphrinomycotina and Basidiomycota, with up to 58 core genes predicted for *Aspergillus terreus* [4]. It also revealed that fungal biotrophy tends to be associated with a highly restricted SM production capacity [2,6]. Indeed, SMs with necrogenic activities might be detrimental to biotrophic fungi that need living host cells to complete their lifecycle. Comparative genomics studies can facilitate the identification of gene clusters involved in the biosynthesis of previously characterized compounds. For example, the genome of *Stagonospora nodorum* contains only one hybrid PKS-NRPS, which is likely the enzyme responsible for the production of pramanicin that displays a typical hybrid polyketide structure [4]. However, only a few SMs have been characterized for a given fungus because common laboratory growth conditions are only conducive to the production of a restricted number of SMs. Thus, most of the gene clusters identified *in silico* encode cryptic or silent pathways. In *Aspergillus* species, several genetic tools that mainly rely on global gene expression modification have led to gene cluster activation and discovery of SMs like terrequinone A, nygerone and endocrocin produced by *Aspergillus nidulans*, *Aspergillus niger* and *Aspergillus fumigatus*, respectively [7-9]. Prior to experimental studies, phylogenetic and comparative genomics analyses are very informative as the number of fungal genomes and characterized SM pathways increases. *In silico* studies can provide new information about the organization of conserved gene clusters, their borders and evolution. Such approaches are very helpful to

identify gene clusters that are involved in the production of SMs that have been characterized in other fungal species and allow subsequent predictions of identical or related compounds that a particular fungal species might produce.

Cladosporium fulvum is a Dothideomycete fungus responsible for tomato leaf mould disease worldwide. This fungus is a biotroph that only colonizes the apoplastic space of tomato leaves [10]. The genome of *C. fulvum* has been sequenced and bioinformatic analyses revealed that it contains 23 SM core genes, an exceptionally high number for a biotroph [11]. This finding questioned the proposed correlation between restricted SM production capacity and fungal biotrophy. In this study, we have analysed the full manifest of SM biosynthetic gene clusters in *C. fulvum* and link these to actual production of SMs and their putative role in pathogenicity. This is the first thorough study of fungal secondary metabolism that provides new insights into SM gene cluster evolution in the context of fungal biotrophy.

2.2 Materials and Methods

2.2.1 Fungal growth conditions

The sequenced strain race 0WU was grown and conidia suspensions (5×10^5 conidia.mL^{-1}) were prepared as previously described [11]. For SM detection, 500 mL of B5 medium (Gamborg B5 medium supplemented with 20 g.L^{-1} sucrose; Duchefa Biochemie B.V., The Netherlands) or PDB (Sigma-Aldrich, Saint-Louis, MO) were inoculated with 5 mL of conidia suspension and grown as shaking culture (200 rpm) for ten days at 20°C. For expression analysis, 50 mL of PDB were inoculated with 0.5 mL of conidia suspension and grown as shaking culture for seven days. Subsequently, mycelium was harvested by filtration over Miracloth, washed once with fresh medium (PDB, B5 (pH4), B5 adjusted to pH7, B5 without carbon source, B5 without nitrogen source or B5 without $FeSO_4$) and transferred to flasks containing 50 mL of the corresponding medium for 48h. Alternatively, 50 mL of B5 medium were inoculated with 0.5 mL of conidia suspensions and incubated for 12 days. Mycelium was harvested by filtration over Miracloth. Both mycelium and culture filtrate were freeze-dried. Twenty-five grams of freshly picked four-week old tomato leaves were autoclaved in flasks, inoculated with 5 mL of conidia suspension and incubated for 7 days. Taking off the leaf epidermis retrieved fungal biomass.

2.2.2 Tomato inoculation and collection of apoplastic fluid

Four-week-old Heinz tomato plants, grown under standard greenhouse conditions, were sprayed with a conidia suspension (10 mL at 5×10^5 conidia.mL^{-1}) as previously described [12]. Apoplastic fluids were collected at 10 days post-inoculation following the protocol of de Wit and Spikman [13].

2.2.3 Functional annotation of secondary metabolism genes

The predicted protein sequence of core enzymes was used to perform a BlastP search in the NCBI non-redundant protein database (www.ncbi.nlm.nih.gov). Conserved domains were identified using InterproScan (www.ebi.ac.uk), the PKS/NRPS analysis website [14] and ASMPKS (gate.smallsoft.co.kr:8008/~hstae/asmpks/pks_prediction.pl; SmallSoft Co. Ltd). For each core gene, the genomic locus was inspected for upstream and downstream genes. BlastP and InterproScan analyses confirmed functional annotation of flanking genes. Borders of the gene clusters were defined when three consecutive genes did not encode proteins with a predicted function associated with secondary metabolism, or when two annotated genes were separated by more than 5 kb.

2.2.4 Phylogenetic analysis

Amino acid sequences (Table S1 in File S1) of full length PKSs, KS and AT domains of hybrid PKS-NRPSs and A domains of NRPSs were aligned using T-Coffee [15]. Alignments were manually edited in Genedoc (www.psc.edu/biomed/genedoc) and poorly aligned regions were removed with Gblocks, allowing smaller final blocks, gap positions within the final blocks and less strict flanking positions [16]. Phylogenetic trees were constructed using the maximum likelihood algorithm with default parameters apart from the JTT amino acid substitution matrix and were edited in MEGA5 [17].

2.2.5 Gene expression analysis

Total RNA from infected tomato leaves was already available [11]. New biological repeats were performed following the same protocol and the same methods were used to extract total RNA from mycelium grown under *in vitro* conditions. As a negative control, RNA was also extracted from healthy plants. cDNA synthesis was performed using 2 µg of total RNA and the First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) or M-MLV reverse transcriptase (Promega, Madison, WI), following the manufacturer's protocol. For quantitative PCR, primers were designed with Primer3Plus [18] (Table S2) and their efficiency was tested on a genomic DNA dilution series. Quantitative PCR was performed with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA). Raw data were analysed following the $2^{-\Delta Ct}$ method [19]. Expression of the actin gene was used to normalize the expression of each gene. This normalization was assessed using the ß-tubulin gene as a control. Results are average of two to three biological repeats, and up to two technical repeats.

2.2.6 Clamped Homogenous Electric Fields (CHEF) gel electrophoresis and Southern blotting

C. fulvum protoplasts were prepared as previously described [20], using 5 $mg.mL^{-1}$ Kitalase (Wako Pure Chemicals) and 10 $mg.mL^{-1}$ Lysing Enzyme (Sigma-Aldrich, Saint-Louis, MO), incubating mycelium overnight at 30°C with gentle shaking. Chromosome-sized DNA molecules were extracted as previously described [20], and separated in 0.8% agarose gels (SeaKem Gold Agarose; Lonza, Rockland, ME) at 8°C using 0.5x TBE buffer. CHEF electrophoresis was carried out with a CHEF-DRII apparatus (Bio-Rad, Hercules, CA) with the following settings (duration/voltage/linear gradient of switching time): 115 hrs / 50 V / 3600-1800 sec; 24 hrs / 50 V / 1800-1300 sec; 28 hrs / 60 V / 1300-800 sec; 28 hrs / 80 V / 800-600 sec. The gel was stained with ethidium bromide for 30 min. DNA gel blotting was performed using standard methods [21]. DNA was transferred onto Hybond N+ nylon membranes (GE Healthcare, Little Chalfont, UK) and subjected to hybridization and detection using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche, Basel, CH) according to the manufacturer's instructions.

2.2.7 Secondary metabolite extraction and thin layer chromatography

Freeze-dried culture filtrate and mycelium were extracted twice in 25 mL of ethyl acetate, incubating the samples at room temperature for 10 min in an ultrasound bath, then shaking for 30 min in a hood. The organic phase was collected by centrifugation for 10 min at 4,000xg and evaporated under nitrogen flow. Concentrated extracts dissolved in acetonitrile were spotted on TLC Silica Gel 60 plates (Merck Millipore, Billerica, MA). The mobile phase was composed of toluene/ethyl acetate/formic acid (5:4:1; v/v/v). Fluorescent compounds were detected at 254 nm and 365 nm wavelengths using a ChemiDoc™ XRS+ System (Bio-Rad) and a Black-Ray® long wave UV lamp model B 100AP (Mineralogical Research Co., San Jose, CA), respectively. Plates were stained using iodine vapour from crystals in a closed tank. Cladofulvin was located by spraying the plates with magnesium acetate (4% w/v in MeOH).

2.2.8 Purification and characterisation of cladofulvin

Extracts of *C. fulvum* were purified using a mass-directed chromatography system consisting of a Waters 2767 auto-sampler, Waters 2545 pump system, Phenomenex LUNA column (5µ, C_{18}, 100 Å, 10 × 250 mm) equipped with a Phenomenex Security Guard pre-column (Luna C_5 300 Å) eluted at 4 $mL{\cdot}min^{-1}$. Solvent **A**, HPLC grade H_2O + 0.05% formic acid; Solvent **B**, HPLC grade MeOH + 0.045% formic acid; solvent **C**, HPLC grade CH_3CN + 0.045% formic acid. The post-column flow was split (100:1) and the minority flow was made up with solvent **B** to 1 $mL{\cdot}min^{-1}$ for simultaneous analysis by diode array detector (Waters 2998), evaporative light scattering (Waters 2424) and ESI mass spectrometry in positive and negative modes (Waters Quatro Micro). The following chromatographic programme was used (the

balance of solvent used was **A**): 0 min, 20% **C**; 10 min 90% **C**; 26 min 95% **C**; 28.5 min 95% **C**; 29 min 20% **C**; 30 min 20% **C**. Fractions containing cladofulvin were combined and evaporated to afford 2.2 mg of a bright orange solid. *m/z* (ES^+) 539.1 $[M]H^+$, 561.1 $[M]Na^+$; (ES^-) 537.2 $[M - H]^-$, 559.2 $[M - 2H + Na]^-$. uv max (CH_3CN) 235.1, 269.1. ^1H NMR: 500 MHz, CD_3OD/CD_3CN; ^{13}C NMR: 125 MHz, CD_3OD/CD_3CN (Table S3). HRMS calc for $C_{30}H_{18}O_{10}$ 537.0822 observed 537.0801 $[M-H]^-$.

2.2.9 Biological activity assays

Purified cladofulvin was solubilised in CH_3CN to 10 mM. Dilution series of pure cladofulvin were applied onto paper discs displayed on LB or PDA plates. CH_3CN (10% v/v in water) was used as negative control. An agar overlay was immediately poured, containing either cells of *Pseudomonas fluorescens*, *Streptomyces coelicolor* (OD_{600} = 1), or spores of *Botrytis cinerea* B05.10 strain or *Hansfordia pulvinata* (10^4 spores). Plates were incubated for two days at 25°C for *P. fluorescens* and at 30°C for *S. coelicolor*. Plates were incubated for eight days at 18°C and 22°C for *B. cinerea* and *H. pulvinata*, respectively. The same diluted samples were infiltrated in leaves of *Solanum lycopersicum* and *Nicotiana benthamiana* plants. Infiltrated leaves were monitored for seven days. Each experiment was performed under light and dark conditions at least twice.

2.3 Results

2.3.1 Inventory of *Cladosporium fulvum* secondary metabolism core genes

The genome of *C. fulvum* contains 23 core SM genes (10 PKSs, 10 NRPSs, 2 hybrid PKS-NRPSs and 1 DMATS, no TC). Of these, 15 are likely to encode functional enzymes, but seven appear to be pseudogenes and/or truncated genes (*PKS4*, *PKS9*, *NPS1*, *NPS5*, *NPS7*, *NPS10* and *HPS2*) [11]. Five PKSs (PksA, Pks1, Pks6, Pks7 and Pks9) show the typical organization of non-reducing PKSs (starter unit (SAT)-keto synthase (KS)-acyl transferase (AT)-product template (PT)-acyl carrier protein (ACP) domains) (Fig. S1 and Table S4 in File S1), suggesting that they produce aromatic compounds [22]. The other five PKSs share the organization of partially- and highly-reducing PKSs with the additional DH-(ER)-KR (dehydratase-(enoyl reductase)-keto reductase) domains, suggesting that they may produce reduced (possibly linear) polyketide chains [22]. PksA, Pks1 and Pks7 contain a TE domain possibly involved in the release of the polyketide chain (Fig. S1 in File S1) [23]. The other PKSs do not appear to contain thiolesterase (TE) domains, suggesting other mechanisms for product release [23].

Of the NRPSs, six are mono/bi-modular enzymes (Nps1, Nps4, Nps5, Nps6, Nps8 and Nps10), while Nps2, Nps3, Nps7 and Nps9 are multi-modular enzymes (Fig. S1 in File S1). However, pseudogenes or truncated genes encode half of them. All predicted functional

NRPSs have a terminal condensation (C) domain that might be involved in the release of the peptide from the enzyme [24]. Nps2 shows the typical organization of type IV fungal siderophore synthetases and Nps9 that of type II enzymes (Table S4 in File S1) [25,26]. Signature specificities of the adenylation (A) domains of these two synthetases are consistent with siderophore biosynthesis.

2.3.2 Phylogenetic analysis of *Cladosporium fulvum* secondary metabolism core enzymes

Phylogenetic analyses that resolve orthologous relationships can predict the structural type of SM that will be synthesized by a given conserved core enzyme [27-29]. Phylogenetic trees were constructed using deduced amino acid sequences of core proteins characterized in other fungal species (*i.e.* they were shown to be involved in the biosynthesis of characterized SMs), of enzymes representing conserved phylogenetic clades and of those of *C. fulvum* that are not truncated. Sequences of fungal non-reducing PKSs, reducing PKSs, and the KS and AT domains of hybrid PKS-NRPSs were aligned to construct the respective maximum likelihood phylogenetic trees. Three out of the five non-reducing PKSs are orthologues of characterized enzymes (PksA, Pks1 and Pks7; Fig. 1A). It was previously reported that PksA is related to the dothistromin synthase of *Dothistroma septosporum*, which is itself orthologous to synthases that are responsible for the biosynthesis of the highly toxic aflatoxins and sterigmatocystins [11]. Pks1 and Pks7 are orthologues of the elsinochrome synthase EfPKS1 from *Elsinoë fawcettii* [30] and the cercosporin synthase CTB1 from *Cercospora nicotianae* [31], respectively (Fig. 1A). The two remaining non-reducing PKSs, Pks6 and Pks9, belong to a clade that comprises synthases responsible for the production of atrochrysone [32], monodictyphenone [33] and endocrocin [9].

The phylogenetic tree for reducing PKSs shows that Pks2 belongs to a clade that includes Dep5 and BCBO9, which are involved in the production of depudecin and botcinic acid, respectively (Fig. S2A in File S1) [34,35]. Because the biosynthesis of botcinic acid requires a second PKS that is not present in *C. fulvum*, it is likely that Pks2 produces a compound with a backbone related to depudecin. Pks3 and Pks5 are closely related to the alternapyrone synthase, PksN, of *Alternaria solani* and the T-toxin synthase, Pks2, of *Cochliobolus heterostrophus*, respectively [36,37]. In contrast, Pks8 is not related to any characterized PKS. The only functional PKS-NRPS hybrid in *C. fulvum*, Hps1, belongs to a clade that includes hybrids involved in cyclopiazonic acid biosynthesis (Fig. S2B in File S1) [38].

The A-domain sequences of *C. fulvum* NRPS enzymes could be aligned with A-domains sequences representative of the different NRPS classes described by Bushley and Turgeon [39]. The phylogenetic tree shows that *C. fulvum* NRPS A domains group to three clades only: SID (siderophore synthetases), EAS (Euascomycete clade synthetases) and CYCLO

(cyclosporin synthetases) (Fig. 1B). Nps1 belongs the CYCLO clade and Nps8 is a hybrid protein containing A domains that belong to the CYCLO and EAS clades. Nps3, Nps4 and Nps6 belong to the EAS clade. Several NRPSs of the CYCLO clade are recombinant proteins containing an A-domain that belongs to the EAS clade [39]. This suggests that *NPS1* might be a truncated gene, which is consistent with its unusual domain organization. The Nps6 sole A-domain is related to the peramine synthetase of *Epichloë festucae*.

Both enzymes carry a *N*-MeT domain, but the PerA synthetase is bi-modular, while Nps6 is mono-modular [40]. Similar to *NPS1*, *NPS6* could also be a truncated gene. Nps2 and Nps9 A-domains are closely related to cognate A-domains of synthetases responsible for the biosynthesis of siderophores of the ferricrocin and ferrichrome type (Fig. 1B).

Although this analysis predicts the type of SMs that might be produced by *C. fulvum* (reduced/non-reduced polyketides, siderophores), it does not provide any information on the precise chemical structure.

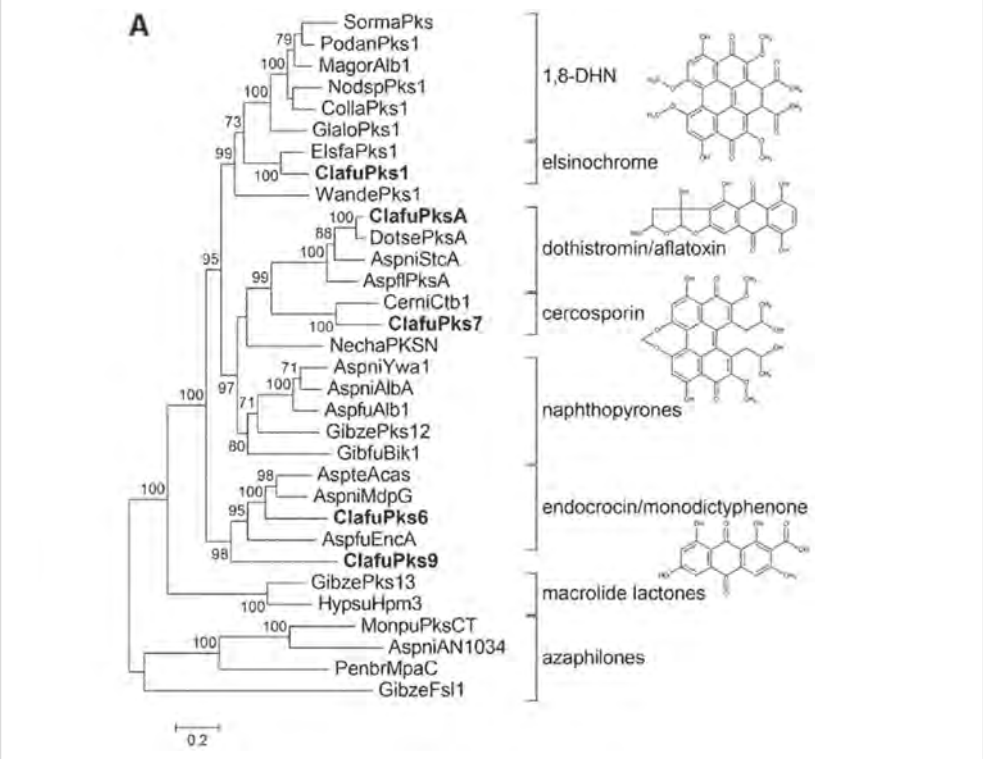


Figure 1. Phylogenetic analysis of *Cladosporium fulvum* PKS and NRPS enzymes. Alignments of **(A)** full-length protein sequences of non-reducing polyketide synthases (PKSs).

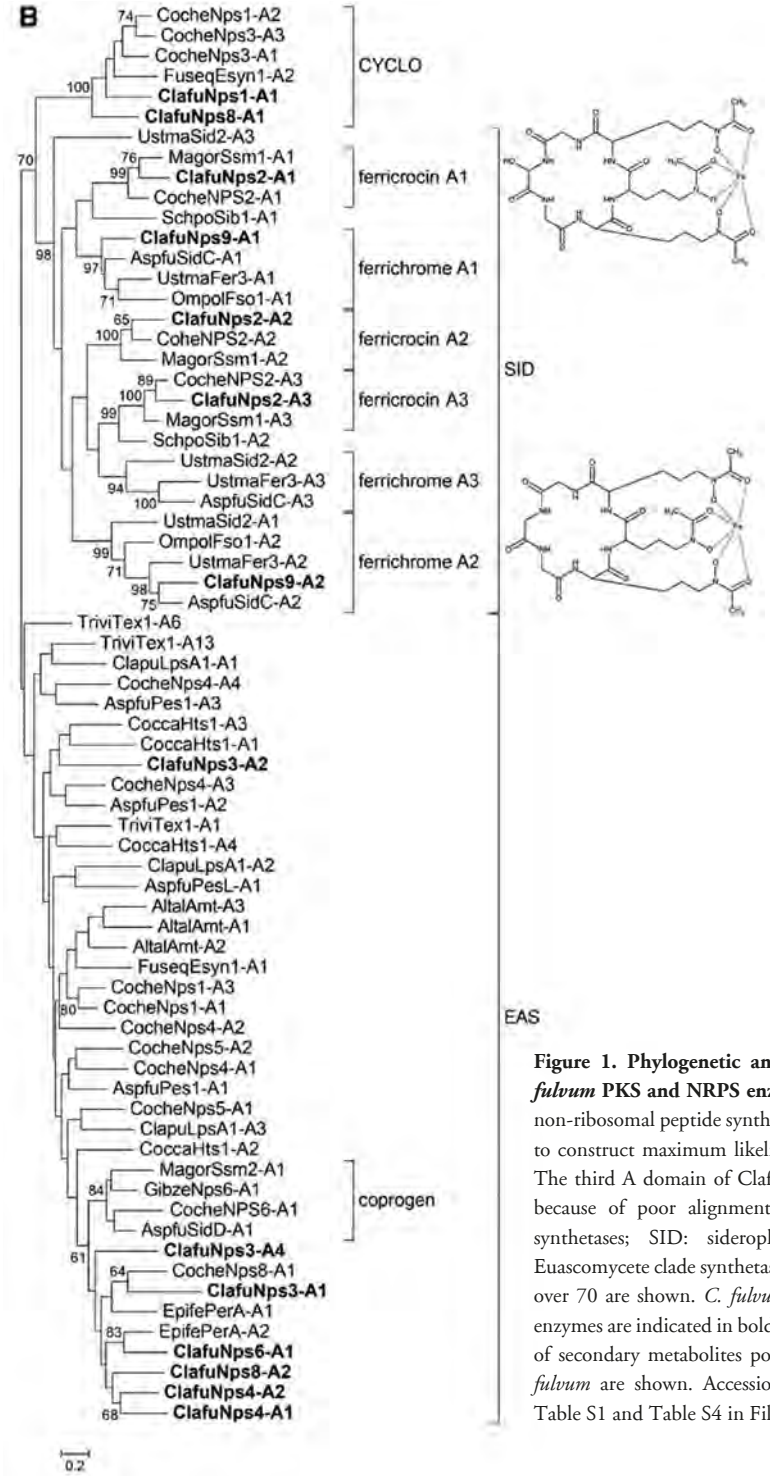


Figure 1. Phylogenetic analysis of *Cladosporium fulvum* PKS and NRPS enzymes. (B) A-domains of non-ribosomal peptide synthetases (NRPSs) were used to construct maximum likelihood phylogenetic trees. The third A domain of ClafuNps9 was not included because of poor alignment. CYCLO: cyclosporine synthetases; SID: siderophore synthetases; EAS: Euascomycete clade synthetases. Only bootstrap values over 70 are shown. *C. fulvum* secondary metabolism enzymes are indicated in bold. The chemical structures of secondary metabolites potentially produced by *C. fulvum* are shown. Accession numbers are given in Table S1 and Table S4 in File S1.

2.3.3 Rearrangements in secondary metabolism gene clusters in the *Cladosporium fulvum* genome

Fifteen putative SM gene clusters have been identified in the *C. fulvum* genome (Fig. S3 and S4 in File S1). Five core genes (two pseudogenes and one likely truncated gene) do not belong to any predicted gene cluster (*PKS5*, *HPS1*, *HPS2*, *NPS1* and *NPS7*), but they could have been part of gene clusters that have been disrupted by transposable elements, which are abundant in the *C. fulvum* genome [12]. The *PKSA* gene cluster has already been described in detail and is homologous to the complete dothistromin biosynthetic gene cluster in *D. septosporum* [12]. However in *C. fulvum*, *HEXA* and *NOR1* are pseudogenes, resulting in a non-functional pathway [41]. Indeed, these two genes encode enzymes responsible for the synthesis of the first (hexanoate) and third (averantin) intermediates in the dothistromin biosynthetic pathway. In *D. septosporum*, this cluster is fragmented over six loci on chromosome 12 [42]. In *C. fulvum*, three loci are located on a small chromosome of 0.8 Mb and the other three loci are located on a 1.8 Mb chromosome (Fig. S5 in File S1), suggesting that inter-chromosomal rearrangements further disrupted the putative ancestral dothistromin cluster in this fungus.

The organization of all other SM loci in the *C. fulvum* genome is fully depicted (Fig. S3 and S4 in File S1). All 15 predicted gene clusters contain genes that encode typical accessory enzymes including dehydrogenases, cytochrome P450 mono-oxygenases and methyltransferases. Several clusters also comprise MSF/ABC transporter and transcription factor encoding genes that might be involved in SM secretion and local gene cluster regulation, respectively. Small chromosomes do not tend to be enriched in SM gene clusters in *C. fulvum* because *PKS6* is located on a 4 Mb chromosome and both *PKS1* and *NPS9* are found on the same 2.9 Mb chromosome, while *PKS7* is located on a 1.8 Mb chromosome (Fig. S5 in File S1).

All orthologues of *C. fulvum* core genes belong to predicted or characterized gene clusters in other fungal species. However in *C. fulvum*, no conserved gene clusters could be found at the *PKS2*, *PKS3*, *PKS5*, *PKS9* and *NPS6* loci. This suggests that these pathways are involved in the biosynthesis of SMs that differ from those identified in other fungi. Thus, it is unlikely that *C. fulvum* can produce depudecin, alternapyrone, T-toxin or peramine. In contrast, conserved gene clusters were identified at the *PKS1*, *PKS6*, *PKS7*, *NPS2* and *NPS9* loci, which are responsible in other fungi for the biosynthesis of elsinochrome, monodictyphenone, cercosporin and siderophore production, respectively.

The gene cluster for elsinochrome biosynthesis in *E. fawcettii* comprises six genes (*PKS1*, *PRF1*, *RDT1*, *TSF1*, *ECT1* and *OXR1*) [43]. Four additional flanking genes (*EfHP1* to *EfHP4*) that encode hypothetical proteins are located at the same locus, but none are present in the genome of *C. fulvum* (Fig. 2A). The elsinochrome gene cluster organization shows conserved synteny between both species for all genes, but in *C. fulvum OXR1* has been replaced by a transposable element and a gene encoding a hypothetical protein (Fig. 2A). Transposon insertion at this locus could have resulted in the loss of *OXR1* and insertion of another gene in *C. fulvum*. A homologue

of *ECT1* is present on another scaffold in *C. fulvum*, suggesting that rearrangements might have occurred at the border of this gene cluster.

Monodictyphenone, atrochrysone and endocrocin share a similar biosynthetic pathway that requires conserved PKS and β-lactamase-type thiolesterase enzymes [9,32,33]. The *PKS6* gene cluster contains seven genes that are part of the monodictyphenone gene cluster in *A. nidulans* (Fig. 2B) [33]. This cluster in *C. fulvum* shows rearrangements associated with the presence of many transposable elements. Remarkably, genes located within the monodictyphenone gene cluster but not involved in the biosynthesis of this SM are all lost in *C. fulvum*. In addition, *mdpH* and *mdpJ* are also absent, while an *mdpL* homologue is found on another scaffold. The *PKS6* gene cluster comprises two additional genes that putatively encode a cytochrome P450 mono-oxygenase and a dehydrogenase (Fig. 2B).

PKS7 is an orthologue of the cercosporin synthase gene, *CTB1*, in *C. nicotianae*, which belongs to a gene cluster of eight genes (*CTB1* to *CTB8*), flanked by genes with a different transcriptional regulation (*ORF9* to *ORF12*) [44]. Homologues of six genes from the cercosporin gene cluster are present at the *PKS7* locus in *C. fulvum*. *PKS7*/*CTB1* and *CTB3* form a core set of genes and rearrangements occurred on both sides in *C. fulvum* (Fig. 2C). Although *CTB6* and *CTB7* are not present at this locus, homologues were identified on different scaffolds. Despite extensive rearrangements, there is no evidence for transposon activity that could be associated with rearrangements at this locus.

The biosynthesis of ferricrocin requires two genes that are located at the same locus in the genome of *Schizosaccharomyces pombe*, which encode an L-ornithine N5-oxygenase and an NRPS [25]. Similarly, in *C. fulvum*, a homologue of the L-ornithine N5-oxygenase gene is located next to *NPS2* (Fig. 2D). In addition, a gene encoding an ABC transporter that was either inserted in *C. fulvum* or lost in *S. pombe* is located in between these two genes.

The biosynthesis of ferrichrome siderophores has been extensively studied in the Basidiomycota *Ustilago maydis*. Sid1 is an L-ornithine N5-oxygenase involved in the production of both ferrichrome and ferrichrome-A [45], while Sid2 and Fer3 are NRPSs involved in the production of ferrichrome and ferrichrome-A, respectively [45,46]. In *U. maydis*, *SID1* and *SID2* are located at the same locus and *FER3* belongs to another gene cluster that comprises six genes (*FER3* to *FER8*) required for ferrichrome-A biosynthesis. The *NPS9* gene cluster identified in *C. fulvum* seems to be a combination of both loci present in *U. maydis* because it contains homologues of *SID1* and *FER5* in addition to the NRPS gene (Fig. 2E). Other homologous genes of the *FER3* gene cluster are present scattered on different scaffolds in *C. fulvum*. Likely, siderophores produced by *C. fulvum* are different from those produced by *U. maydis*, but they likely can sequester iron. Indeed, differences in NRPS post-assembly steps will result in varied compounds that are still functional siderophores as exemplified by those of the ferrichrome family [47].

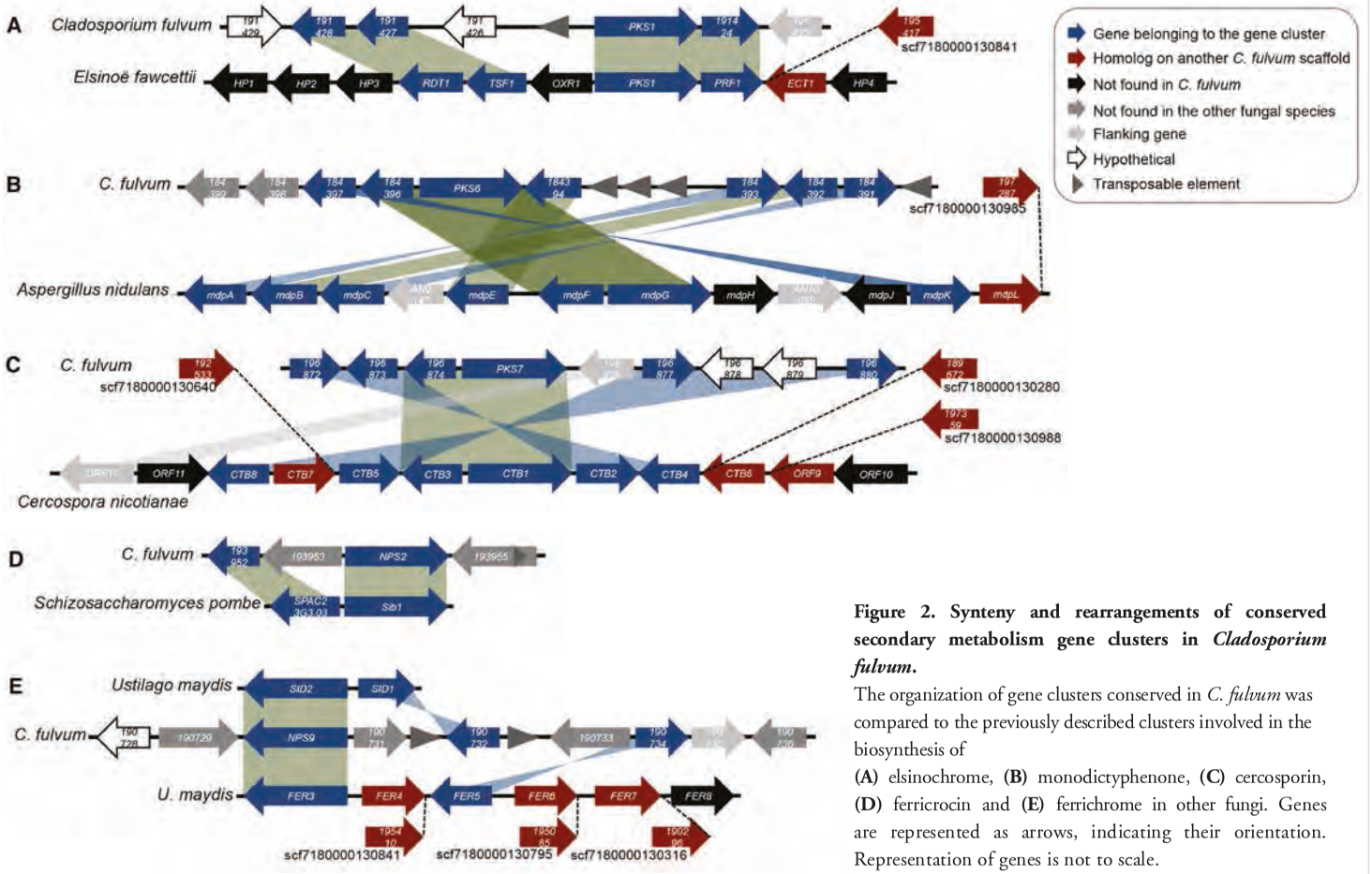


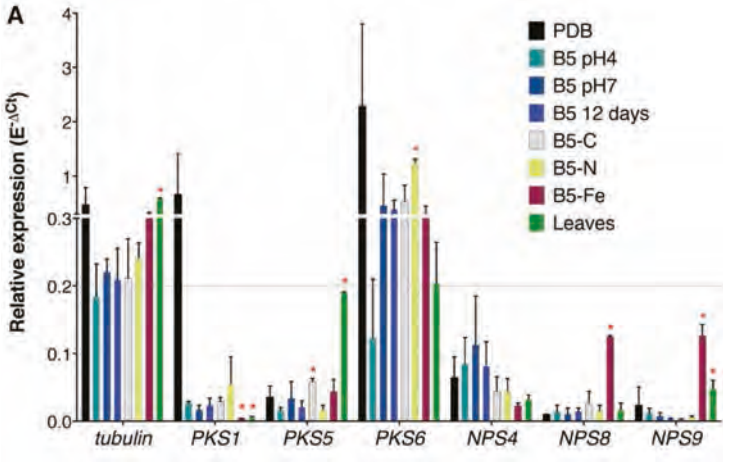
Figure 2. Synteny and rearrangements of conserved secondary metabolism gene clusters in *Cladosporium fulvum*.

The organization of gene clusters conserved in *C. fulvum* was compared to the previously described clusters involved in the biosynthesis of

(A) elsinochrome, **(B)** monodictyphenone, **(C)** cercosporin, **(D)** ferricrocin and **(E)** ferrichrome in other fungi. Genes are represented as arrows, indicating their orientation. Representation of genes is not to scale.

2.3.4 Most SM functional core genes are weakly expressed or down-regulated during colonization of tomato leaves

To further characterize the functional core genes, expression analysis was performed under eight different *in vitro* growth conditions and during infection of tomato using Reverse Transcription quantitative real-time PCR (RT-qrtPCR). Only *PKS6* exhibited higher expression than the tubulin gene in almost all *in vitro* conditions tested (Fig. 3A). *PKS1* was highly expressed in PDB medium only, *NPS4* exhibited clear expression in all conditions, and *PKS5* and *NPS9* genes were induced when *C. fulvum* was grown on autoclaved leaves. *NPS8* and *NPS9* were significantly induced under iron depletion, which is consistent with the prediction that Nps9 is a siderophore synthetase. All other genes exhibited a very low expression level that is about ten times below that of the tubulin gene (Fig. S6A in File S1). During tomato infection (from 0 days post-inoculation (dpi) to 16 dpi), only *PKS6* and *NPS9* exhibited high expression at the early stages of infection when runner hyphae were growing on the leaf surface (Fig. 3B). Remarkably, their expression dropped after penetration when the fungus was colonizing the apoplastic space between mesophyll cells. The expression of *PKS6* was induced at later stages of infection when conidiophores emerged from the plant and produced conidia. A similar expression profile was observed for most genes located in the predicted *PKS6* gene cluster, except for two of them whose expression was only detected from 12 or 16 dpi (Fig. S7 in File S1). These genes encode homologues of the mdpB reductase and mdpE transcription factor of the monodictyphenone pathway. In contrast, the effector genes *Avr4* and *Avr9* were strongly induced during colonization of tomato leaves (Fig. 3B). Expression of all other core genes remained low or even barely detectable during the whole infection cycle (Fig. S6B in File S1), although a few genes might show weak down- or up-regulation (Fig. S6C and S6D in File S1). Thus, it appears that most of the potentially functional SM pathways are silent under the tested *in vitro* conditions and only show very low expression when the fungus is growing inside its host.



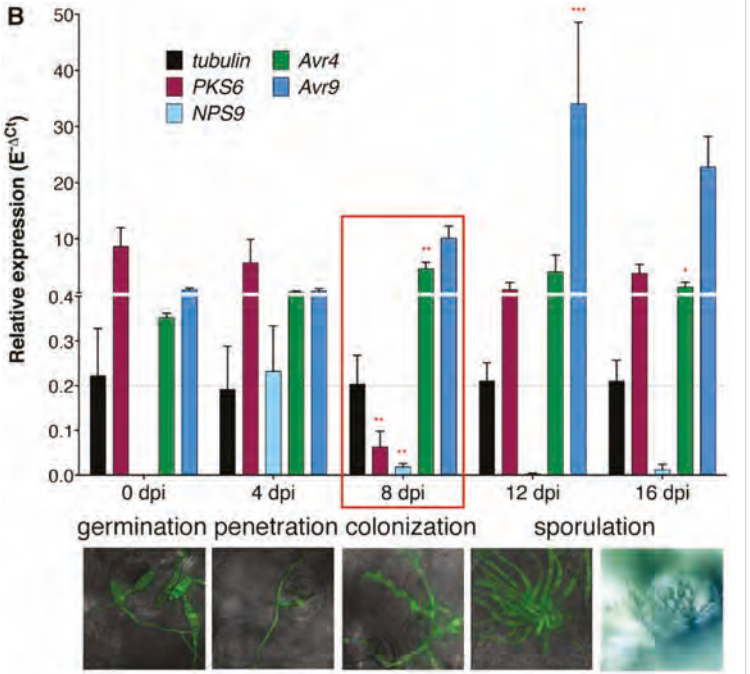


Figure 3. Expression profile of *Cladosporium fulvum* secondary metabolism functional core genes. Expression profiles were measured by RT-qrtPCR using RNA isolated from mycelium grown in diverse *in vitro* growth conditions: PDB, B5 with different pHs, B5 without carbon source (B5-C), B5 without nitrogen source (B5-N), B5 without $FeCl_3$ (B5-Fe), stationary phase (B5-12days) and autoclaved tomato leaves; and from tomato plants inoculated with the sequenced *C. fulvum* race 0WU strain from 0 to 16 days post-inoculation (dpi). Results are shown relative to the actin gene expression according to the $E^{-\Delta Ct}$ method, where E is the efficiency of a given primer pair. Tubulin gene was used as a control for calibration and the effector genes *Avr4* and *Avr9* were used as positive controls for the tomato infection experiment. The grey dotted line indicates the tubulin expression level. Values are the mean of three biological repeats and the error bars represent standard deviations (SD). **(A)** Only six genes show expression during *in vitro* growth, while **(B)** two genes are down-regulated during colonization of tomato. Pictures of tomato infected by a GFP-tagged *C. fulvum* strain are shown below to indicate the development of the fungus at the different time points of infection. Expression in each *in vitro* condition was compared to that in B5 pH4 medium using multiple t-tests, not assuming consistent SD, correcting for multiple comparisons with the Holm-Sidak method. For each gene, each *in planta* time point was compared to the previous one using a Two-way ANOVA followed by a multiple comparison test corrected with the Holm-Sidak method. All statistical tests were performed at the alpha significance threshold of 0.05. Red asterisks indicate significant differences only (* $p<0.01$; ** $p<0.001$; *** $p<0.0001$).

2.3.5 Production of secondary metabolites by *Cladosporium fulvum*

The present combined phylogenetic and comparative genomics analyses suggest that *C. fulvum* has the genetic potential to produce at least two polyketides (elsinochrome- and cercosporin-related compounds) and two non-ribosomal peptides (ferricrocin- and ferrichrome-related siderophores). In addition, *C. fulvum* was shown to produce the bianthraquinone pigment cladofulvin [48]. Production of these compounds by the sequenced wild type race 0WU strain was assessed under different *in vitro* conditions (12 days in PDB and B5 media). Culture filtrates and mycelium were separated by filtration prior to extraction and thin layer chromatography (TLC) and liquid chromatography/mass spectrometry (LC-MS) analyses. LC-MS profiling revealed that mycelium produced only two abundant metabolites that eluted at ca. 8.60 and 11.60 minutes, respectively (Fig. 4). The first LC-MS peak corresponds to a compound with a mass *m/z* of 538 $g.mol^{-1}$, which is the molecular weight of cladofulvin. This SM is secreted because it is also detected in the culture filtrate. It corresponds to the sole yellow spot observed on silica TLC plates viewed under visible light, which turns into violet when treated with magnesium acetate as previously reported for cladofulvin (Fig. S8 in File S1) [48]. Nuclear magnetic resonance (NMR) analysis confirmed the structure of cladofulvin after purification of the corresponding LC-MS peak (Table S3 in File S1). The second LC-MS peak was identified as linoleic acid, a common unsaturated fatty acid, based on the fragmentation spectrum and UV absorbance.

Extracts from culture filtrate contain additional early-eluted peaks for which no *m/z* could be assigned. Accordingly, TLC analysis revealed the presence of many additional compounds when viewed under UV light and after iodine staining (Fig. S8 in File S1). Metabolites were also extracted from apoplastic fluids collected from healthy and infected tomato plants, but the metabolite concentration in these samples did not allow assigning any mass and subsequent identification (data not shown).

Toxicity of the purified cladofulvin was tested on *Solanaceae* plants (*Solanum lycopersicum* and *Nicotiana benthamiana*) grown under light and dark conditions. While the positive control dothistromin triggered light-dependent necrosis (toxicity was much weaker on tomato), cladofulvin did not induce any necrosis (Fig. S9 in File S1), even at concentrations in the mM range (data not shown). Cladofulvin was also tested for toxicity against bacteria (*Pseudomonas fluorescens* and *Streptomyces coelicolor*) and fungi (*Botrytis cinerea* and *Hansfordia pulvinata*, a fungal antagonist of *C. fulvum*), but no antimicrobial activity against any of these organisms was observed (data not shown). The biological role of cladofulvin is unlikely related to pathogenicity or colonization of an ecological niche. Consistent with only cladofulvin being produced by *C. fulvum in vitro*, crude organic extracts from mycelium or culture filtrate did not show any antimicrobial activity against all these organisms grown under light or dark conditions (data not shown).

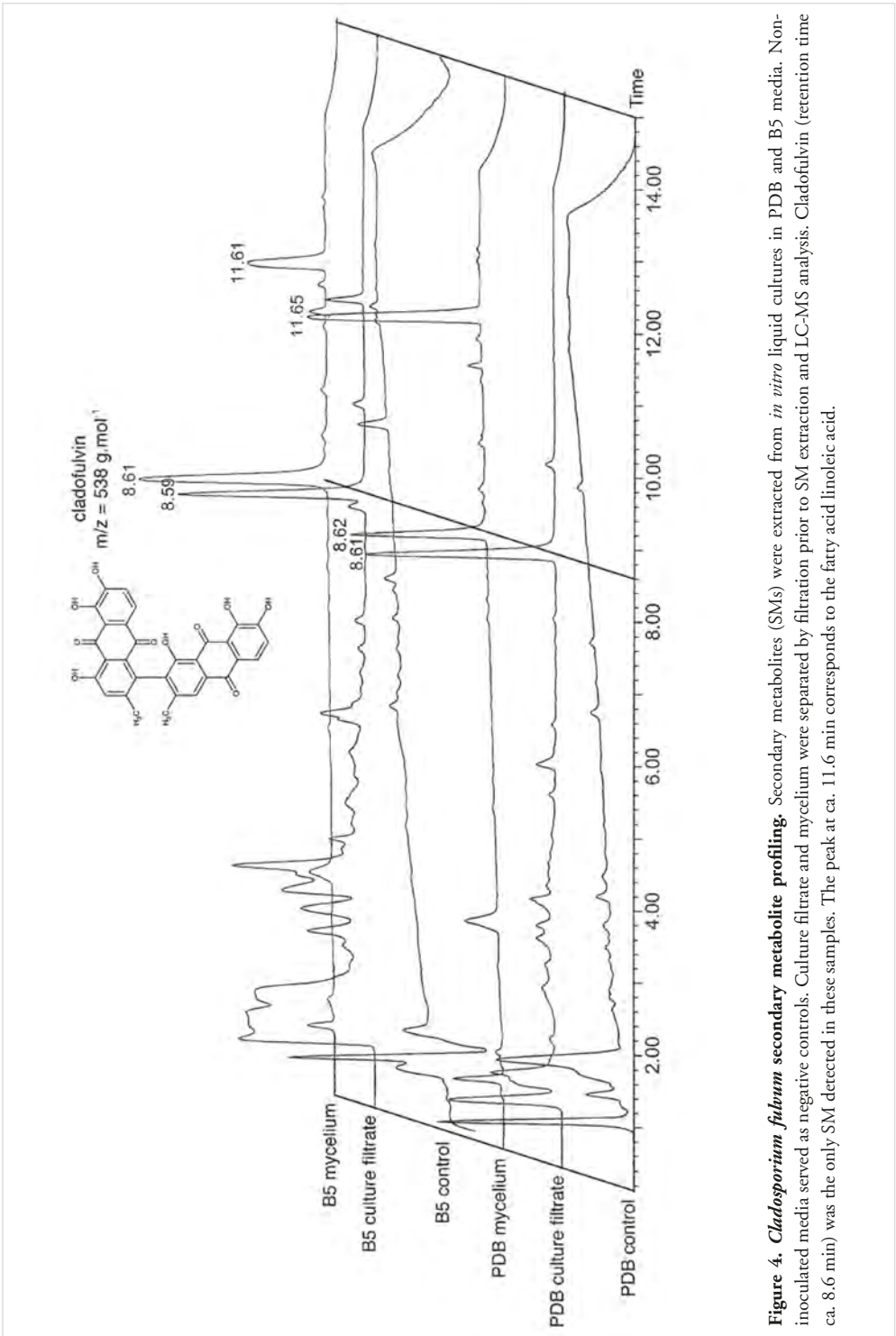


Figure 4. *Cladosporium fulvum* secondary metabolite profiling. Secondary metabolites (SMs) were extracted from *in vitro* liquid cultures in PDB and B5 media. Non-inoculated media served as negative controls. Culture filtrate and mycelium were separated by filtration prior to SM extraction and LC-MS analysis. Cladofulvin (retention time ca. 8.6 min) was the only SM detected in these samples. The peak at ca. 11.6 min corresponds to the fatty acid linoleic acid.

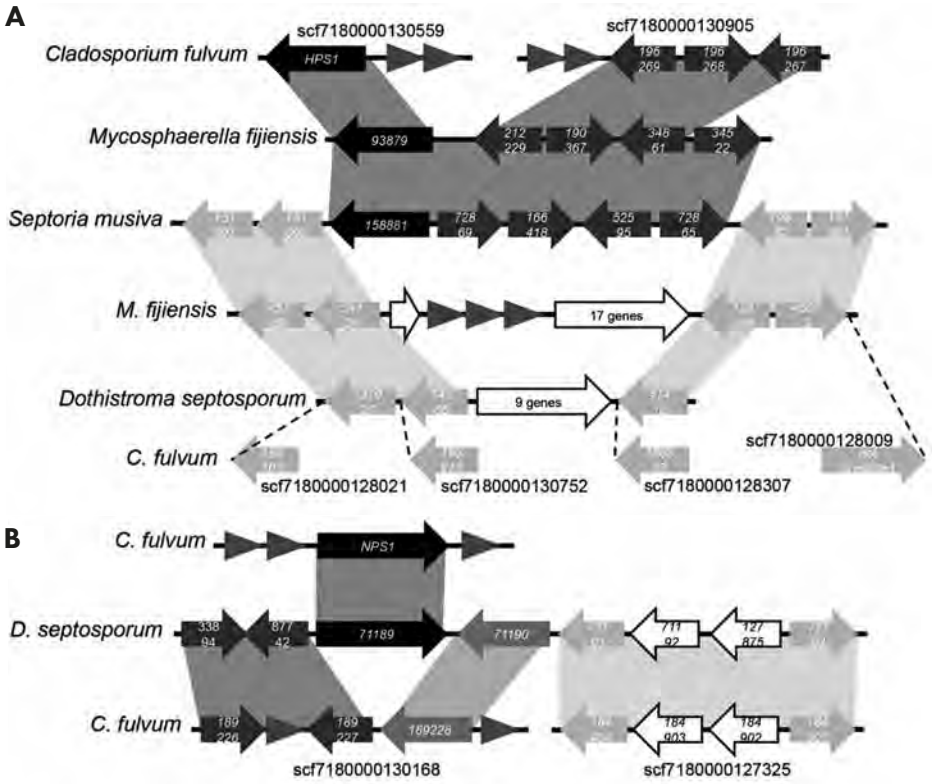


Figure 5. Disruption of *Cladosporium fulvum* gene clusters by transposable elements. (A) The *HPS1* and **(B)** *NPS1* genes in *C. fulvum* are orthologs of core genes that belong to conserved gene clusters in related fungal species. Genes are represented as arrows, indicating their orientation. Black arrows are genes with conserved orientation. Dark grey arrows are genes in the gene clusters that have undergone rearrangements. Light grey arrows are genes that border the gene cluster. Triangles represent transposable elements. Representation of genes is not to scale.

2.4 Discussion

2.4.1 Transposable elements have shaped the *Cladosporium fulvum* secondary metabolome

Loss of SM clusters has been suggested to possibly involve presence/activity of transposable elements. Yet, clear evidence is still missing. For example, *Sclerotinia sclerotiorum* and *B. cinera* are closely related fungi, with *S. sclerotiorum* containing more transposons in its genome [49]. The latter species has lost several SM gene clusters that are present in *B. cinerea*, but these losses are not associated with transposable elements. In contrast, comparison of all SM loci between *C. fulvum* and *D. septosporum* suggested transposable element-mediated gene losses in *C. fulvum* [12]. The present study also suggests that transposition events might have shaped the gene content and organization of SM gene clusters in *C. fulvum*. Indeed, all core genes except *PKSA*

and *PKS7* are located at loci that contain at least one transposable element. Moreover, five gene clusters are located at scaffold borders, which are composed of multiple repeats, and five other gene clusters reside on small scaffolds that contain no other genes.

The *C. fulvum* genome contains at least two striking examples of gene cluster disruption that is likely the result of transposon activity. *HPS1* and *NPS1* do not belong to gene clusters and they are located on small scaffolds delimited by many repeats. However, orthologues of these two core genes were found in related fungal species. *HPS1* orthologues in *Mycosphaerella fijiensis* and *Septoria musiva* are part of a conserved gene cluster that contains four additional genes (Fig. 5A). Interestingly, homologues of three of these genes are located at a single locus on another scaffold in *C. fulvum*. This suggests that in *C. fulvum HPS1* was also part of this conserved gene cluster. Moreover, the gene cluster borders in *S. musiva* are conserved at a different locus in *M. fijiensis* and *D. septosporum*, while they are scattered over different scaffolds in *C. fulvum*. The gene content in between these border genes in *M. fijiensis* and *D. septosporum* is different. This observation suggests that this conserved locus is prone to rearrangements, which might have led to the loss of the *HPS1* gene cluster in *D. septosporum* and to its re-localization to another locus in *M. fijiensis*. *NPS1* has an orthologue in *D. septosporum* where it belongs to a predicted gene cluster (Fig. 5B). The other genes from this gene cluster are located at two different loci in the *C. fulvum* genome. Similarly to *HPS1*, it is likely that transposable elements led to rearrangements in this cluster and to the re-localization of *NPS1* to another locus. These examples show that *C. fulvum* gene clusters suffered from severe rearrangements likely due to transposon activity, which may have led to inactivation of several SM biosynthetic pathways. Remarkably, the conserved gene clusters that might be involved in the biosynthesis of elsinochrome-, cercosporin- and ferricrocin-like compounds are not associated with many transposable elements and are located in the middle of scaffolds, although some rearrangements have occurred. Retention of these gene clusters might point to an important role of their products at specific stages of the *C. fulvum* lifecycle.

2.4.2 Prediction of a fungal secondary metabolome

In this study, we made use of phylogenetics and comparative genomics analyses to predict SMs that *C. fulvum* might produce. All genes involved in the dothistromin biosynthetic pathway present in *D. septosporum* have homologues located on two chromosomes in *C. fulvum*. The *PKS7* gene cluster is homologous to the cercosporin gene cluster in *C. nicotianae*. However, both *PKSA* and *PKS7* are among the core genes with the lowest expression level under all conditions tested. In addition, homologues of several genes required for early steps in dothistromin biosynthesis are pseudogenes in *C. fulvum* [41]. Accordingly, TLC and LC-MS analyses could detect neither dothistromin nor cercosporin under the growth conditions tested.

It is hypothesized that plant pathogenic fungi secrete siderophores for iron acquisition from the external environment during the infection process and synthesize intracellular siderophores

for iron storage [50]. Ferricrocin was reported to be an intracellular siderophore in *S. pombe* and *Magnaporthe oryzae* [51], in which no transporter has been reported at the NRPS gene locus. *NPS2* and *NPS9* belong to gene clusters predicted to be involved in the biosynthesis of ferricrocin- and ferrichrome-type siderophores. In *S. pombe*, the biosynthesis of ferricrocin requires another gene located at the same locus [25], which is also present in *C. fulvum*. However in this latter species, a gene encoding an ABC transporter is located in between the two genes, which might be required for secretion of the predicted siderophore. Iron depletion is known to induce the production of siderophores [50]. Consistent with this, *NPS9* was induced under such condition, but *NPS2* remained barely expressed. Surprisingly, *NPS8* was also induced, suggesting that it might encode a novel type of siderophore synthetase. It is noteworthy that *C. fulvum* does not have any NRPS in the *NPS6* clade that comprises the synthetases responsible for biosynthesis of the external siderophore coprogen. The siderophore system in *C. fulvum* might differ from other fungi such as *C. heterostrophus* and *M. oryzae* by using the putative Nps8 siderophore for iron acquisition or storage. *NPS9* was expressed in runner hyphae of *C. fulvum* prior to penetration of the host and was down-regulated at later stages of infection, suggesting that siderophore production is not crucial for colonization of the host.

Only one core gene, *PKS6*, exhibited high expression under most *in vitro* conditions, consistent with the detection of only cladofuvin as the major compound in liquid shaking cultures. *PKS6* belongs to a gene cluster that is conserved in *Aspergillus* species, which is involved in the production of the related compounds atrochrysone, monodictyphenone and endocrocin. The production of endocrocin anthrone homodimer was reported in *A. oryzae* [32], which shows a structure reminiscent to the one described for cladofulvin. Remarkably, in some *Aspergillus* species, the *mdpH* gene that is missing in *C. fulvum* is involved in directing the biosynthetic pathway towards the production of atrochrysone, and subsequently monodictyphenone, rather than towards the production of endocrocin anthrone [33]. This could explain the similarity in structure between cladofulvin and endocrocin. The two genes of this gene cluster that are specific to *C. fulvum* might be involved in the asymmetrical dimerization of cladofulvin. Altogether, these results suggest that Pks6 is the enzyme responsible for the biosynthesis of cladofulvin, which was the most abundant metabolite detected in all conditions. Functional analysis of *PKS6* is needed to confirm its requirement in cladofulvin production, but attempts to disrupt this gene have been unsuccessful so far.

PKS1 belongs to a gene cluster homologous to the elsinochrome gene cluster in *E. fawcettii*. All genes except *OXR1* are located within the gene cluster present in *C. fulvum*. The complete elsinochrome biosynthetic pathway has yet to be fully elucidated and there is no function assigned to the hypothetical protein encoded by *OXR1* [43]. It is likely that *C. fulvum* is able to produce elsinochrome or a highly similar compound. Despite its expression in PDB, LC-MS analysis did not detect such a compound. It is possible that the method used to extract SMs was not adapted to the isolation of this type of compound, although this is unlikely because the procedure did extract cladofulvin, which has a chemical structure similar to that of elsinochrome.

Functional analysis of *PKS1* will elucidate whether it is responsible for the biosynthesis of an elsinochrome-like compound. Surprisingly, the genome of *C. fulvum* does not contain any gene that belongs to the 1,8-DHN-melanin PKS clade, which suggests that *C. fulvum* is not able to produce DHN-melanin, although it might produce melanin from tyrosine through the DOPA pathway [52]. Alternatively, it was found that a PKS involved in DHN-melanin production in *A. niger* is also responsible for the biosynthesis of several naphtho-γ-pyrones [53]. Similarly, Pks1 could be responsible for the production of elsinochrome and/or DHN-melanin in *C. fulvum*.

2.4.3 Secondary metabolism and biotrophic growth of *Cladosporium fulvum*

Comparative genomics analyses of necrotrophic and biotrophic fungi suggested that a biotrophic lifestyle is associated with loss of SM genes [2,6]. Indeed, *Blumeria graminis* and *Tuber melanosporum*, two unrelated biotrophic fungi (an obligate biotrophic pathogen of barley and a symbiont, respectively), share common genomic features: a large genome size due to invasion of transposable elements and a significant reduction of the total number of genes, including SM genes [6,54]. Sequencing of two rust pathogens, *Melampsora larici-populina* and *Puccinia graminis f. sp. tritici*, revealed two large genomes, but did not show any reduction in gene number [55]. On the contrary, lineage-specific gene families are expanded in these fungi, but surprisingly their genomes contain only one SM core gene for the biosynthesis of a non-ribosomal peptide. These features are similar to those observed in the symbiont *Laccaria bicolor*, which shows expansion of gene families but not of PKS and NRPS gene families [2].

The biotrophic pathogen *C. fulvum* has a large genome size consistent with all these biotrophic fungal species, but does not show any reduction in gene number and has a higher SM production potential than some hemi-biotrophic or necrotrophic fungi [12]. However, EST sequencing and quantitative PCR analyses showed that, apart from *PKS6*, most of the SM genes have a low level of expression *in vitro*. Remarkably, expression levels are even lower during colonization of tomato, including expression of *PKS6*. This gene is highly expressed again when the fungus starts to produce conidiophores and sporulates on the leaf surface. Induction of gene expression at this stage suggests a role for the corresponding SM in survival of the fungus outside its host plant. In accordance with the expression data, only one major compound could be detected and no phytotoxic activity could be observed for SM extracts. Altogether, these data suggest that most SM biosynthetic pathways in *C. fulvum* are cryptic and that low expression and down-regulation of SM gene clusters during infection of its host might be novel mechanisms associated with fungal biotrophy. In addition, pseudogenization appears to have inactivated several core genes and the dothistromin pathway in *C. fulvum* [12,41]. Likely, *C. fulvum*'s cryptic SM pathways are induced at specific stages of its lifecycle outside its host when the fungus has to survive on the leaf surface or as a saprophyte on dead tissues. Further research is required to determine which environmental or growth conditions can activate these cryptic pathways.

Acknowledgments

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References

1. Stergiopoulos I, Collemare J, Mehrabi R, de Wit PJGM (2013) Phytotoxic secondary metabolites and peptides produced by plant pathogenic *Dothideomycete* fungi. FEMS Microbiol Rev 37:67-93.
2. Collemare J, Lebrun MH (2012) Fungal secondary metabolites: ancient toxins and novel effectors in plant-microbe interactions. In: Martin F, Kamoun S, editors. Effectors in plant-microbe interactions. Chichester, UK: John Wiley and Sons, Inc. pp. 377-399.
3. Keller NP, Turner G, Bennett JW (2005) Fungal secondary metabolism - from biochemistry to genomics. Nat Rev Microbiol 3:937-947.
4. Collemare J, Billard A, Böhnert HU, Lebrun MH (2008) Biosynthesis of secondary metabolites in the rice blast fungus *Magnaporthe grisea*: the role of hybrid PKS-NRPS in pathogenicity. Mycol Res 112:207-215.
5. Keller NP, Hohn TM (1997) Metabolic pathway gene clusters in filamentous fungi. Fungal Genet Biol 21:17-29.
6. Spanu PD, Abbott JC, Amselem J, Burgis TA, Soanes DM, *et al.* (2010) Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. Science 330:1543-1546.
7. Bok JW, Hoffmeister D, Maggio-Hall LA, Murillo R, Glasner JD, *et al.* (2006) Genomic mining for *Aspergillus* natural products. Chem Biol 13:31-37.
8. Henrikson JC, Hoover AR, Joyner PM, Cichewicz RH (2009) A chemical epigenetics approach for engineering the *in situ* biosynthesis of a cryptic natural product from *Aspergillus niger*. Org Biomol Chem 7:435-438.
9. Lim FY, Hou Y, Chen Y, Oh JH, Lee I, *et al.* (2012) Genome-based cluster deletion reveals endocrocin biosynthetic pathway in *Aspergillus fumigatus*. Appl Environ Microbiol 78:6395.
10. Thomma BP, van Esse HP, Crous PW, de Wit PJGM (2005) *Cladosporium fulvum* (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic *Mycosphaerellaceae*. Mol Plant Pathol 6:379-393.
11. de Wit PJGM, van der Burgt A, Ökmen B, Stergiopoulos I, Abd-Elsalam KA, *et al.* (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 Genet 8:e1003088.
12. de Wit PJGM (1977) A light and scanning-electron microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium fulvum*. Neth J Plant Pathol 83:109-122.
13. de Wit PJGM, Spikman G (1982) Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. Physiol Plant Pathol 21:1-11.
14. Bachmann BO, Ravel J (2009) Chapter 8. Methods for *in silico* prediction of microbial polyketide and nonribosomal peptide biosynthetic pathways from DNA sequence data. Method Enzymol 458:181-217.
15. Notredame C, Higgins DG, Heringa J (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol 302:205-217.
16. Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17:540-552.
17. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, *et al.* (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731-2739.
18. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, *et al.* (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 35:W71-W74.

19. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Method 25:402-408.
20. Westerink N, Brandwagt BF, de Wit PJGM, Joosten MHAJ (2004) *Cladosporium fulvum* circumvents the second functional resistance gene homologue at the *Cf-4* locus (*Hcr9-4E*) by secretion of a stable avr4E isoform. Mol Microbiol 54:533-545.
21. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. 3rd ed. Cold Springer Harbor, NY: Cold Springer Laboratory Press.
22. Cox RJ (2007) Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. Org Biomol Chem 5:2010-2026.
23. Du L, Lou L (2010) PKS and NRPS release mechanisms. Nat Prod Rep 27:255-278.
24. Kopp F, Marahiel MA (2007) Macrocyclization strategies in polyketide and nonribosomal peptide biosynthesis. Nat Prod Rep 24:735-749.
25. Schwecke T, Göttling K, Durek P, Dueñas I, Käufer NF, *et al.* (2006) Nonribosomal peptide synthesis in *Schizosaccharomyces pombe* and the architectures of ferrichrome-type siderophore synthetases in fungi. ChemBioChem 7:612-622.
26. Bushley KE, Ripoll DR, Turgeon BG (2008) Module evolution and substrate specificity of fungal nonribosomal peptide synthetases involved in siderophore biosynthesis. BMC Evol Biol 8:328.
27. Kroken S, Glass NL, Taylor JW, Yoder OC, Turgeon BG (2003) Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. P Natl Acad Sci USA 100:15670-15675.
28. Baker SE, Perrone G, Richardson NM, Gallo A, Kubicek CP (2012) Phylogenomic analysis of polyketide synthase-encoding genes in *Trichoderma*. Microbiol 158:147-154.
29. Brown DW, Butchko RA, Baker SE, Proctor RH (2012) Phylogenomic and functional domain analysis of polyketide synthases in *Fusarium*. Fungal Biol 116:318-331.
30. Liao HL, Chung KR (2008) Genetic dissection defines the roles of elsinochrome Phytotoxin for fungal pathogenesis and conidiation of the citrus pathogen *Elsinoë fawcettii*. Mol Plant Microbe In 21:469-479.
31. Choquer M, Dekkers KL, Chen HQ, Cao L, Ueng PP, *et al.* (2005) The *CTB1* gene encoding a fungal polyketide synthase is required for cercosporin biosynthesis and fungal virulence of *Cercospora nicotianae*. Mol Plant Microbe Int 18:468-476.
32. Awakawa T, Yokota K, Funa N, Doi F, Mori N, *et al.* (2009) Physically discrete beta-lactamase-type thioesterase catalyzes product release in atrochrysone synthesis by iterative type I polyketide synthase. Chem Biol 16:613-623.
33. Chiang YM, Szewczyk E, Davidson AD, Entwistle R, Keller NP, *et al.* (2010) Characterization of the *Aspergillus nidulans* monodictyphenone gene cluster. Appl Environ Microbiol 76:2067-2074.
34. Wight WD, Kim KH, Lawrence CB, Walton JD (2009) Biosynthesis and role in virulence of the histone deacetylase inhibitor depudecin from *Alternaria brassicicola*. Mol Plant Microbe In 22:1258-1267.
35. Dalmais B, Schumacher J, Moraga J, Le Pêcheur P, Tudzynski B, *et al.* (2011) The *Botrytis cinerea* phytotoxin botcinic acid requires two polyketide synthases for production and has a redundant role in virulence with botrydial. Mol Plant Pathol 12:564-579.
36. Fujii I, Yoshida N, Shimomaki S, Oikawa H, Ebizuka Y (2005) An iterative type I polyketide synthase PKSN catalyzes synthesis of the decaketide alternapyrone with regio-specific octa-methylation. Chem Biol 12:1301-1309.
37. Baker SE, Kroken S, Inderbitzin P, Asvarak T, Li BY, *et al.* (2006) Two polyketide synthase-encoding genes are required for biosynthesis of the polyketide virulence factor, T-toxin, by *Cochliobolus heterostrophus*. Mol Plant Microbe In 19:139-149.
38. Tokuoka M, Seshime Y, Fujii I, Kitamoto K, Takahashi T, *et al.* (2008) Identification of a novel polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) gene required for the biosynthesis of cyclopiazonic acid in *Aspergillus oryzae*. Fungal Genet Biol 45:1608-15.
39. Bushley KE, Turgeon BG (2010) Phylogenomics reveals subfamilies of fungal nonribosomal peptide synthetases and their evolutionary relationships. BMC Evol Biol 10:26.
40. Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B (2005) A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol Microbiol 57:1036-1050.

41. Chettri P, Ehrlich KC, Cary JW, Collemare J, Cox MP, *et al*. (2013) Dothistromin genes at multiple separate loci are regulated by AflR. Fungal Genet Biol 51:12-20.
42. Bradshaw RE, Slot JC, Moore GG, Chettri P, de Wit PJ, *et al*. (2013) Fragmentation of an aflatoxin-like gene cluster in a forest pathogen. New Phytol 198:525-535.
43. Chung KR, Liao HL (2008) Determination of a transcriptional regulator-like gene involved in biosynthesis of elsinochrome phytotoxin by the citrus scab fungus, *Elsinoë fawcettii*. Microbiol 154:3556-3566.
44. Chen H, Lee MH, Daub ME, Chung KR (2007) Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae*. Mol Microbiol 64:755-770.
45. Winterberg B, Uhlmann S, Linne U, Lessing F, Marahiel MA, *et al*. (2010) Elucidation of the complete ferrichrome A biosynthetic pathway in *Ustilago maydis*. Mol Microbiol 75:1260-1271.
46. Yuan WM, Gentil GD, Budde AD, Leong SA (2001) Characterization of the *Ustilago maydis sid2* gene, encoding a multidomain peptide synthetase in the ferrichrome biosynthetic gene cluster. J Bacteriol 183:4040-4051.
47. Munawar A, Marshall JW, Cox RJ, Bailey AM, Lazarus CM (2013) Isolation and characterisation of a ferrirhodin synthetase gene from the sugarcane pathogen *Fusarium sacchari*. ChemBioChem 14:388-394.
48. Davies DG, Hodge P (1974) Chemistry of quinones. 5. Structure of cladofulvin, a bianthraquinone from *Cladosporium fulvum* Cooke. J Chem Soc Perk T 1:2403-2405.
49. Amselem J, Cuomo CA, van Kan JA, Viaud M, Benito EP, *et al*. (2011) Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. PLoS Genet 7:e1002230.
50. Johnson L (2008) Iron and siderophores in fungal-host interactions. Mycol Res 112:170-183.
51. Hof C, Eisfeld K, Welzel K, Antelo L, Foster AJ, *et al*. (2007) Ferricrocin synthesis in *Magnaporthe grisea* and its role in pathogenicity in rice. Mol Plant Pathol. 8:163-172.
52. Langfelder K, Streibel M, Jahn B, Haase G, Brakhage AA (2003) Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet Biol 38:143-158.
53. Chiang YM, Meyer KM, Praseuth M, Baker SE, Bruno KS, *et al*. (2011) Characterization of a polyketide synthase in *Aspergillus niger* whose product is a precursor for both dihydroxynaphthalene (DHN) melanin and naphtho-γ-pyrone. Fungal Genet Biol 48:430-437.
54. Martin F, Kohler A, Murat C, Balestrini R, Coutinho PM, *et al*. (2010) Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature 464:1033-1038.
55. Duplessis S, Cuomo CA, Lin YC, Aerts A, Tisserant E, *et al*. (2011) Obligate biotrophy features unraveled by the genomic analysis of rust fungi. P Natl Acad Sci USA 108:9166-9171.

Supporting information

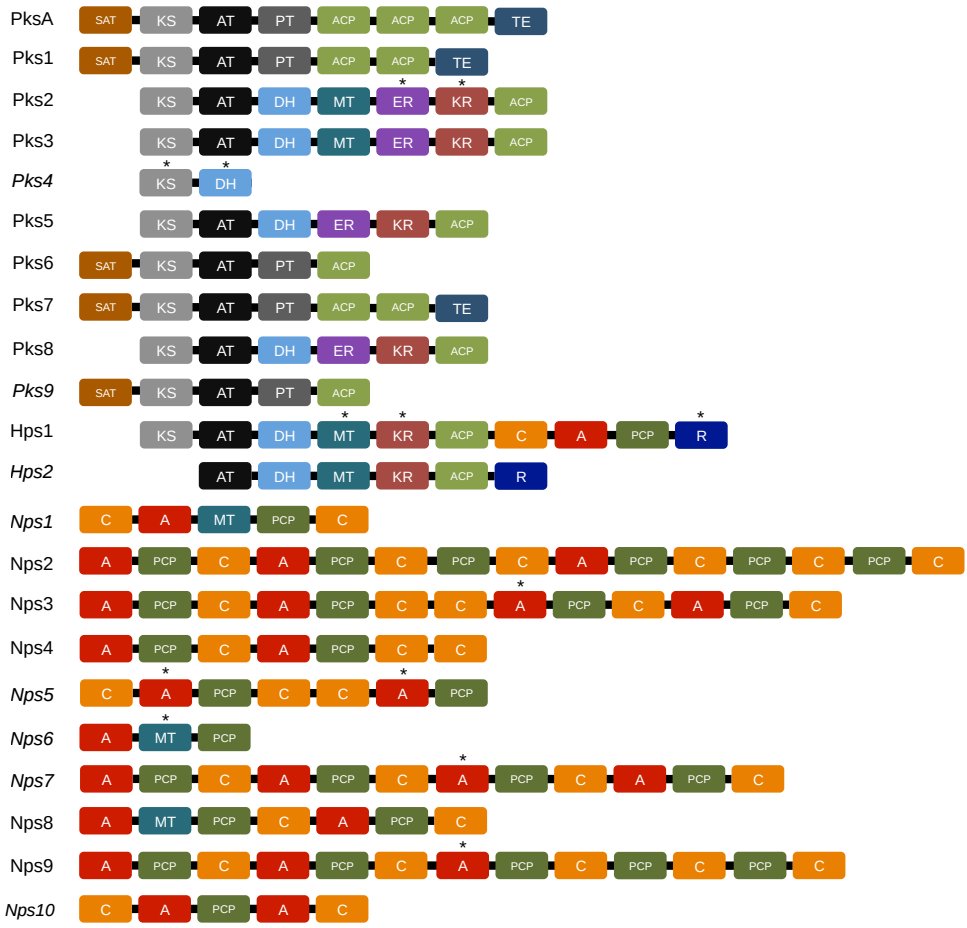

Figure S1. Domain organization of *Cladosporium fulvum* polyketide synthases and non-ribosomal peptide synthetases. SAT: starter unit:ACP transacylase; KS: keto-synthase; AT: acyl transferase; PT: product template; ACP: acyl carrier protein; TE: thiolesterase; DH: dehydratase; ER: enoyl reductase; KR: keto-reductase; MT: C- or N-methyl transferase; R: reductase; A: adenylation; PCP: peptidyl carrier protein; C: condensation. Predicted non-functional enzymes are indicated in italics. Stars indicate domains that are likely not functional because conserved catalytic residues are mutated (data not shown).

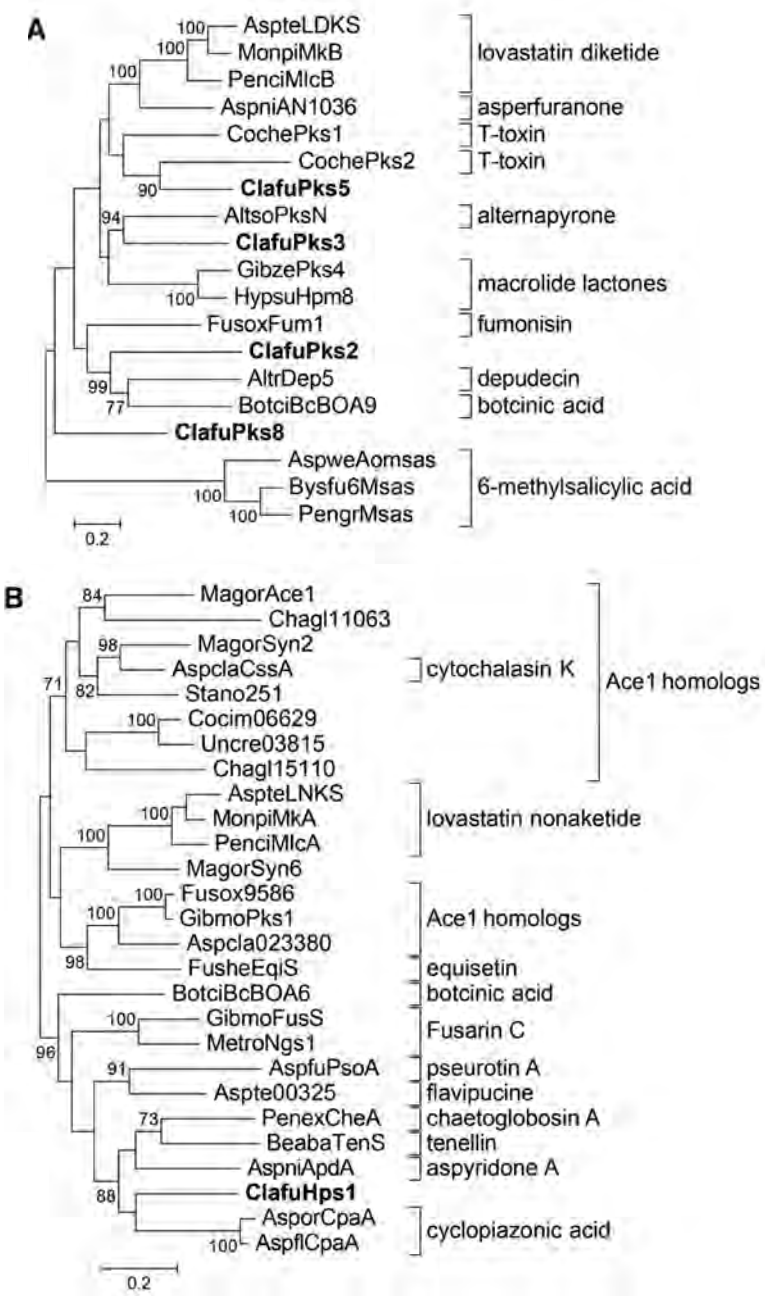

Figure S2. Phylogenetic analysis of *Cladosporium fulvum* PKS and hybrid PKS-NRPS enzymes. Alignments of (**A**) full-length protein sequences of reducing polyketide synthases (PKS) and (**B**) KS and AT domains of hybrid polyketide synthase-non-ribosomal peptide synthetases (PKS-NRPS) were used to construct maximum likelihood phylogenetic trees. Only bootstrap values over 70 are shown. *C. fulvum* secondary metabolism enzymes are indicated in bold. Accession numbers are given in Table S1 and Table S4.

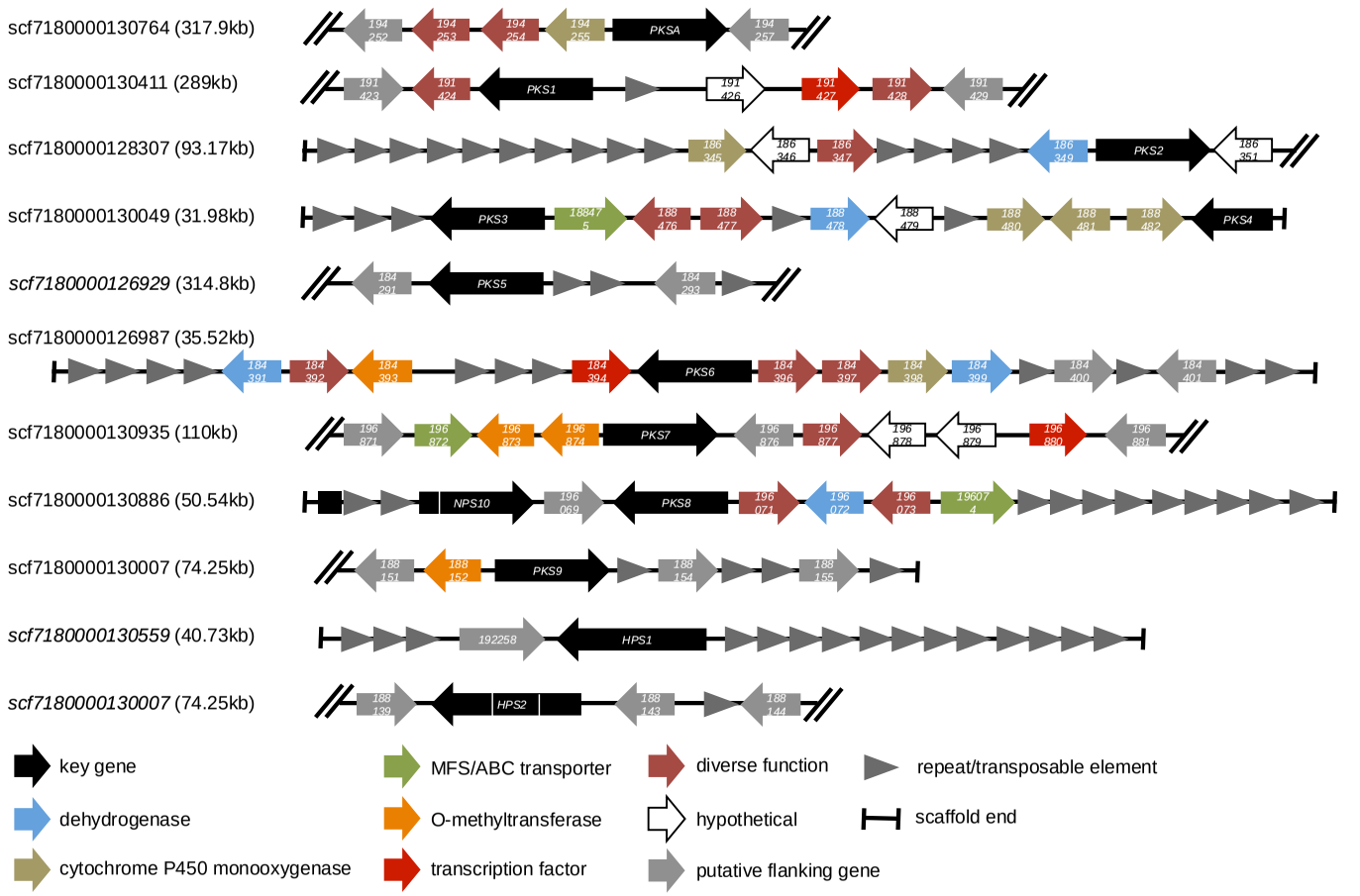

Figure S3. Locus organization of polyketide synthase and hybrid genes in the *Cladosporium fulvum* genome. White lines within key genes indicate pseudogenization. The PKSA gene cluster is fully depicted in De Wit *et al.* (2012) and Chettri *et al.* (2013).

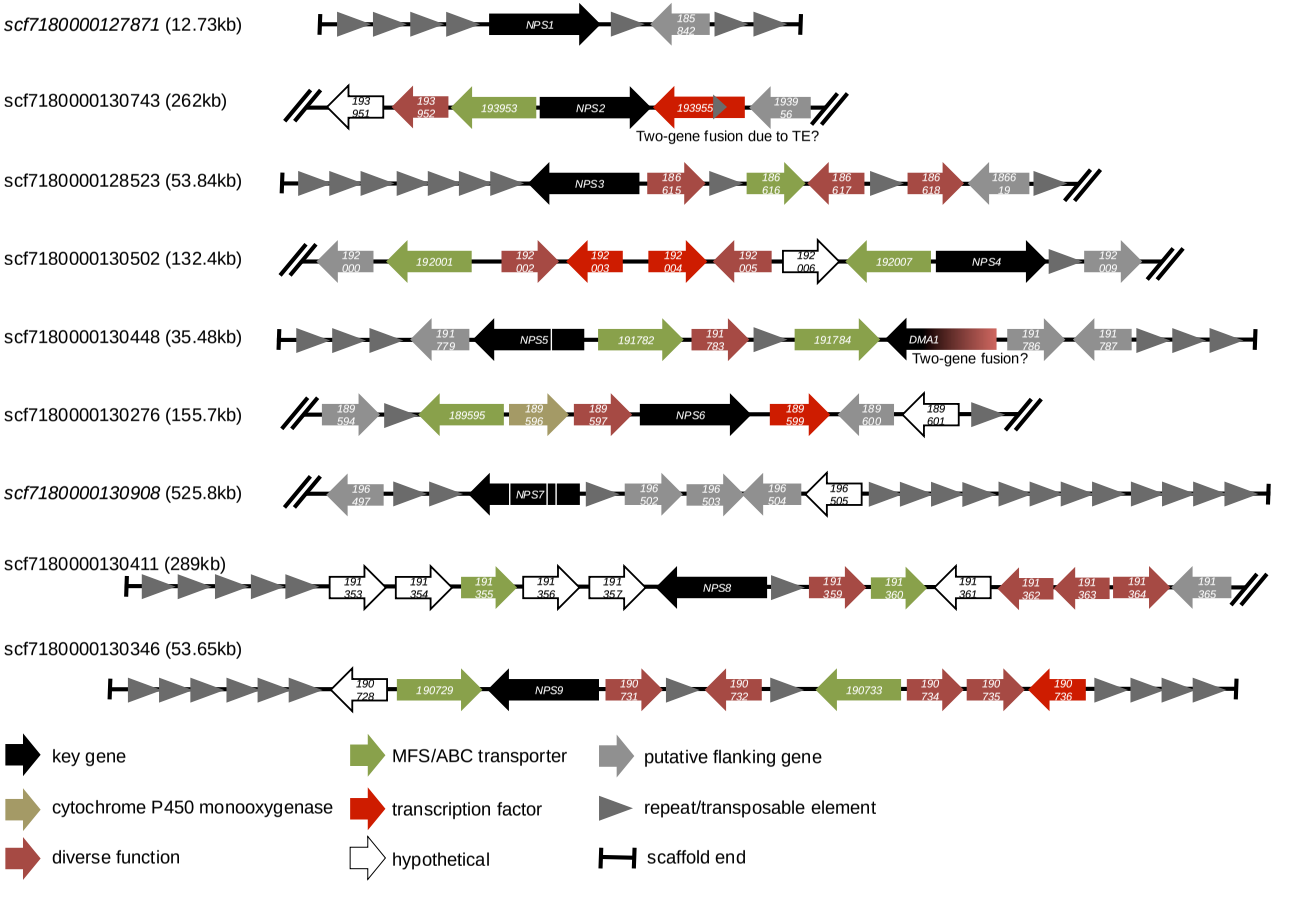

Figure S4. Locus organization of non-ribosomal peptide synthetase genes in the *Cladosporium fulvum* genome. White lines within key genes indicate pseudogenization.

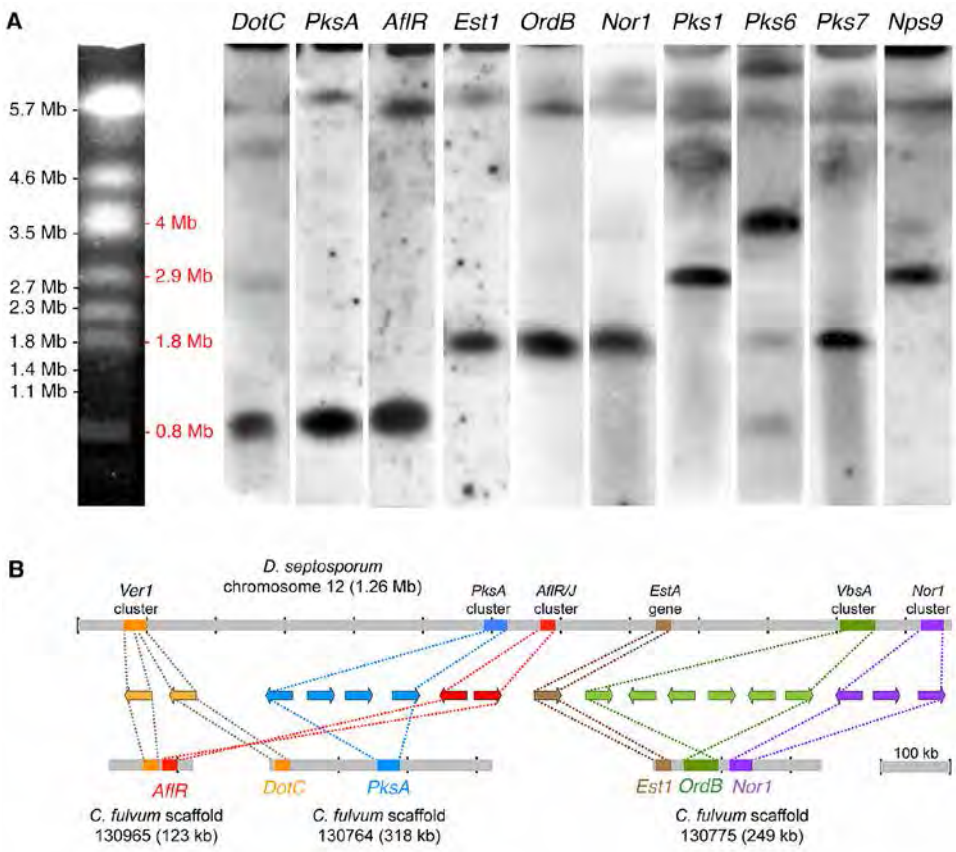

Figure S5. Localization of secondary metabolism genes on chromosomes of *Cladosporium fulvum*. (**A**) CHEF gel electrophoresis was performed to separate chromosomes of *C. fulvum* (left lane) and large size DNA was transferred onto nylon membrane. Specific probes corresponding to genes from different clusters were hybridized to identify chromosomes that carry them. Size standards indicated on the left are from chromosome preparations of *Schizosaccharomyces pombe* and *Hansenula wingei*. In red are indicated the size of the *C. fulvum* chromosomes that carry secondary metabolism genes. (**B**) Organization of the dothistromin gene cluster is shown in *Dothistroma septosporum* and *C. fulvum* (adpated from de Wit *et al.*, 2012).

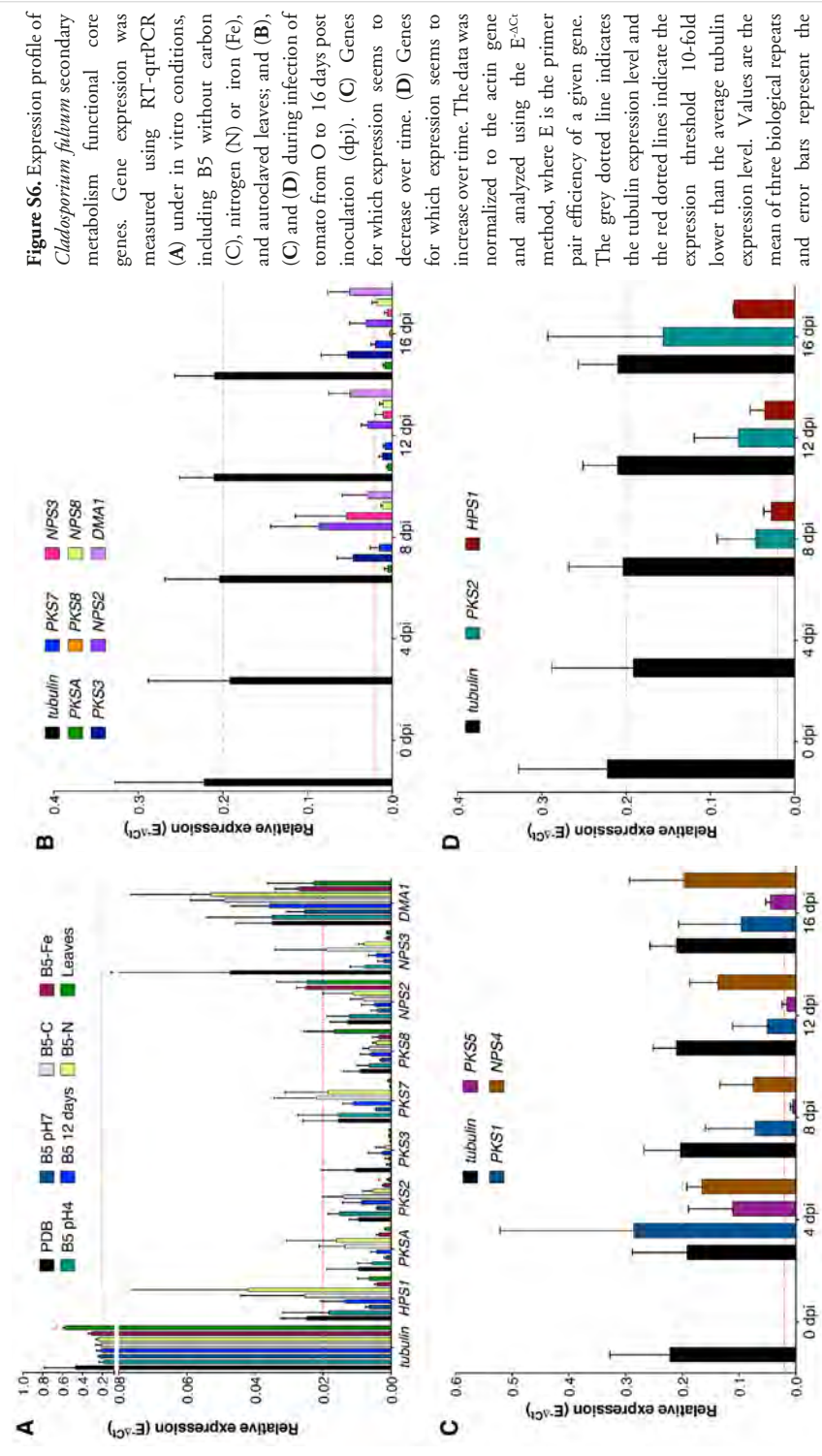

Figure S6. Expression profile of *Cladosporium fulvum* secondary metabolism functional core genes. Gene expression was measured using RT-qrtPCR (**A**) under in vitro conditions, including B5 without carbon (C), nitrogen (N) or iron (Fe), and autoclaved leaves; and (**B**), (**C**) and (**D**) during infection of tomato from O to 16 days post inoculation (dpi). (**C**) Genes for which expression seems to decrease over time. (**D**) Genes for which expression seems to increase over time. The data was normalized to the actin gene and analyzed using the $E^{-\Delta Ct}$ method, where E is the primer pair efficiency of a given gene. The grey dotted line indicates the tubulin expression level and the red dotted lines indicate the expression threshold 10-fold lower than the average tubulin expression level. Values are the mean of three biological repeats and error bars represent the standard deviation.

Expression in each *in vitro* condition was compared to expression in B5 pH4 using multiple t-tests , not assuming consistent SD, correcting for multiple comparisons with the Holm-Sidak method, at the alpha significance threshold of 0.05. For each gene, each *in planta* time point was compared to the previous one using a Two-way ANOVA followed by a multiple comparison test corrected with the Holm-Sidak method at the alpha significance threshold of 0.05. Significant differences are indicated by red stars.

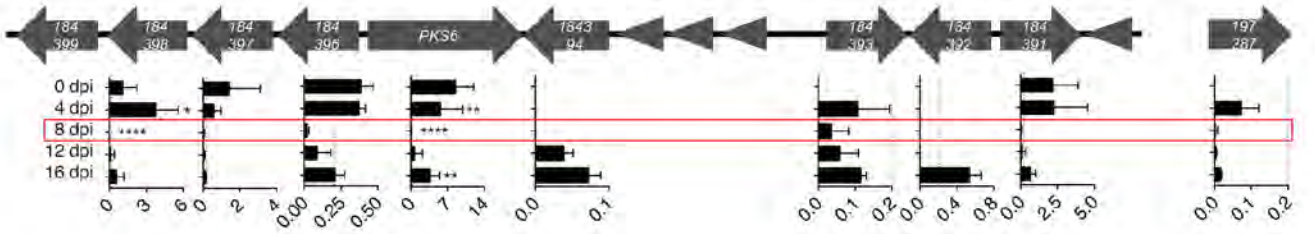

Figure S7. Expression profile of genes located at the *PKS6* gene locus.
Gene expression was measured using RT-qrtPCR during infection of tomato from 0 to 16 days post inoculation (dpi). The data was normalized to the actin gene and relative expression is indicated on the X axis using the $E^{-\Delta Ct}$ method, where E is the primer pair efficiency of a given gene. The grey dotted line indicates the tubulin expression level. The red square indicates the time point when the expression of genes is at the lowest level.

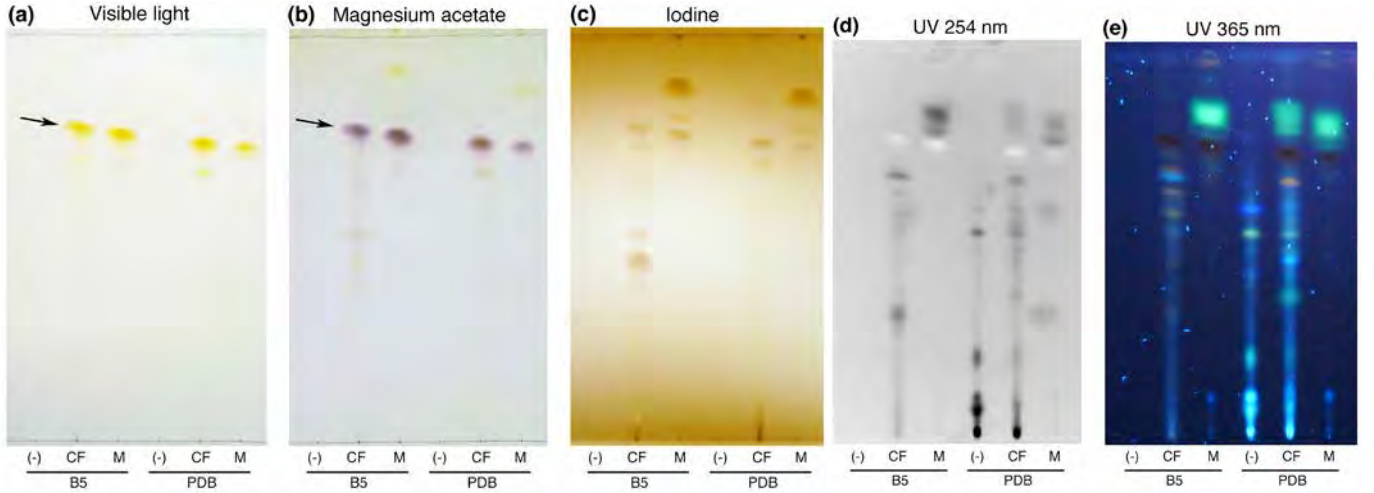

Figure S8. Thin layer chromatography (TLC) analysis of *Cladosporium fulvum* secondary metabolites. TLC plate pictures were taken (**a**) under visible light, (**b**) after staining with 4% magnesium acetate in methanol, (**c**) after iodine staining, (**d**) under 254 nm UV light and (**e**) under 365 nm UV light. Arrows indicate the position of cladofulvin. (-) control (sterile medium); CF, culture filtrate ; M, mycelium.

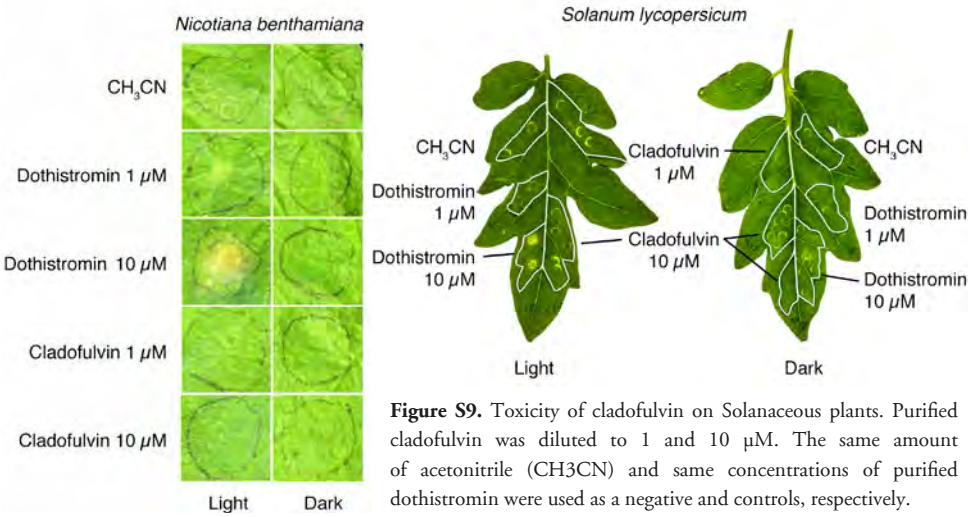

Figure S9. Toxicity of cladofulvin on Solanaceous plants. Purified cladofulvin was diluted to 1 and 10 μM. The same amount of acetonitrile (CH3CN) and same concentrations of purified dothistromin were used as a negative and controls, respectively.

Table S1. Accession numbers of protein sequences used in phylogenetic analyses.

Core enzyme	Fungal species	GenBank accession number
SormaPks	*Sordaria macrospora*	CAM35471.1
PodanPks1	*Podospora anserina*	XP_001910795.1
MagorAlb1	*Magnaporthe oryzae*	XP_003715434.1
NodspPks1	*Nodulisporium sp. ATCC74245*	AAD38786.1
CollaPks1	*Colletotrichum lagenaria*	BAA18956.1
GlaloPks1	*Glarea lozoyensis*	AAN59953.1
ElsfaPks1	*Elsinoe fawcettii*	ABU63483.1
WandePks1	*Wangiella dermatidis*	AAD31436.3
DotsePksA	*Dothistroma septosporum*	EME39092.1
AspniStcA	*Aspergillus nidulans*	AAC49191.1
AspflPksA	*Aspergillus flavus*	AAS90093.1
CerniCtb1	*Cercospora nicotianae*	AAT69682.1
NechaPKSN	*Nectria haematococca*	AAS48892.1
AspniYwa1	*Aspergillus nidulans*	CAA46695.2
AspniAlbA	*Aspergillus niger*	EHA28527.1
AspfuAlb1	*Aspergillus fumigatus*	AAC39471.1
GibzePks12	*Gibberella zeae*	AAU10633.1
GibfuBik1	*Gibberella fujikuroi*	CAB92399.1
AspteAcas	*Aspergillus terreus*	XP_001217072.1
AspniMdpG	*Aspergillus nidulans*	XP_657754.1
AspfuEncA	*Aspergillus fumigatus*	XP_746435.1
GibzePks13	*Gibberella zeae*	ABB90282.1
HypsuHpm3	*Hypomyces subiculosus*	ACD39762.1
MonpuPksCT	*Monascus purpureus*	BAD44749.1
AspniAN1034	*Aspergillus nidulans*	EAA65602.1
PenbrMpaC	*Penicillium brevicompactum*	ADY00130.1
GibzeFsl1	*Gibberella zeae*	XP_390640.1
CocheNps1	*Cochliobolus heterostrophus*	AAX09983.1
CocheNps3	*Cochliobolus heterostrophus*	AAX09985.1
FuseqEsyn1	*Fusarium equiseti*	Q00869.2
UstmaSid2	*Ustilago maydis*	AAB93493.1
MagorSsm1	*Magnaporthe oryzae*	XP_003719607.1
CocheNps2	*Cochliobolus heterostrophus*	AAX09984.1
SchpoSib1	*Schizosaccharomyces pombe*	CAB72227.1
AspfuSidC	*Aspergillus fumigatus*	XP_753088.1
UstmaFer3	*Ustilago maydis*	DAA04939.1
OmpolFso1	*Omphalotus olearius*	AAX49356.1
TriviTex1	*Trichoderma virens*	AAM78457.1
ClapuLpsA1	*Claviceps purpurea*	AET79183.1
CocheNps4	*Cochliobolus heterostrophus*	AAX09986.1
AspfuPes1	*Aspergillus fumigatus*	XP_752404.1
CoccaHts1	*Cochliobolus carbonum*	Q01886.2
AspfuPesL	*Aspergillus fumigatus*	XP_751084.1
AltalAmt	*Alternaria alternata*	AAF01762.1
CocheNps5	*Cochliobolus heterostrophus*	AAX09987.1
MagorSsm2	*Magnaporthe oryzae*	XP_003714007.1

Table S1 (continued). Accession numbers of protein sequences used in phylogenetic analyses.

Core enzyme	Fungal species	GenBank accession number
GibzeNps6	*Gibberella zeae*	XP_383923.1
CocheNPS6	*Cochliobolus heterostrophus*	AAX09988.1
AspfuSidD	*Aspergillus fumigatus*	XP_748662.1
CocheNps8	*Cochliobolus heterostrophus*	AAX09990.1
EpifePerA	*Epichloe festucae*	BAE06845.2
AspteLDKS	*Aspergillus terreus*	AAD34559.1
MonpiMkB	*Monascus pilosus*	ABA02240.1
PenciMlcB	*Penicillium citrinum*	BAC20566.1
AspniAN1036	*Aspergillus nidulans*	EAA65604.1
CochePks1	*Cochliobolus heterostrophus*	AAB08104.3
CochePks2	*Cochliobolus heterostrophus*	ABB76806.1
AltsoPksN	*Alternaria solani*	BAD83684.1
GibzePks4	*Gibberella zeae*	ABB90283.1
HypsuHpm8	*Hypomyces subiculosus*	ACD39767.1
FusoxFum1	*Fusarium oxysporum*	ACB12550.1
AltbrDep5	*Alternaria brassicicola*	ACZ57548.1
BotciBcBOA9	*Botrytis cinerea*	CBX87032.1
AspweAomsas	*Aspergillus westerdijkiae*	AAS98200.1
Bysni6Msas	*Byssochlamys nivea*	AAK48943.1
PengrMsas	*Penicillium griseofulvum*	P22367.1
MagorAce1	*Magnaporthe oryzae*	CAG28797.1
Chagl11063	*Chaetomium globosum*	XP_001220460.1
MagorSyn2	*Magnaporthe oryzae*	CAG28798.1
AspclaCssA	*Aspergillus clavatus*	XP_001270543.1
Stano251	*Stagonospora nodorum*	XP_001790998.1
Cocim06629	*Coccidioides imitis*	XP_001242733.1
Uncre03815	*Uncinocarpus reesii*	EEP78969.1
Chagl15110	*Chaetomium globosum*	XP_001221381.1
AspteLNKS	*Aspergillus terreus*	Q9Y8A5.1
MonpiMkA	*Monascus pilosus*	ABA02239.1
PenciMlcA	*Penicillium citrinum*	BAC20564.1
MagorSyn6	*Magnaporthe oryzae*	CAG29113.1
Fusox9586	*Fusarium oxysporum*	EGU88865.1
GibmoPks1	*Gibberella moniliformis*	AAR92208.1
Aspcla023380	*Aspergillus clavatus*	XP_001269050.1
FusheEqiS	*Fusarium heterosporum*	AGO86662.1
BotciBcBOA6	*Botrytis cinerea*	CAP58786.1
GibmoFusS	*Gibberella moniliformis*	AAT28740.1
MetroNgs1	*Metarhizium robertsii*	ACS68554.1
AspfuPsoA	*Aspergillus fumigatus*	ABS87601.1
Aspte00325	*Aspergillus terreus*	EAU38971.1
PenexCheA	*Penicillium expansum*	CAO91861.1
BeabaTenS	*Beauveria bassiana*	CAL69597.1
AspniApdA	*Aspergillus nidulans*	XP_681681.1
AsporCpaA	*Aspergillus oryzae*	BAK26562.1
AspflCpaA	*Aspergillus flavus*	BAI43678.1

Table S2. Oligonucleotides used in this study.

Name	Sequence (5' to 3')	Name	Sequence (5' to 3')
qHPS1_F	CTTGCCAGTGGGTCTACCAT	*q197287_F*	AGATCCGGCGTGAATACAAC
qHPS1_R	TAGGATCACTTCGCCTGCTT	*q197287_R*	TTCCTGCCAGCTTGACTTTT
qPKSA_F	TCCCGGCTCAGATTGATAAC	*q184391_F*	AGCTTCGGTCATCTCAAGGA
qPKSA_R	CCGACGTATAGAGGCTGCTC	*q184391_R*	CGAGTGTCGAGGAACACTGA
qPKS1_F	GTGATGCACTGAAGGCTCAA	*q184392_F*	CAGTTCCAAAGCCTGCCTAC
qPKS1_R	AGCAAGTTGGTCGAGCTGAT	*q184392_R*	AGGAAGTGTGGACTGGATGC
qPKS2_F	TGTGGCTATTGCACTCGAAG	*q184393_F*	GTGGTGGATTTCAGCCTGTT
qPKS2_R	TCCATTGATCTGATGCCGTA	*q184393_R*	TCATCGACGATTGTGGTGTT
qPKS3_F	GCGTAGGTCAGGCTGCTATC	*q184394_F*	TCTGTCTAGACGGCGAGGAT
qPKS3_R	CGAGTGAGTTGAGGACGACA	*q184394_R*	CTTCGAAGATCCGTTTCGAG
qPKS5_F	TGCTGGTATCGTGGGTAACA	*q184396_F*	GGACCTGGAGCATCACATCT
qPKS5_R	CAGAGTTCTCGGCCAGGTAG	*q184396_R*	GGATGGTGTCACCCGTAAAC
qPKS6_F	CTGCATATCGGAGCAGTGAA	*q184397_F*	CTTGTCAAGGTCATGCGAGA
qPKS6_R	TTGCGTTTCTTGAAGTCGTG	*q184397_R*	GGCAGATCGCTTGAGTATCC
qPKS7_F	AGCTGAAGAACGGAAGTGGA	*q184398_F*	GATGATCGGACACTGGACCT
qPKS7_R	GTGTTCTGTTGGGCACAATG	*q184398_R*	CACACCCAAAGGCGTAAGAT
qPKS8_F	TGACATCGCAGACTTCCTTG	*q184401_F*	AACAGCAGAAAGGACGGAAA
qPKS8_R	AGGGCAAAGGAAGCGATATT	*q184401_R*	GAGTTCCTGGGTTCCTCTCC
qNPS2_F	TGGACTCACAGCGCACTATC	*q184402_F*	AACAGCAGAAAGGACGGAAA
qNPS2_R	ATACGGACGGTCTTGTCTGG	*q184402_R*	GAGTTCCTGGGTTCCTCTCC
qNPS3_F	GTACACTTGTGGCGGATGTG		
qNPS3_R	TCATGTACGCTGGAAGCAAG		
qNPS4_F	GTGGCTCTAGCGGCATACTC		
qNPS4_R	TCCTGCCAGTAGCTGGTCTT		
qNPS6_F	AATGGCTAAAACACGCCATC		
qNPS6_R	TCCAACGAATTCCAGACTCC		
qNPS8_F	ACTCTCTTCGTTTGGCAGGA		
qNPS8_R	CTCATGAGCTTGCGTTGGTA		
qNPS9_F	ATATGGCCCGACTCACTACG		
qNPS9_R	CAGTGAGCGATTCTGTTGGA		
qDMA1_F	TGTGCTGGTACTGCCTTCAG		
qDMA1_R	CCGTCTTGTTGACATGTTGC		

Oligonucleotides used to measure *actin*, *tubulin*, *Avr4* and *Avr9* gene expression are the same as in de Wit *et al.* (2012).

Table S3. ^{1}H and ^{13}C NMR data for cladofulvin.

The NMR data collected for the purified compound is identical to the values reported for cladofulvin in the literature.

Position	δC	δH
1	129.3	-
2	149.3	-
3	124.7	7.35, s, 1H
4	162.3	-
5	115.2	-
6	181.4	-
7	115.9	-
8	149.8	-
9	152.1	-
10	121.1	7.11, d, 1H, J = 8.2 Hz
11	121.4	7.46, d, 1H, J = 8.2 Hz
12	125.1	-
13	193.3	-
14	131.3	-
15	19.6	2.03, s, 3H
16	136.0	-
17	146.3	-
18	121.5	7.77, s, 1H, J = 8.1 Hz
19	132.3	-
20	180.9	-
21	124.9	-
22	121.4 (2xC)	7.76, d, 1H
23	120.9	7.21, d, 1H, J = 8.1 Hz
24	150.4	-
25	152.3	-
26	116.4	-
27	193.4	-
28	114.1	-
29	162.5	-
30	19.5	2.02, s, 3H

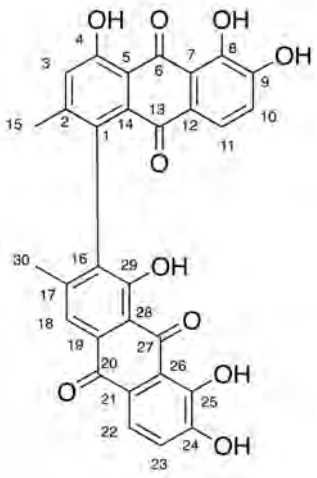

Table S4. Functional annotation of *Cladosporium fulvum* core secondary metabolism enzymes.

Gene	Protein ID[a]	Best BlastP hit (NCBI)[b]	e-value	IPS domains[c]	IGS domains[c]	ASMPKS domains[c]	Predicted precursor[d]
PKSA	194256	AAZ95017.1 polyketide synthase *Mycosphaerella pini*	0.0	KS-AT-ACP-ACP-ACP-TE	KS-AT-?-ACP-ACP-ACP-TE	KS-AT-ACP-ACP-ACP-TE	Malonyl-CoA
PKS1	191425	EFQ92987.1 hypothetical protein PTT_09773 *Pyrenophora teres*	0.0	KS-AT-ACP-ACP-TE	KS-AT-?-ACP-ACP-TE	KA-AT-ACP-ACP-TE	Malonyl-CoA
PKS2	186350	CBI52337.1 putative polyketide synthase *Sordaria macrospora*	0.0	KS-AT-ME-ER-KR-ACP	KS-AT-DH-?-?-KR-ACP	KS-AT-DH-KR-ACP	Methylmalonyl-CoA
PKS3	188474	AAR90260.1 polyketide synthase *Cochliobolus heterostrophus*	0.0	KS-AT-ME-ER-KR-ACP	KS-AT-DH-?-ER-KR	KS-AT-DH-ER-KR	Malonyl-CoA
PKS4	188483	XP_002482968.1 putative polyketide synthase *Talaromyces stipitatus*	1e-62	KS-DH	none	none	n.d.
PKS5	184292	XP_003042842.1 hypothetical NECHADRAFT_106474 *Nectria haematococca*	0.0	KS-AT-ER-KR-ACP	KS-AT-?-ER-KR	KS-AT-DH-ER-KR-ACP	Malonyl-CoA
PKS6	184395	XP_002482968.1 putative polyketide synthase *Talaromyces stipitatus*	0.0	KS-AT-ACP	KS-AT-?-ACP	KS-AT-ACP	Malonyl-CoA
PKS7	196875	ADO14690.1 cercosporin polyketide synthase *Mycosphaerella coffeicola*	0.0	KS-AT-ACP-ACP-TE	KS-AT-ACP-ACP-TE	KS-AT-ACP-ACP-TE	Malonyl-CoA
PKS8	196070	AAR90246.1 PKS10 *Botryotinia fuckeliana*	0.0	KS-AT-ER-KR-ACP	?-?-ER-KR-ACP	KS-AT-DH-ER-KR-ACP	Methylmalonyl-CoA
PKS9	188153	XP_657754.1 hypothetical protein AN0150.2 *Aspergillus nidulans*	0.0	KS-AT-ACP	KS-?-ACP	KS-AT-ACP	Malonyl-CoA
HPS1	192259	XP_002486604.1 putative PKS-NRPS *Talaromyces stipitatus*	0.0	KS-AT-ME-KR-ACP-C-A-PCP	KS-AT-?-?-KR-?-A-PCP	KS-AT-DH-KR-ACP	Malonyl-CoA / DMAFASVI new signature
HPS2	188140 / 188141 / 188142	XP_002153037.1 PMAA_009380 *Penicillium marneffei*	0.0	KS-AT-ME-KR-ACP	?-?-KR-ACP	AT-DH-KR-ACP	Malonyl-CoA
NPS1	185841	ACJ04424.1 aureobasidin A1 biosynthesis complex *Aureobasidium pullulans*	0.0	C-A-PCP-C	?-A-ME-PCP	-	DAWLYVAV CssA-M1-D-Ala|Cyclosporine synthetase CssA Val
NPS2	193954	AAD00581.2 peptide synthetase *Aureobasidium pullulans*	0.0	A-PCP-C-A-PCP-C-PCP-C-A-PCP-C-PCP-C-PCP-C	A1-PCP-?-A2-PCP-?-PCP-?-A3-PCP-?-PCP-?-PCP-?	-	A1: DVFELIMI Gly / A2: DVFSVAXX Ser/Ala / A3: DVLDIGGI N^5-hydroxy-N^5-acetyl-L-Orn

Table S4 (continued). Functional annotation of *Cladosporium fulvum* core secondary metabolism enzymes.

Gene	Protein ID[a]	Best BlastP hit (NCBI)[b]	e-value	IPS domains[c]	IGS domains[c]	ASMPKS domains[c]	Predicted precursor[d]
NPS3	186614	XP_002380231.1 putative NRPS *Aspergillus flavus*	0.0	A-PCP-C-A-PCP-C-C-A-PCP-C-A-PCP-C	A1-PCP-?-A2-PCP-?-?-PCP-?-A3-PCP	-	A1: DVWNLSTF EntF/SyrE Ser / A2: DVICVA-V new signature / A3: DVSYAGXX new signature
NPS4	192008	EFY94582.1 NRPS *Metarhizium anisopliae*	0.0	A-PCP-C-A-PCP-C-C	A1-PCP-?-A2-PCP-?	-	A1: DASDIAVP new signature / A2: DVSDVGPP new signature
NPS5	191780 / 191781	XP_002843341.1 NRPS *Arthroderma otae*	0.0	C-A-PCP-C-C-A-PCP	?-PCP-?-?-PCP	-	n.d.
NPS6	189598	XP_001267502.1 putative NRPS *Neosartorya fischeri*	8e-141	A-PCP	A-?-PCP	-	A: DAGDIGFP new signature
NPS7	196498 / 196499 / 196500 / 196501	Q01886.2 HC-toxin synthetase *Cochliobolus carbonum*	2e-84	A-C-A-A	A1-?-A2-A3	-	A1: DASFVIGF new signature / A2: DNQ-VGAI new signature / A3: DVYARGXX new signature
NPS8	191358	AAX09983.1 NPS1 *Cochliobolus heterostrophus*	0.0	A-PCP-C-A-PCP-C	A1-ME-PCP-?-A2-PCP	-	A1: DAMVVGGV TycB-M2-L-Phe/L-Trp|tyrocidine synthetase / A2: DGFFEGIP BacA/FenB Ile
NPS9	190730	XP_001821068.1 SidC *Aspergillus oryzae*	0.0	A-PCP-C-A-PCP-C-A-PCP-C-PCP-C-PCP-C	A1-PCP-?-A2-PCP-?-A3-PCP-?-PCP-?-PCP-?	-	A1: DPMMWMAI Ser / A2: DVQHTITV Gly / A3: DVGSSGAI N^5-hydroxy-N^5-acetyl-L-Orn
NPS10	196066 / 196067 / 196068	XP_002486632.1 putative aminoadipate-semialdehyde dehydrogenase *Talaromyces stipitatus*	2e-56	C-A-PCP-A-C	PCP	-	n.d.
DMA1	191785	XP_003298803.1 hypothetical protein PTT_09620 *Pyrenophora teres f. teres*	3e-128	IPR017795 Aromatic prenyltransferase, DMATS type	-	-	-

[a] Protein ID from the Doe Joint Genome Institute resource (www.jgi.doe.gov).

[b] BlastP was performed using the non-redundant protein database of NCBI (www.ncbi.nlm.nih.gov).

[c] Search for conserved domains was carried out with InterproScan (IPS; www.ebi.ac.uk), PKS/NRPS analysis website (IGS; nrps.igs.umaryland.edu) and ASMPKS (gate.smallsoft.co.kr:8008/~hstae/asmpks/pks_prediction.pl). KS: keto-synthase; AT: acyl transferase; DH: dehydratase; ER: enoyl reductase; KR: keto-reductase; ME: methyl transferase; ACP: acyl carrier protein; TE: thio-esterase; A: adenylation; C: condensation; PCP: peptidyl carrier protein.

[d] Prediction made by ASMPKS for acyl specificity and by PKS/NRPS analysis website for amino acid specificity. Signatures of A domains were also compared to those described for siderophore synthetases and bacterial synthetases. n.d.: not determined.

Chapter 3

Regulation of secondary metabolite production in the fungal tomato pathogen *Cladosporium fulvum*

Scott Griffiths[a]
Benedetta Saccomanno[a]
Pierre J.G.M. de Wit[a,*]
Jérôme Collemare[a,b,*]

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a Laboratory of Phytopathology, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands
b UMR1345 IRHS-INRA, 42 rue Georges Morel, 49071 Beaucouzé Cedex, France
* Corresponding authors: j.collemare@gmail.com; pierre.dewit@wur.nl

Abstract

Cladosporium fulvum is a non-obligate biotrophic fungal tomato pathogen for which fifteen secondary metabolite (SM) gene clusters were previously identified in its genome, with several possibly implicated in the production of toxins. However, most of these SM biosynthetic pathways remain cryptic during growth *in planta* and in different *in vitro* conditions. The sole SM produced *in vitro* is the pigment cladofulvin. In this study, we attempted to activate cryptic pathways in order to identify new compounds produced by *C. fulvum*. For this purpose, we manipulated orthologues of the global regulators VeA, LaeA and HdaA known to regulate SM biosynthesis in other fungal species. In *C. fulvum*, deleting or over-expressing these regulators yielded no new detectable SMs. Yet, quantification of cladofulvin revealed that CfHdaA is an activator while CfVeA and CfLaeA seemed to act as repressors of cladofulvin production. In the wild type strain, cladofulvin biosynthesis was affected by the carbon source, with highest production under carbon limitation and traces only in presence of saccharose. Repression of cladofulvin production by saccharose was dependent on both CfVeA and CfLaeA. Deletion of *CfVeA* or *CfLaeA* caused production of sterile mycelia, while Δ*cfhdaa* deletion mutants conidiated, suggesting that cladofulvin production is not linked to asexual reproduction. Profiling the transcription of these regulators showed that *CfHdaA*-mediated regulation of cladofulvin production is independent of both *CfVeA* and *CfLaeA*. Our data suggest CfLaeA directly affects cladofulvin production while the effect of CfVeA is indirect, suggesting a role for CfLaeA outside of the Velvet complex. In conclusion, our results showed that regulation of SM production in *C. fulvum* is different from other fungi and indicate that manipulation of global regulators is not a universal tool to discover new fungal natural products.

Key words: cladofulvin, cryptic biosynthetic pathways, LaeA, VeA, HdaA, global regulator

3.1 Introduction

From genomic analyses it has become clear that fungi are an under-exploited source of secondary metabolites (SMs). Many gene clusters related to SM biosynthesis have been identified in the genomes of filamentous fungi, yet this genetic potential rarely translates into an equivalent known chemical diversity because most SM biosynthetic pathways remain cryptic during *in vitro* cultivation (Collemare *et al.*, 2008; Brakhage & Schroeckh, 2011; Chiang *et al.*, 2011; Gressler *et al.*, 2011). Indeed, the potency and context-specific nature of SMs is such that their respective biosynthetic genes are tightly regulated by a complex and hierarchical network of regulatory proteins. Pathway-specific transcription factors affect the expression of individual gene clusters, and typically such regulators are encoded within the gene clusters they regulate (Keller & Hohn, 1997; Yu & Keller, 2005). In contrast, global regulators typically act on multiple gene clusters simultaneously, coordinating a global response to environmental stimuli (Yu and Keller, 2005). This global regulation includes responses to carbon source, by transcription factor CreA (Espeso & Peñalva, 1992); light, by VeA (Calvo *et al.*, 2004), VelB (Bayram *et al.*, 2008), FphA (Blumenstein *et al.*, 2005), and WC1-LreA (Atoui *et al.*, 2010); nitrogen, by transcription factor AreA (Cary *et al.*, 2006); pH, by transcription factor PacC (Ehrlich *et al.*, 1999); and oxylipins, by fatty acid oxygenases PpoA, B and C (Tsitsigiannis & Keller, 2006). In addition, SM gene clusters seem to be under control of chromatin modifying enzymes, such as histone methyltransferases Kmt6 (Connolly *et al.*, 2013) and LaeA (Bok & Keller, 2004); histone deacetylase, HdaA (Lee *et al.*, 2009); and chromatin-associated repressor, RcoA (Hicks *et al.*, 2001). Finally, signalling pathways that involve G-proteins and kinases are known to link development and SM production (Rosén *et al.*, 1999; Shimizu & Keller, 2001; Han *et al.*, 2004).

Several strategies have been employed to activate silent gene clusters. The over-expression of a local, pathway-specific regulator (*ApdR*) in *Aspergillus nidulans* resulted in the production of two novel polyketide synthase-non-ribosomal peptide synthetase (PKS–NRPS) hybrid metabolites, aspyridone A and B (Bergmann *et al.*, 2007). Similarly in *Aspergillus flavus*, over-expression of the transcription factor-expressing gene *LepE* resulted in the activation of a cryptic biosynthetic pathway that produces PKS-NRPS hybrid SMs leporins (Cary *et al.*, 2015). Co-cultivation of *Nigrospora sphaerica* and *Alternaria tenuissima* induced the production of the anti-fungal polyketide stemphyperylenol (Chagas *et al.*, 2013). Similarly, the interaction between *Streptomyces rapamycinus* and *Aspergillus fumigatus* induced the production of the meroterpenoids fumicyclin A and B (König *et al.*, 2013). In the search for new fungal SMs, epigenetic-based approaches are particularly appealing, as they tend to affect multiple SM gene clusters simultaneously. In *Fusarium graminearum*, deletion of the methyltransferase *KMT6* induced 19 out of 45 SM gene clusters, with a clear correlation between level of methylation and gene expression (Connolly *et al.*, 2013). In all species of *Aspergillus* tested, LaeA is a positive regulator of SM production. Approximately half of the SM gene clusters in *A. nidulans*, *A. fumigatus*, and *A. flavus* are controlled by *LaeA* (Sarikaya-Bayram *et al.*, 2015). Deletion of *LaeA* in *A. fumigatus* impairs gliotoxin production

(Bok *et al.*, 2005). In *A. nidulans,* the deletion of *LaeA* abolished sterigmatocystin, penicillin, and lovastatin biosynthesis, whereas its over-expression increased penicillin and lovastatin production (Bok & Keller, 2004). Furthermore, *LaeA* positively regulates the production of endocrocin in *A. nidulans* (Lim *et al.*, 2012). In *Penicillium citrinum*, *LaeA* is required for the production of the polyketide ML-236B (Baba *et al.*, 2012).

The action of LaeA appears to depend on another global regulator, VeA, a molecular hub that brings together functionally related proteins that control fungal development in response to light (Kim *et al.*, 2002). Similar to LaeA, VeA regulates SM production in a species-specific manner (Jain & Keller, 2013). Inactivation of *DsVeA* in *Dothistroma septosporum*, impaired the production of dothistromin (Chettri *et al.*, 2012). Deletion of *AcVeA* in *Acremonium chrysogenum* and *PcVelA* in *Penicillium chrysogenum*, impaired cephalosporin and penicillin production, respectively (Dreyer *et al.*, 2007; Kopke *et al.*, 2013). In contrast, deletion or over-expression of *FfVel1* in *Fusarium fujikuroi* stimulated and impaired the production of bikaverin, respectively (Wiemann *et al.*, 2010).

HdaA is a predicted histone deacetylase (HDAC) that regulates SM gene clusters by removing acetyl groups from the amino-terminal tail of core histones, and the effects inversely mirror those of LaeA (Lee *et al.*, 2009). Indeed, the deletion of *HdaA* in *A. nidulans* increased the biosynthesis of sterigmatocystin and penicillin (Lee *et al.*, 2009). Similarly, treatment of *Alternaria alternata* and *Penicillium expansum* with the HDAC-inhibitor trichostatin A resulted in the accumulation of several unidentified SMs, suggesting that this epigenetic regulation is common to divergent fungal species (Shwab *et al.*, 2007). In *A. fumigatus*, however, gliotoxin production was negatively affected by the deletion of *HdaA* and stimulated in over-expression mutants (Lee *et al.*, 2009). Furthermore, a protein complex involving the HDAC Rdp3 is implicated in the direct activation of stress-response genes in yeast (Nadal *et al.*, 2004), thus *HdaA* might be a positive and negative regulator. Although they mainly show opposite effects on SM gene clusters, it was found that LaeA- and HdaA-dependent regulations are not linked (Shwab et al., 2007). In addition to regulating the production of SMs, the velvet complex is also known to play an important role in fungal development. In *A. parasiticus*, the loss of *VeA* blocked the formation of sexual structures (sclerotia) and decreased conidiation (Calvo *et al.*, 2004). In *A. nidulans*, the loss of *VeA* also blocked sexual development, but conidial production was increased (Kato *et al.*, 2003).

Cladosporium fulvum is a non-obligate biotrophic tomato pathogen with a reported capacity to produce a large arsenal of diverse SMs (de Wit *et al.*, 2012). Despite the presence of seventeen predicted functional core SM genes, the anthraquinone cladofulvin is the only SM produced in quantifiable amounts during growth *in vitro* (Agosti *et al.*, 1962; Davies, 1974; Collemare *et al.*, 2014). Based on a phylogenetic analysis and the transcriptional profiling of SM core genes during cladofulvin production, the *PKS6* locus was strongly associated with biosynthesis of this metabolite (Collemare et al., 2014). This was confirmed in deletion mutants of the developmental regulator *CfWor1* in which cladofulvin is not detected and *PKS6* is down regulated (Ökmen et

al., 2014). The *PKS1* gene is the only core SM gene that was induced in these deletion mutants and was associated with the production of an unknown black pigment (Ökmen et al., 2014). Although *NPS8* and *NPS9* genes were expressed under iron starvation conditions and the *PKS5* gene was expressed under carbon starvation, their expression levels remain significantly lower to that of *PKS6* under all conditions (Collemare *et al.*, 2014). Other SM core genes remained silent during all tested conditions and no compounds other than cladofulvin could be isolated from *C. fulvum*. Homologues of *VeA*, *LaeA* and *HdaA* were identified in the genome of *C. fulvum* (de Wit *et al.*, 2012) and we hypothesized that manipulating them might activate cryptic biosynthetic pathways. We generated deletion mutants and transformants over-expressing these three global regulators and analysed their metabolic profiles in culture media supplemented with different carbon sources. We discuss the interplay between global regulators in *C. fulvum* on the regulation of cladofulvin production, and the suitability of global regulator manipulation as a general tool to activate cryptic SM biosynthetic pathways.

3.2 Materials and methods

3.2.1 Fungal strain used in this study

The sequenced *C. fulvum* race 0WU strain (CBS131901; de Wit et al., 2012) was used as wild type for fungal transformation. *C. fulvum* was propagated on half-strength potato dextrose agar (19.5 g L^{-1} PDA, Difco, and 15 g L^{-1} technical agar, Oxoid) at 20°C for two weeks to allow the production of conidia. Stocks of conidia were maintained in 25% glycerol at –80°C.

3.2.2 Nucleic acid methods

Genomic DNA was extracted from fungal mycelia prepared by growing conidia on potato dextrose broth (PDB, Difco) for seven days. The biomass was separated using Miracloth and ground using a pestle and mortar chilled with liquid nitrogen. DNA was then isolated using the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. All PCR amplifications were performed using Phusion Flash high-fidelity polymerase master mix (Thermo Scientific) according to the standard protocol given and using oligonucleotides from Table S1. Total RNA isolation and cDNA synthesis were performed as previously described (Mesarich *et al.*, 2014). Oligonucleotides for quantitative real-time PCR (qrtPCR) were designed using Primer3 Plus (Table S1) (Rozen & Skaletsky, 1999). Their efficiency and specificity were determined using *C. fulvum* gDNA dilutions. qrtPCR was performed with the Applied Biosystems 7300 Real Time PCR System and treated with the SDS v1.4.1 software (Applied Biosystems, Foster City, USA). Reactions were performed in 25 µL containing 100 ng (in 1 µL) of cDNA or genomic DNA as template, 12.5 µL Sensimix (Bioline, London, UK), 1 µL of each forward and reverse primer (7.5 µM) and 9.5 µL ddH_2O. The programme used was an initial 95°C denaturation step for

10 min followed by denaturation for 15 s at 95°C and annealing/extension for 45 s at 60°C for 40 cycles. Results of at least three biological repeats were analysed using the $2^{-\Delta\Delta}$Ct method (Livak & Schmittgen, 2002). The number of insertion events of the deletion cassette in transformants, reflected by the number of inserted hygromycin gene copies, was calculated using the single copy actin and tubulin genes as previously described (Mesarich et al., 2014).

3.2.3 Construction of plasmids for targeted gene deletion

Gene replacement constructs for Δ*cfhdaa*, Δ*cflaea* and Δ*cfvea* were prepared using the MultiSite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) according to the manufacturer's instructions. The upstream (US) and downstream (DS) regions flanking *CfHdaA*, *CfLaeA* and *CfVeA* were amplified using primers given in Table S1 and gel extracted using a Zymogen Gel DNA Recovery Kit (Baseclear). Purified US fragments were cloned into *pDONR P4-P1R using the BP reaction, yielding pP4-P1R_US_hdaA, pP4-P1R_US_laeA and pP4-P1R_US_veA.* Purified DS fragments were cloned into *pDONR P2-P3 using the BP reaction, yielding pP2R-P3_DS_hdaA, pP2R-P3_DS_laeA and pP2R-P3_DS_veA. To generate the final mutagenizing vector for each targeted gene, the plasmid pairs carrying each respective US and DS region for a given gene were used in an LR reaction with p221_GFP_HYG* (pDONR containing a cassette with *GFP* and hygromycin resistance marker genes; Ökmen et al., 2013) and the destination vector *pDEST R4-R3*. The LR reaction was catalyzed by the LR clonase II plus enzyme mix (Invitrogen), and 1 µL from each reaction was used to transform electrocompetent *E. coli* DH5α. Positive clones were grown, and their plasmids were extracted using a Zymogen Zyppy Plasmid Miniprep Kit (Baseclear) for verification by restriction digest. One correct plasmid was chosen from each reaction and named *pDest43-*Δ*hdaA*, *pDest43-*Δ*laeA*, or *pDest43-*Δ*veA*.

3.2.4 Construction of plasmids for gene over-expression

The *toxA* promoter fragment was removed from *pFBT029* (kindly provided by Pr. B.P.H.J. Thomma, Wageningen University) by digestion with *Sac*I and *Asc*I (Fermentas). The fragments were separated by electrophoresis on a 1% agarose gel, and the backbone was excised for purification with a Zymogen Gel DNA Recovery Kit (Baseclear), blunt ended using T4 DNA ligase and circularised using T4 DNA ligase (Promega). The *CfAvr9* promoter region (813nt upstream of the *CfAvr9* start codon) was amplified using the *AscI_Avr9_Promoter_Forward/PacI_Avr9_Promoter_Reverse* primer pair (Table S1). The amplicon and the promoterless-*pFBT029* plasmid were independently restricted using *Asc*I and *Pac*I (New-England Biolabs), purified using a Zymogen Gel DNA Recovery Kit (Baseclear), ligated using T4 DNA ligase (Promega), and used to transform chemically competent *E. coli* DH5α using the standard heat shock method. Plasmids were extracted from positive clones and correct insertion of the *CfAvr9* promoter region was verified by restriction digesting and sequencing (Macrogen). One correct plasmid was selected and named *pFBTS3*. Each global regulator gene (*CfHdaA*, *CfLaeA* and

CfVeA) was amplified from *C. fulvum* gDNA using the corresponding primers given in Table S1. Each forward primer contained a *Pac*I restriction site, and each reverse primer carried a *Not*I restriction site. Each gene was amplified, run on a 1% agarose gel and then excised for cleaning using a Zymogen Gel DNA Recovery Kit (Baseclear). Each amplicon and *pFBTS3* was restricted in a *Pac*I/*Not*I double digest (Fermentas FD), purified using a Zymogen Gel DNA Recovery Kit (Baseclear), and used in a subsequent ligation reaction with T4 DNA ligase (Promega). Each ligation reaction was used to transform chemically competent *E. coli* DH5α using the standard heat shock protocol. Positive clones were grown for plasmid extraction using a Zymogen Zyppy Plasmid Miniprep Kit (Baseclear). Correct plasmids were confirmed by a restriction digest and their inserts sequenced by Macrogen. One correct plasmid was chosen for each gene and named *pFBTS3-CfHdaA*, *pFBTS3-CfLaeA,* or *pFBTS3-CfVeA*.

3.2.5 *Agrobacterium tumefaciens*-mediated *Cladosporium fulvum* transformation and transformant screening

Agrobacterium tumefaciens AGL1 strain was transformed by electroporation with plasmids constructed to generate *C. fulvum* deletion mutants and over-expression transformants. Positive transformants were selected using LB agar with spectinomycin (100 μg mL^{-1}). *A. tumefaciens*-mediated transformation of *C. fulvum* was performed as previously described (Ökmen *et al.*, 2014). Selection of transformants was performed on PDA supplemented with hygromycin (100 μg mL^{-1}). Primers used to verify transformants and the double crossover authentic deletion mutants are given in Table S1. Three independent deletion mutants were chosen for further analyses, in addition to one single ectopic control for each genotype. One over-expressing transformant from *C. fulvum OE.CfHdaA, C. fulvum OE.CfLaeA,* and *C. fulvum OE.CfVeA* was chosen for further analyses.

3.2.6 Secondary metabolite extraction from *Cladosporium fulvum* strains grown on potato dextrose agar (PDA)

C. fulvum wild type, ectopic transformants and deletion mutants were grown on PDA plates for two weeks in the dark at 22°C. The agar was removed from each plate, divided with a scalpel and placed into two 50 mL tubes. Twenty-five mL of ethyl acetate was added to each sample, and the tubes were placed on a rotary shaker for 4 hours in a fume hood at room temperature. The organic extracts originating from the same samples were combined, passed through a Schleicher & Schuell 595½ folded paper and recovered into a clean glass tube. The extracts were concentrated to approximately 1 mL using a nitrogen flow, transferred to a micro-centrifuge tube and centrifuged at 14, 000 x *g* for 10 min. The ethyl acetate phase was recovered to a clean glass tube and then dried under a nitrogen flow. The residue was dissolved in 1 mL acetonitrile (ACN) and transferred to a 2 mL micro-centrifuge tube. The samples were centrifuged at 14, 000 x *g* for 10 min then transferred to a 1 mL clear glass shell vial (Waters) for analysis by HPLC.

3.2.7 Secondary metabolite extraction from *Cladosporium fulvum* strains grown on liquid media

Wild type *C. fulvum* and deletion mutants Δ*cfhdaa*, Δ*cflaea* and Δ*cfvea*, along with their respective ectopic controls were grown on *Aspergillus* complete medium (ACM) without any additional carbon source, and on ACM supplemented with 25 mM of either glucose, mannitol, saccharose, or xylose. In addition, the wild type was also grown on ACM supplemented with 25 mM fructose, 12.5 mM saccharose, or mixed 12.5 mM glucose and 12.5 mM fructose. The fungal strains were grown in a Cellstar 6-well cell culture plate (Greiner bio-one). Each well was filled with 3 mL of each medium containing 1x 10^6 conidia mL^{-1}, or in the case of the sterile strains (Δ*cflaea* and Δ*cfvea*), 100 µL of mycelia ground in water with a pestle and mortar. The cultures were incubated in the dark at 22°C for 12 days without agitation.

Conidia of the wild type and *OE.CfHdaA*, *OE.CfLaeA* and *OE.CfVeA* transformants were pre-cultured on 3 mL of ACM xylose for 14 days in 6-well plates as described above. Each pre-culture was filtered through Miracloth to recover mycelia, which were washed with water and then transferred to 3 mL of nitrogen limited Gambourg B5 medium to induce the *CfAvr9* promoter (Van den Ackerveken *et al.*, 1994). The cultures were incubated in at 22°C in the dark for 48 hours.

The biomass from each liquid culture was filtered through Miracloth, freeze-dried and weighed. The biomass and culture filtrate were combined in a 50 mL tube, and twice extracted with 10 mL ethyl acetate (Collemare et al., 2014). The organic phases were recovered, transferred to a clean glass tube and dried using a nitrogen flow. The residue was dissolved in 1 mL ACN and transferred to a 1 mL clear glass shell vial (Waters) for analysis by HPLC.

3.2.8 Detection of secondary metabolites and cladofulvin quantification using UV-HPLC

Prior to analysis by UV-HPLC, the samples dissolved in ACN were centrifuged at 20,000 x *g* for 5 min in a micro-centrifuge tube, and then transferred to a 1 mL clear glass shell vial (Waters). HPLC analysis with a Waters Symmetry reverse phase (RP) C18 column (Waters) was carried out on a Waters 600S system. Samples were eluted with a gradient of solvents (A) H_2O and (B) ACN (both containing 0.1% trifluoroacetic acid) at a flow rate of 1 mL min^{-1}. The following gradient was used: 0 min, A (95%); 10 min, A (10%); 12 min, A (10%), 15 min, A (0%), 16 min, A (95%), 20 min, A (95%). UV spectra were obtained using a 996 photodiode array detector set to detect absorbance in the 200 – 800 nm range. All reagents and solvents were of analytical and HPLC grade, respectively.

The identification of cladofulvin in SM extracts was guided by the retention time (RT) and UV spectrum of an authentic cladofulvin standard (Collemare *et al.*, 2014). Once identified, the concentration of cladofulvin was determined by measuring the surface area under each corresponding peak using the Empower Software (build 1154) and dividing by the response factor (RF) of pure cladofulvin. The concentration of cladofulvin was normalised to fungal dry weight and expressed as µg mg^{-1}.

3.3 Results

3.3.1 Identification of orthologues of global regulators of secondary metabolism and development in *Cladosporium fulvum*

Orthologues of *VeA* (Joint Genome Institute protein ID 188542), *LaeA* (protein ID 186126) and *HdaA* (protein ID 192224) were previously identified during the analysis of the *C. fulvum* genome (de Wit *et al.*, 2012). Gene predictions were confirmed by RT-PCR for *Clafu192224* only (Fig. S1). Gene prediction of *Clafu188542* has been revised and confirmed by RT-PCR and sequencing (Fig. S1), for it contained an initially wrongly predicted intron at the 3' end. Gene prediction of *Clafu186126* has also been revised based on RT-PCR and sequencing (Fig. S1), for it lacked the first intron and its third intron was wrongly predicted. Orthology of these three genes to characterized global regulators was confirmed by protein alignments and, for *Clafu186126*, by a phylogenetic analysis (Fig. S2 and Table S2). All three proteins contain predicted conserved motifs as described in characterized VeA, LaeA and HdaA proteins in other fungi (Fig. S2) (Kim *et al.*, 2002; Bok & Keller, 2004; Chettri *et al.*, 2012). Hereafter, we name these protein-encoding genes from *C. fulvum CfVeA*, *CfLaeA* and *CfHdaA* respectively.

Data from previous RNA-seq experiments showed that all three genes are expressed when the fungus grows in the dark in different synthetic media (Fig. 1; Mesarich et al., 2014; Ökmen et al., 2014). For each gene, expression levels are similar in wild type grown in the different media tested. *CfVeA* and *CfLaeA* are up-regulated in deletion mutants of the global regulator *CfWor1* grown on PDB medium (Fig. 1), in which *PKS6* is down-regulated and *PKS1* is highly up-regulated (Ökmen *et al.*, 2014). This suggests that these two PKS core genes at least might exhibit a *CfVeA*- and *CfLaeA*-dependent expression. In contrast, *CfHdaA* is not differentially expressed in this mutant compared to wild type.

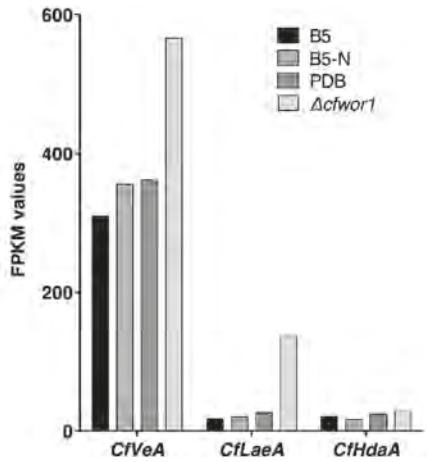


Figure 1. *In vitro* expression of global regulator genes *CfVeA*, *CfLaeA* and *CfHdaA*. *Cladosporium fulvum* wild type strain was grown on PDB for 7 days prior to cultivation in fresh PDB, B5 or B5-N (nitrogen starvation) media for 2 additional days. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values as determined with Cufflinks were previously obtained (Mesarich et al., 2014). FPKM values from Δ*cfwor1* deletion mutants grown on PDB were also previously obtained (Ökmen et al., 2014).

3.3.2 Manipulation of global regulators affects *Cladosporium fulvum* development and production of cladofulvin

In an attempt to activate cryptic SM biosynthetic pathways and to investigate the role of *CfVeA*, *CfLaeA* and *CfHdaA* in cladofulvin production, all three regulators were individually replaced by the hygromycin resistance selection marker by targeted gene replacement, yielding *C. fulvum* Δ*cfvea*, Δ*cflaea*, and Δ*cfhdaa*. Three independent deletion mutants and one ectopic transformant from each transformation experiment were confirmed and selected for further analyses (Fig. S3). Although no regulator was essential for vegetative growth on PDA (Fig. 2A), Δ*cfvea* and Δ*cflaea* deletion mutants were developmentally defective as they were not able to differentiate conidia. They produced sterile and flocculent colonies only.

The loss of *CfHdaA* did not overtly alter the morphology of *C. fulvum* mutants or their ability to conidiate (Fig. 2A). Each deletion mutant background gave rise to differently coloured colonies during propagation on PDA, suggesting that SM production was affected (Fig. 2A). The wild type strain and ectopic transformants were green-brown (Fig. 2A and Fig. S4). In contrast, Δ*cfhdaa* deletion mutants were blue-grey, and Δ*cfvea* and Δ*cflaea* deletion mutants were light and dark yellow, respectively (Fig. 2A). SMs were extracted from these PDA cultures directly using ethyl acetate and analysed by HPLC. The HPLC traces for wild type and ectopic transformants showed only a single major peak corresponding to cladofulvin based on its retention time and UV spectrum of a pure cladofulvin standard (Fig. 2B, Fig. S4 and Fig. S5). Cladofulvin was detected in all genetic backgrounds except for Δ*cfhdaa*, suggesting that *CfHdaA* positively regulates cladofulvin production on PDA (Fig. 2B and Fig. S5). Cladofulvin exhibits a yellow colour under acidic pH conditions, but turns dark purple under neutral to basic pH conditions (Davies, 1974; Collemare et al., 2014). Addition of a drop of 1M HCl provoked a slight yellow coloration of wild type and Δ*cfvea* deletion mutant (Fig. 2A). In contrast, addition of a drop of 1M HCl NaOH triggered a strong colour change from yellow to purple of the Δ*cfvea* and Δ*cflaea* deletion mutants only (Fig. 2A). It suggests that high amounts of cladofulvin are produced in both genetic backgrounds and that both mutants strongly acidified their growth medium. Upon these treatments, no colour change was observed for Δ*cfhdaa* deletion mutants, which is consistent with no cladofulvin produced (Fig. 2A). The absence of new compounds indicated that other SM biosynthetic pathways remained cryptic in these genetic backgrounds (Fig. S5).

In a species- and gene cluster-specific manner, *VeA*, *LaeA* and *HdaA* are known to induce or repress the production of SMs. Because of these opposite effects, wild type *C. fulvum* was also transformed with a plasmid containing each regulator fused to the promoter region of the nitrogen-inducible *CfAvr9* gene (Van den Ackerveken *et al.*, 1994), yielding *C. fulvum OE.CfVeA*, *OE.CfLaeA*, or *OE.CfHdaA*. Wild type and each transformant were grown in *Aspergillus* Complete Medium (ACM) supplemented with 25mM xylose liquid medium until mid-exponential phase (7 days), and then transferred to B5-N liquid medium to induce the *CfAvr9* promoter.

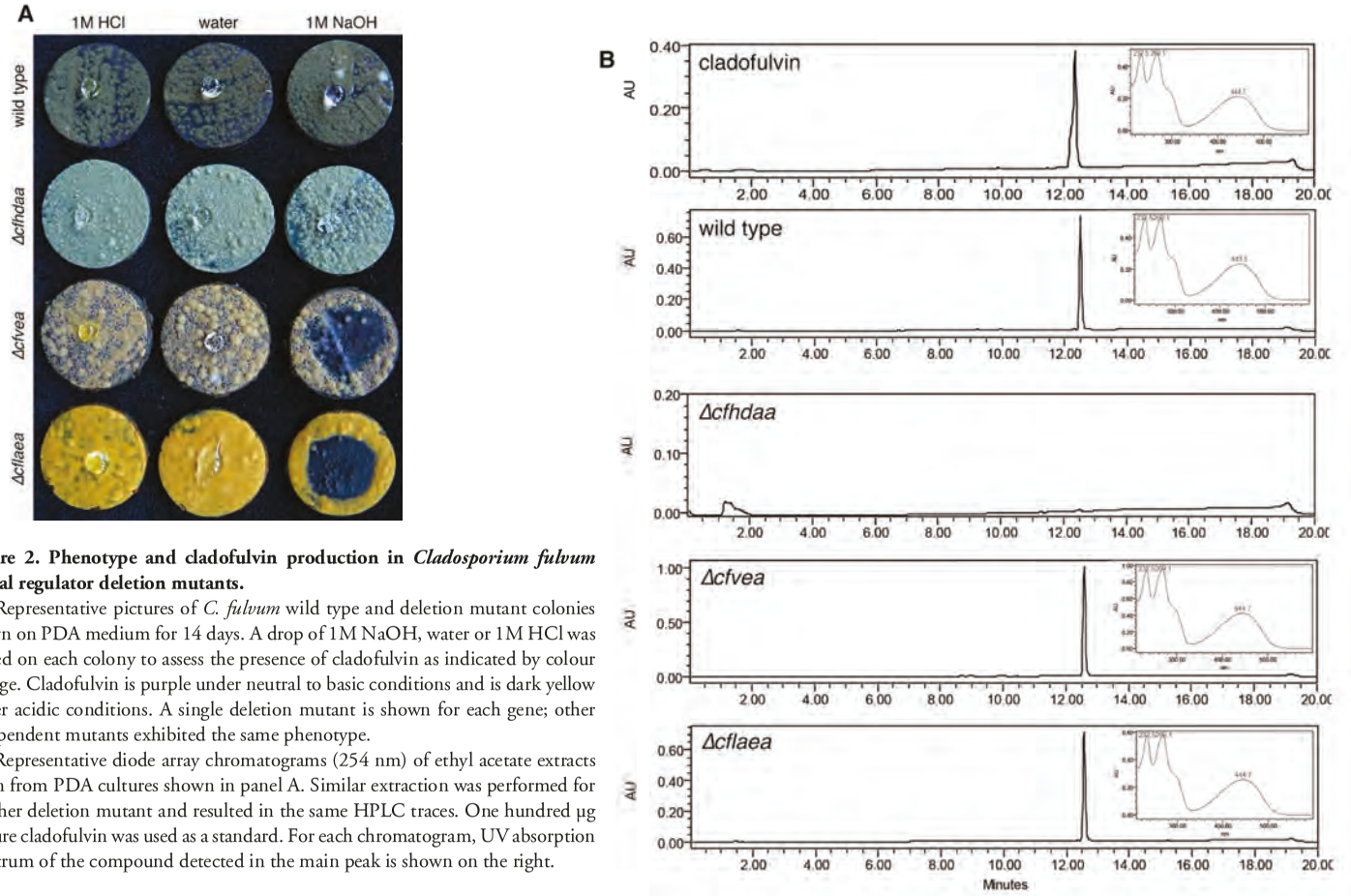


Figure 2. Phenotype and cladofulvin production in *Cladosporium fulvum* global regulator deletion mutants.

(A) Representative pictures of *C. fulvum* wild type and deletion mutant colonies grown on PDA medium for 14 days. A drop of 1M NaOH, water or 1M HCl was placed on each colony to assess the presence of cladofulvin as indicated by colour change. Cladofulvin is purple under neutral to basic conditions and is dark yellow under acidic conditions. A single deletion mutant is shown for each gene; other independent mutants exhibited the same phenotype.

(B) Representative diode array chromatograms (254 nm) of ethyl acetate extracts taken from PDA cultures shown in panel A. Similar extraction was performed for another deletion mutant and resulted in the same HPLC traces. One hundred µg of pure cladofulvin was used as a standard. For each chromatogram, UV absorption spectrum of the compound detected in the main peak is shown on the right.

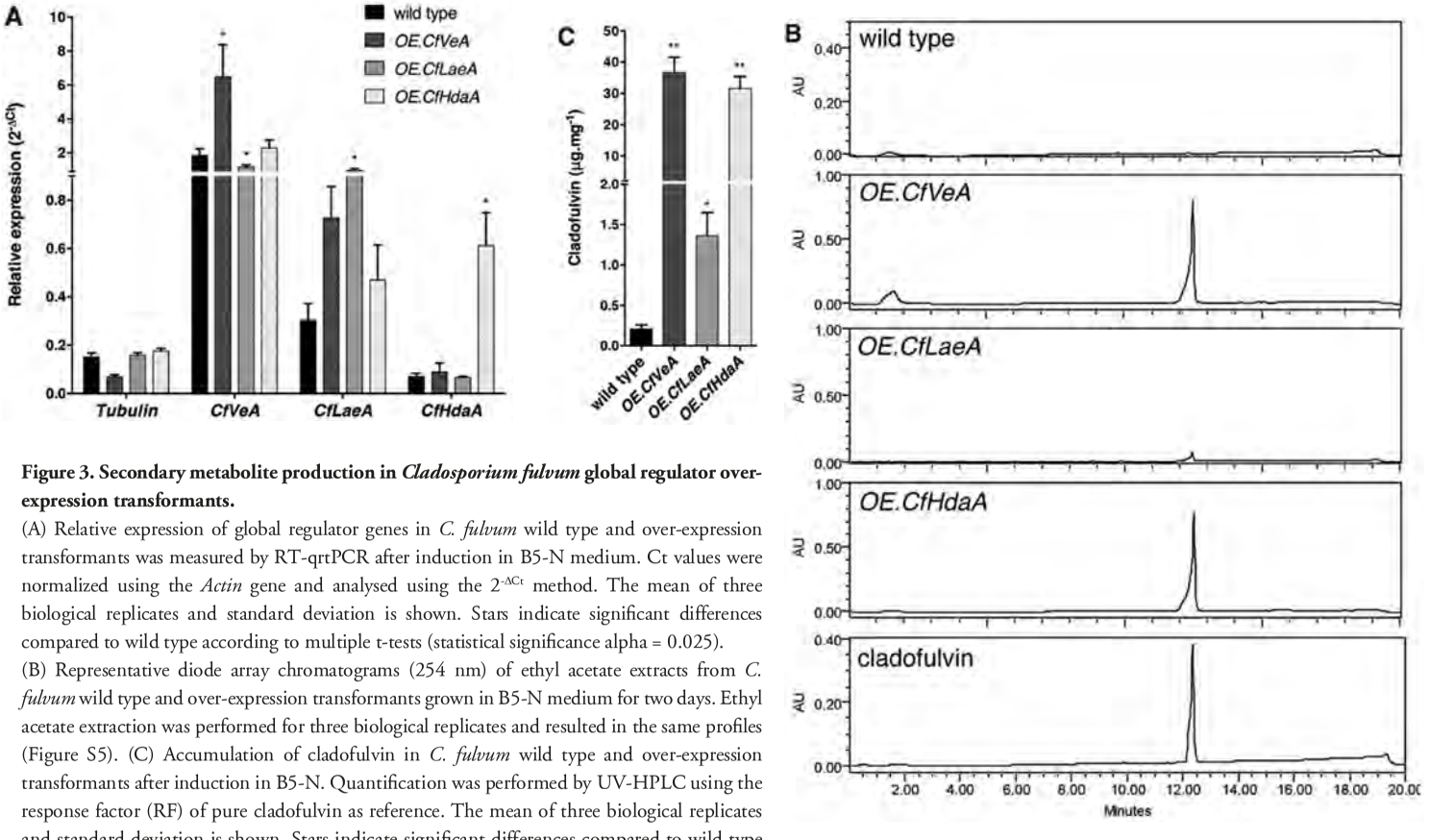


Figure 3. Secondary metabolite production in *Cladosporium fulvum* global regulator over-expression transformants.

(A) Relative expression of global regulator genes in *C. fulvum* wild type and over-expression transformants was measured by RT-qrtPCR after induction in B5-N medium. Ct values were normalized using the *Actin* gene and analysed using the $2^{-\Delta Ct}$ method. The mean of three biological replicates and standard deviation is shown. Stars indicate significant differences compared to wild type according to multiple t-tests (statistical significance alpha = 0.025).

(B) Representative diode array chromatograms (254 nm) of ethyl acetate extracts from *C. fulvum* wild type and over-expression transformants grown in B5-N medium for two days. Ethyl acetate extraction was performed for three biological replicates and resulted in the same profiles (Figure S5). (C) Accumulation of cladofulvin in *C. fulvum* wild type and over-expression transformants after induction in B5-N. Quantification was performed by UV-HPLC using the response factor (RF) of pure cladofulvin as reference. The mean of three biological replicates and standard deviation is shown. Stars indicate significant differences compared to wild type according to an unpaired two-tailed t-test with Welch's correction (statistical significance alpha = 0.025) (* P-value < 0.025; ** P-value < 0.01).

Total RNA was isolated from each culture 48 hours after incubation to confirm the induction of each respective regulator by RT-qrtPCR. The expression of *CfHdaA*, *CfLaeA*, and *CfVeA* was indeed induced by 8.8 (t-test P-value = 3.497897e-007), 3.1 (t-test P-value = 7.820044e-005) and 3.5-fold (t-test P-value = 0.00985062) in the respective transformants when compared to wild type (Fig. 3A). Again, HPLC analysis of ethyl acetate extracts from each transformant showed that no new SM compounds were detected after induction (Fig. 3B and Fig. S5). However, the level of cladofulvin production greatly differed, with the highest concentration of cladofulvin found in cultures of *OE.CfHdaA* (32 µg mg^{-1} fungal dry weight) and *OE.CfVeA* (37 µg mg^{-1}), a lower concentration in *OE.CfLaeA* (1.3 µg mg^{-1}) and a trace in the wild type (0.2 µg mg^{-1}) (Fig. 3C). The data suggest that in B5-N medium, all three global regulators, but especially CfVeA and CfHdaA, behave as dominant positive regulators of cladofulvin biosynthesis when their expression is induced.

3.3.3 Cladofulvin production is influenced by the type of carbon source

Carbon sources affect the production of SM genes in fungal species (Espeso & Peñalva, 1992; Bluhm & Woloshuk, 2005; Brakhage, 2013). In addition, the regulation of SM production by VeA in *A. nidulans* is affected by the concentration of the carbon source (Atoui *et al.*, 2010). To test the effect of different carbon sources on the metabolic profile of wild type *C. fulvum* and global regulator deletion mutants, each background was grown for 12 days in ACM medium without additional carbon sources, and in ACM supplemented with one of the four carbohydrates: glucose, mannitol, saccharose, or xylose (each at 25 mM). No new SMs could be identified by HPLC in extracts from any strain grown on these different carbon sources, suggesting that *C. fulvum* SM biosynthetic pathways remained cryptic under these conditions too (Fig. S5). However, the amount of cladofulvin produced greatly varied (Fig. 4 and Fig. S6). In the wild type, accumulation was highest in ACM without additional carbon source (59 ± 15 µg mg^{-1}), much reduced by growth on glucose (15.6 ± 5.5 µg mg^{-1}), mannitol (13.3 ± 2.1 µg mg^{-1}), or xylose (14.1 ± 2.0 µg mg^{-1}), and almost completely abolished by saccharose (0.92 ± 0.2 µg mg^{-1}) (Fig. 4). Saccharose is found within the apoplastic spaces of tomato leaves, and during infection by *C. fulvum* it is rapidly hydrolysed into fructose and glucose by a secreted fungal invertase (Joosten *et al.*, 1990). At the same time mannitol accumulates, suggesting that glucose and fructose are quickly converted into mannitol (Joosten *et al.*, 1990).

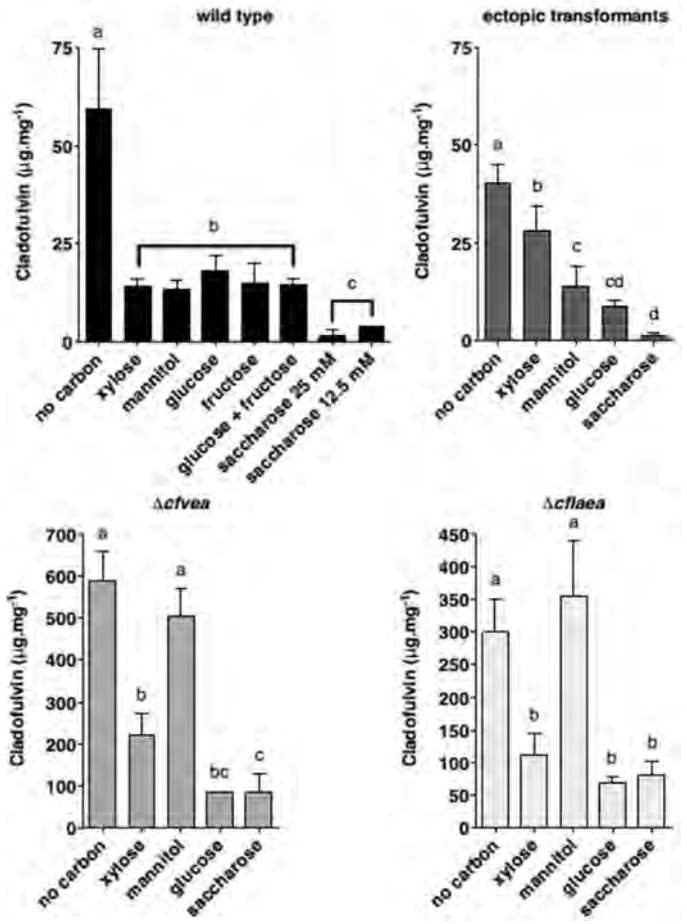


Figure 4. Effect of the type of carbon source on the production of cladofulvin by *Cladosporium fulvum* wild type and global regulator deletion mutants.

Secondary metabolites were extracted using ethyl acetate from wild type, Δ*cfvea*, Δ*cflaea* and Δ*cfhdaa* deletion mutants grown in *Aspergillus* Complete Medium (ACM) without carbon (no carbon), or supplemented with either 25 mM glucose, mannitol, saccharose or xylose. Wild type was also grown on ACM supplemented with 25 mM fructose, 12.5 mM saccharose or 12.5 mM glucose and 12.5 mM fructose mix. Quantification of cladofulvin production was performed by UV-HPLC using the response factor (RF) of pure cladofulvin as reference. The mean of three biological replicates and standard deviation is shown. Statistical groups are given according to the results of ordinary one-way ANOVAs and *post-hoc* Tukey's multiple comparisons test (statistical significance alpha = 0.05).

Therefore, the observed repression might be caused by fructose or a combination of fructose and glucose, rather than saccharose. To test this, we grew *C. fulvum* on 25 mM fructose, 12.5 mM glucose and 12.5 mM fructose and measured the cladofulvin concentration. Cladofulvin was not strongly repressed by fructose (14.9 ± 2.0 µg mg^{-1}), or glucose and fructose (14.3 ± 1.6 µg mg^{-1}) (Fig. 4). Furthermore, lowering the concentration of saccharose to 12.5 mM did not significantly increase cladofulvin production (3.9 ± 3.7 µg mg^{-1}) (Fig. 4). These results show that saccharose specifically inhibits cladofulvin production.

Highest production in the absence of carbon source and strong inhibition by saccharose were also observed in ectopic transformants. Significantly more cladofulvin was produced by Δ*cfvea* and Δ*cflaea* relative to the wild type, irrespective of the carbon source (Fig. 4). Cladofulvin was not produced in Δ*cfhdaa* when grown in any culture condition (Fig. S5 and Fig. S6). However in Δ*cfvea* and Δ*cflaea* deletion mutants, repression by mannitol and saccharose were completely abolished because cladofulvin production in ACM containing these carbon sources reaches the levels of those obtained on ACM without carbon and with glucose, respectively (Fig. 4). Altogether, these results indicate that repression of cladofulvin production by mannitol and saccharose are mediated by both CfVeA and CfLaeA.

3.3.4 Interplay between global regulators in *Cladosporium fulvum*

The metabolomic data so far have shown that cladofulvin production is regulated by all three global regulators and by the carbon source in a CfVeA- and CfLaeA-dependent manner. It was shown that LaeA and HdaA act antagonistically on the regulation of SM gene clusters in *A. nidulans* (Shwab *et al.*, 2007). In addition, VeA and LaeA are components of the same regulatory protein complex (Bayram *et al.*, 2008). To examine the interplay between these three regulators in *C. fulvum*, wild type and each deletion mutant were grown in ACM xylose until mid-exponential phase and total RNA was isolated for expression analysis of regulator genes by RT-qrtPCR (Fig. 5). *OE.CfHdaA*, *OE.CfLaeA*, *OE.CfVeA* and wild type controls were first transferred to B5-N medium and incubated for 48 hours prior to RT-qPCR to induce the *CfAvr9* promoter (Fig. 3A). Expression of *CfHdaA* was not affected by modification in gene expression of *CfVeA* and *CfLaeA*. In contrast, CfHdaA seems to be a repressor of *CfVeA* because expression of *CfVeA* was induced 24-fold in Δ*cfhdaa* mutants (Fig. 5). The expression of *CfVeA* was abolished in Δ*cflaea* mutants while *CfLaeA* was induced in Δ*cfvea* mutants (Fig. 5). Intriguingly, the same pattern was observed in *OE.CfLaeA* and *OE.CfVeA* transformants with *CfVeA* lower expressed and *CfLaeA* higher, although not significantly, expressed, respectively (Fig. 3A).

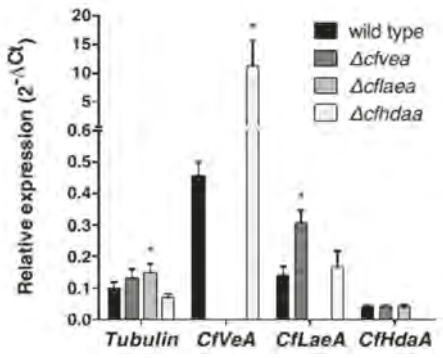


Figure 5. Expression of *CfVeA*, *CfLaeA* and *CfHdaA* in *Cladosporium fulvum* global regulator deletion mutants.
Relative expression of global regulator genes in *C. fulvum* wild type and deletion mutants grown in *Aspergillus* Complete Medium (ACM) with 25 mM xylose was measured by RT-qrtPCR. Ct values were normalized using the *Actin* gene and analysed using the $2^{-\Delta Ct}$ method. The mean of three biological replicates and standard deviation is shown. Stars indicate significant differences compared to wild type according to multiple t-tests (statistical significance alpha = 0.025).

3.4 Discussion

3.4.1 Regulation of secondary metabolism by global regulators in *Cladosporium fulvum*

In filamentous fungi, biosynthesis of SMs in response to environmental stimuli is controlled by a complex network of global regulators (Yu & Keller, 2005; Bayram & Braus, 2012). The scaffold protein, VeA, and the putative histone methyltransferase, LaeA, are both crucial components of the well-characterized Velvet protein complex that controls SM production and colony development in response to light (Kim *et al.*, 2002; Bok & Keller, 2004; Sarikaya-Bayram *et al.*, 2015). The histone deacetylase HdaA has been studied in *A. nidulans*, where it regulates SM gene clusters in an opposite way to LaeA (Shwab *et al.*, 2007; Lee *et al.*, 2009). Manipulating these three global regulators profoundly altered the SM profile in other fungal species (Calvo *et al.*, 2004; Shwab *et al.*, 2007; Rohlfs *et al.*, 2007; Lee *et al.*, 2009; Wiemann *et al.*, 2010; Chettri *et al.*, 2012; Wu *et al.*, 2012). Orthologues of VeA, LaeA and HdaA were identified in *C. fulvum* and they are expressed *in vitro* at a significant level. We hypothesized that manipulating them would deregulate some of the many silent SM gene clusters in the *C. fulvum* genome (de Wit *et al.*, 2012; Collemare *et al.*, 2014). However, ethyl acetate extracts of deletion mutants and over-expression transformants of *CfVeA*, *CfLaeA* and *CfHdaA* showed that production of only a single SM, the anthraquinone cladofulvin, was affected. This suggests that only the gene cluster involved in cladofulvin production is under control of these global regulators. The SM core genes *NPS8* and *NPS9* that are up-regulated under iron starvation are unlikely regulated by any of these global regulators because no link between iron starvation and VeA, LaeA and HdaA homologues has been reported (Collemare et al., 2014). Also, the expression profile of *CfVeA* and *CfLaeA* suggested that they might be positive regulators of the *PKS1* core gene because all three genes are up-regulated in the deletion mutant of the transcription factor *CfWor1* (Ökmen *et al.*, 2014). However, no black pigment as seen in the Δ*cfwor1* mutants could be observed in *OE.CfVeA* and *OE.CfLaeA* over-expression transformants, suggesting that induction of *PKS1* depends on additional factors or is independent of these two regulators.

It is possible that the absence of new SMs in our *C. fulvum* deletion mutants and over-expression transformants is due to the inability to detect them, especially when SMs are produced in very low amounts or when they do not strongly absorb light in the 200-800 nm range. Nevertheless, our results suggest that regulation of SM biosynthetic pathways in *C. fulvum* is different from fungi in which it has been extensively studied, especially in *Aspergillus* species. Such a difference might be linked to the genomic position of SM gene clusters. Indeed, it was reported in *A. nidulans* that LaeA seems to regulate both telomere-proximal and –distal clusters, whereas the regulation by HdaA is confined to sub-telomeric regions (Perrin et al., 2007; Shwab et al., 2007). SM gene clusters do not appear to be enriched in sub-telomeric areas in *C. fulvum* and in its close relative the pine needle pathogen *D. septosporum* (de Wit *et al.*, 2012). In this case, manipulation of VeA, LaeA and HdaA to activate cryptic biosynthetic pathways might be restricted to fungal species with an enrichment of SM gene clusters located in sub-telomeric regions.

3.4.2 Regulation of cladofulvin production by global regulators

Hyperacetylation of core histones is commonly associated with euchromatin and gene activation (Robyr *et al.*, 2002). Thus, the HDAC HdaA is expected to be involved in gene repression by removing acetyl groups from histones and promoting a heterochromatin structure (Trojer *et al.*, 2003). Correspondingly, the deletion of *HdaA* gene in *A. nidulans* stimulated SM production (Shwab *et al.*, 2007). The metabolic profile of *C. fulvum* Δ*cfhdaa* deletion mutants and *OE.CfHdaA* transformants clearly showed that CfHdaA positively regulates cladofulvin production. We hypothesize that CfHdaA silences a region of chromatin that contains a repressor of cladofulvin biosynthesis. Conversely, the activation of this repressor in hyperacetylated Δ*cfhdaa* deletion mutants prevented cladofulvin production under all tested conditions. Because HDACs were also associated with cell responses to oxidative and osmotic stress (Nadal *et al.*, 2004; Lee *et al.*, 2009), it may be speculated that cladofulvin production is linked to a stress response that is mediated by *CfHdaA*.

Deletion of *CfLaeA* resulted in the strong production of cladofulvin, whilst its over-expression barely increased cladofulvin production. These results are consistent with CfLaeA acting as a repressor of cladofulvin production. This is similar to the situation in *Trichoderma reesei*, as several SM genes were up-regulated when the *LaeA* homologue, *Lae1*, was deleted or over-expressed in this fungus (Karimi-Aghcheh *et al.*, 2013). In contrast, the role of *CfVeA* is not clear because both its deletion and over-expression resulted in the over-production of cladofulvin. Increased cladofulvin production in Δ*cfvea* deletion mutants is not due to the observed induction of *CfLaeA* in this background, because the *OE.CfLaeA* transformant produced very low amounts of cladofulvin. Similarly, the enhanced production of cladofulvin in the *OE.CfVeA* transformant cannot be explained by the higher expression of *CfLaeA*. In contrast, increased cladofulvin production in Δ*cflaea* deletion mutants might be linked to the abolishment of *CfVeA* gene expression in this background. This last observation suggests epistasis between *CfVeA* and *CfLaeA*, as the expression of the former requires the latter. This was also described in *T. reesei*, where *Lae1* was required for the expression of the *VeA* homologue, *Vel1* (Karimi-Aghcheh *et al.*, 2013). Our results suggest CfLaeA might have a function outside of the Velvet complex in *C. fulvum*, and this needs further investigation. A previous report hypothesized that LaeA might perform a specific regulation of certain SM gene clusters because it binds to AflR and other AflR-like transcription factors (Ehrlich *et al.*, 2011), which may be performed independently of VeA.

CfVeA expression is highly induced in the Δ*cfhdaa* deletion mutant, showing that CfHdaA also plays a role in regulating *CfVeA*. However, this effect is likely indirect because *CfVeA* expression is not repressed in the *OE.CfHdaA* transformant. This result also confirms that the effect of *CfVeA* over-expression on cladofulvin production is indirect because the Δ*cfhdaa* deletion mutants are not able to produce any cladofulvin.

3.4.3 Cladofulvin production is affected by carbon source type

It has long been established that SM production is subject to catabolite repression. The production of penicillin in the fungus *P. chrysogenum* is dampened by glucose, fructose, galactose, and saccharose, but is stimulated by poorly-metabolised lactose (Brakhage, 1998). In contrast, aflatoxin production is induced in presence of glucose or saccharose, but is repressed in presence of xylose, galactose or lactose (Calvo *et al.*, 2002). The most conducive medium for cladofulvin production was ACM without a carbon source, which suggests that cladofulvin production might be part of a nutrient stress response, or linked to colony maturation that is driven by nutrient depletion. The highly reduced amount of cladofulvin in ACM supplemented with saccharose, but not with other tested carbohydrates, showed that this carbon source forms part of a strong negative signal. It is known that during growth within the tomato apoplast, *C. fulvum* secretes invertase which catalyzes the extra-cellular conversion of saccharose into glucose and fructose (Joosten *et al.*, 1990). However, cladofulvin production in wild type was not strongly inhibited by glucose or fructose, showing that saccharose is the major negative signal. The presumed polyketide synthase involved in cladofulvin biosynthesis (*PKS6*) is poorly expressed during colonization of tomato mesophyll tissue, and cladofulvin could not be detected in apoplastic fluids of *C. fulvum*-infected tomato plants (Collemare *et al.*, 2014). *PKS6* is induced during conidiation on the leaf surface, suggesting a link between conidiation and cladofulvin production. However, both Δ*cfvea* and Δ*cflaea* deletion mutants did not differentiate conidia yet showed enhanced cladofulvin production. In contrast, Δ*cfhdaa* deletion mutants did differentiate conidia but were no longer able to produce cladofulvin. These results suggest that conidiation and cladofulvin production are not inter-dependent in *C. fulvum*. The *in planta* expression pattern of *PKS6* might therefore be due to catabolite repression by saccharose in the tomato apoplast, as observed *in vitro*. Induction of cladofulvin production when the fungus is present on the leaf surface might be a response to nutrient depletion and/or environmental stresses.

The production of cladofulvin in both Δ*cfvea* and Δ*cflaea* deletion mutants was very high during growth on every tested carbon source, including on ACM supplemented with saccharose. Furthermore, both Δ*cfvea* and Δ*cflaea* produced the most cladofulvin in unsupplemented ACM and on ACM supplemented with mannitol. We noticed that fungal growth was clearly reduced under both conditions relative to growth on ACM supplemented with other carbon sources. High cladofulvin production combined with poor fungal growth suggests that that the mutants were carbon-limited in ACM mannitol. In *Alternaria brassicicola*, *Botrytis cinerea* and *C. fulvum*, mannitol is converted into fructose by mannitol dehydrogenase (Noeldner *et al.*, 1994; Dulermo *et al.*, 2010; Calmes *et al.*, 2013). Given that *C. fulvum* Δ*cfvea* and Δ*cflaea* grew well on fructose, we hypothesise that CfVeA and CfLaeA are required for the expression or activity of mannitol dehydrogenase and/or mannitol transporters.

3.5 Conclusions

Collectively, our results on cladofulvin production suggest that it is disconnected from fungal development and might be linked to a general stress response. In particular, it provided new clues on how cladofulvin production is repressed *in planta* and indicated that the biological function of cladofulvin might be linked to survival. Both aspects require further investigations to understand the role of cladofulvin in the biology of *C. fulvum*.

Importantly, the loss or induction of *VeA*, *LaeA* or *HdaA* in *C. fulvum* affected the production of cladofulvin alone, whilst multiple SM gene clusters are simultaneously regulated by these global regulators in other fungal species. Our results indicate that manipulating such regulators might not be a universal tool to discover cryptic SM compounds in all fungal species, even in those that have an extensive SM gene catalogue. These regulators have been extensively studied and used as a tool in *Aspergillus* species, in which SM gene clusters seem to be preferentially located at telomeres (Palmer & Keller, 2010). Thus, these tools might be most useful only in species in which SM gene clusters are preferentially sub-telomeric. From this work, and previous studies, it appears that manipulating culture conditions can be a more effective and inexpensive approach to alter SM production in diverse fungi.

References

Agosti G, Birkinshaw JH, Chaplen P. 1962. Studies in the biochemistry of micro-organisms. 112. Anthraquinone pigments of strains of Cladosporium fulvum Cooke. *The Biochemical Journal* **85**: 528-30.

Atoui A, Kastner C, Larey CM, Thokala R, Etxebeste O, Espeso EA, Fischer R, Calvo AM. 2010. Cross-talk between light and glucose regulation controls toxin production and morphogenesis in Aspergillus nidulans. *Fungal Genetics and Biology* **47**: 962-72.

Baba S, Kinoshita H, Nihira T. 2012. Identification and characterization of Penicillium citrinum VeA and LaeA as global regulators for ML-236B production. *Current Genetics* **58**: 1-11.

Bayram O, Braus GH. 2012. Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiology Reviews* **36**: 1-24.

Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, Braus-Stromeyer S, Kwon N, Keller NP, Yu J, *et al.* 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* **320**: 1504-6.

Bergmann S, Schümann J, Scherlach K, Lange C, Brakhage AA, Hertweck C. 2007. Genomics-driven discovery of PKS-NRPS hybrid metabolites from Aspergillus nidulans. *Nature Chemical Biology* **3**: 213-7.

Bluhm BH, Woloshuk CP. 2005. Amylopectin induces fumonisin B1 production by Fusarium verticillioides during colonization of maize kernels. *Molecular Plant-Microbe Interactions: MPMI* **18**: 1333-9.

Blumenstein A, Vienken K, Tasler R, Purschwitz J, Veith D, Frankenberg-Dinkel N, Fischer R. 2005. The Aspergillus nidulans phytochrome FphA represses sexual development in red light. *Current Biology* **15**: 1833-8.

Bok JW, Keller NP. 2004. LaeA, a regulator of secondary metabolism in Aspergillus spp. *Eukaryotic Cell* **3**: 527-35.

Bok JW, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Keller NP. 2005. LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryotic Cell* **4**: 1574-82.

Brakhage AA. 1998. Molecular regulation of beta-lactam biosynthesis in filamentous fungi. *Microbiology and Molecular Biology Reviews* **62**: 547-85.

Brakhage AA. 2013. Regulation of fungal secondary metabolism. *Nature Reviews Microbiology* **11**: 21-32.

Brakhage AA, Schroeckh V. 2011. Fungal secondary metabolites - strategies to activate silent gene clusters. *Fungal Genetics and Biology* **48**: 15-22.

Calmes B, Guillemette T, Teyssier L, Siegler B, Pigné S, Landreau A, Iacomi B, Lemoine R, Richomme P, Simoneau P. 2013. Role of mannitol metabolism in the pathogenicity of the necrotrophic fungus Alternaria brassicicola. *Frontiers in Plant Science* **4**: 131.

Calvo AM, Bok J, Brooks W, Keller NP. 2004. veA is required for toxin and sclerotial production in Aspergillus parasiticus. *Applied and Environmental Microbiology* **70**: 4733-9.

Calvo AM, Wilson RA, Bok JW, Keller NP. 2002. Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* **66**: 447-459.

Cary JW, Ehrlich KC, Kale SP, Calvo AM, Bhatnagar D, Cleveland TE. 2006. Regulatory elements in aflatoxin biosynthesis. *Mycotoxin Research* **22**: 105-9.

Cary JW, Uka V, Han Z, Buyst D, Harris-Coward PY, Ehrlich KC, Wei Q, Bhatnagar D, Dowd PF, Martens SL, *et al.* 2015. An Aspergillus flavus secondary metabolic gene cluster containing a hybrid PKS–NRPS is necessary for synthesis of the 2-pyridones, leporins. *Fungal Genetics and Biology* **81**: 88-97.

Chagas FO, Dias LG, Pupo MT. 2013. A mixed culture of endophytic fungi increases production of antifungal polyketides. *Journal of Chemical Ecology* **39**: 1335-42.

Chettri P, Calvo AM, Cary JW, Dhingra S, Guo Y, McDougal RL, Bradshaw RE. 2012. The veA gene of the pine needle pathogen Dothistroma septosporum regulates sporulation and secondary metabolism. *Fungal Genetics and Biology* **49**: 141-51.

Chettri P, Ehrlich KC, Cary JW, Collemare J, Cox MP, Griffiths SA, Olson MA, de Wit PJGM, Bradshaw RE. 2012. Dothistromin genes at multiple separate loci are regulated by AflR. *Fungal Genetics and Biology* **51**: 12-20.

Chiang Y, Chang S, Oakley BR, Wang CCC. 2011. Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms. *Current Opinion in Chemical Biology* **15**: 137-43.

Collemare J, Billard A, Böhnert HU, Lebrun M. 2008. Biosynthesis of secondary metabolites in the rice blast fungus Magnaporthe grisea: the role of hybrid PKS-NRPS in pathogenicity. *Mycological Research* **112**: 207-15.

Collemare J, Griffiths S, Iida Y, Jashni MK, Battaglia E, Cox RJ, de Wit PJGM. 2014. Secondary Metabolism and Biotrophic Lifestyle in the Tomato Pathogen Cladosporium fulvum. *PloS One* **9**: e85877.

Connolly LR, Smith KM, Freitag M. 2013. The Fusarium graminearum histone H3 K27 methyltransferase KMT6 regulates development and expression of secondary metabolite gene clusters. *PLoS Genetics* **9**: e1003916.

Davies DG. 1974. Chemistry of quinones. Part V. Structure of cladofulvin, a bianthraquinone from cladosporium fulvum cooke. *Journal of the Chemical Society, Perkin Transactions* **1:** 2403-2405.

Dreyer J, Eichhorn H, Friedlin E, Kürnsteiner H, Kück U. 2007. A homologue of the Aspergillus velvet gene regulates both cephalosporin C biosynthesis and hyphal fragmentation in Acremonium chrysogenum. *Applied and Environmental Microbiology* **73**: 3412-22.

Dulermo T, Rascle C, Billon-Grand G, Gout E, Bligny R, Cotton P. 2010. Novel insights into mannitol metabolism in the fungal plant pathogen Botrytis cinerea. *The Biochemical Journal* **427**: 323-32.

Ehrlich KC, Cary JW, Montalbano BG. 1999. Characterization of the promoter for the gene encoding the aflatoxin biosynthetic pathway regulatory protein AFLR. *Biochimica et Biophysica Acta* **1444**: 412-7.

Ehrlich KC, Mack BM, Cary JW, Bhatnagar D, Kale SP. 2011. A hypothesis to explain how LaeA specifically regulates certain secondary metabolite biosynthesis gene clusters. *World Mycotoxin Journal* **4**: 53-58.

Espeso EA, Peñalva MA. 1992. Carbon catabolite repression can account for the temporal pattern of expression of a penicillin biosynthetic gene in Aspergillus nidulans. *Molecular Microbiology* **6**: 1457-65.

Gallo M, Katz E. 1972. Regulation of secondary metabolite biosynthesis: catabolite repression of phenoxazinone synthase and actinomycin formation by glucose. *Journal of Bacteriology* **109**: 659-67.

Gressler M, Zaehle C, Scherlach K, Hertweck C, Brock M. 2011. Multifactorial induction of an orphan PKS-NRPS gene cluster in Aspergillus terreus. *Chemistry & Biology* **18**: 198-209.

Han K, Seo J, Yu J. 2004. Regulators of G-protein signalling in Aspergillus nidulans: RgsA downregulates stress response and stimulates asexual sporulation through attenuation of GanB (Galpha) signalling. *Molecular Microbiology* **53**: 529-40.

Hicks J, Lockington RA, Strauss J, Dieringer D, Kubicek CP, Kelly J, Keller N. 2001. RcoA has pleiotropic effects on Aspergillus nidulans cellular development. *Molecular Microbiology* **39**: 1482-93.

Jain S, Keller N. 2013. Insights to fungal biology through LaeA sleuthing. *Fungal Biology Reviews* **27**: 51-59.

Joosten MHAJ, Hendrickx LJM, Wit PJGM. 1990. Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of Cladosporium fulvum (syn. Fulvia fulva). *Netherlands Journal of Plant Pathology* **96**: 103-112.

Karimi-Aghcheh R, Bok JW, Phatale PA, Smith KM, Baker SE, Lichius A, Omann M, Zeilinger S, Seiboth B, Rhee C, *et al.* 2013. Functional analyses of Trichoderma reesei LAE1 reveal conserved and contrasting roles of this regulator. *G3: Genes|Genomes|Genetics* **3**: 369-78.

Kato N, Brooks W, Calvo AM. 2003. The expression of sterigmatocystin and penicillin genes in Aspergillus nidulans is controlled by veA, a gene required for sexual development. *Eukaryotic Cell* **2**: 1178-86.

Keller NP, Hohn TM. 1997. Metabolic Pathway Gene Clusters in Filamentous Fungi. *Fungal Genetics and Biology* **21**: 17-29.

Kim H, Han K, Kim K, Han D, Jahng K, Chae K. 2002. The veA gene activates sexual development in Aspergillus nidulans. *Fungal Genetics and Biology* **37**: 72-80.

Kopke K, Hoff B, Bloemendal S, Katschorowski A, Kamerewerd J, Kück U. 2013. Members of the Penicillium chrysogenum velvet complex play functionally opposing roles in the regulation of penicillin biosynthesis and conidiation. *Eukaryotic Cell* **12**: 299-310.

König CC, Scherlach K, Schroeckh V, Horn F, Nietzsche S, Brakhage AA, Hertweck C. 2013. Bacterium induces cryptic meroterpenoid pathway in the pathogenic fungus Aspergillus fumigatus. *ChemBioChem* **14**: 938-42.

Lee I, Oh J, Shwab EK, Dagenais TRT, Andes D, Keller NP. 2009. HdaA, a class 2 histone deacetylase of Aspergillus fumigatus, affects germination and secondary metabolite production. *Fungal Genetics and Biology* **46**: 782-90.

Lim FY, Hou Y, Chen Y, Oh J, Lee I, Bugni TS, Keller NP. 2012. Genome-based cluster deletion reveals an endocrocin biosynthetic pathway in Aspergillus fumigatus. *Applied and Environmental Microbiology* **78**: 4117-25.

Livak KJ, Schmittgen TD. **2002**. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-8.

Mesarich CH, Griffiths SA, van der Burgt A, Ökmen B, Beenen HG, Etalo DW, Joosten MHAJ, de Wit PJGM. **2014**. Transcriptome Sequencing Uncovers the Avr5 Avirulence Gene of the Tomato Leaf Mold Pathogen Cladosporium fulvum. *Molecular Plant-Microbe Interactions* **27**: 846-57.

Nadal E, Zapater M, Alepuz PM, Sumoy L, Mas G, Posas F. **2004**. The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. *Nature* **427**: 370-4.

Noeldner PKM, Coleman MJ, Faulks R, Oliver RP. **1994**. Purification and characterization of mannitol dehydrogenase from the fungal tomato pathogen Cladosporium fulvum (syn. Fulvia fulva). **45**: 281-289.

Palmer JM, Keller NP. **2010**. Secondary metabolism in fungi: does chromosomal location matter? *Current Opinion in Microbiology* **13**: 431-6.

Robyr D, Suka Y, Xenarios I, Kurdistani SK, Wang A, Suka N, Grunstein M. **2002**. Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. *Cell* **109**: 437-46.

Rohlfs M, Albert M, Keller NP, Kempken F. **2007**. Secondary chemicals protect mould from fungivory. *Biology letters* **3**: 523-5.

Rosén S, Yu JH, Adams TH. **1999**. The Aspergillus nidulans sfaD gene encodes a G protein beta subunit that is required for normal growth and repression of sporulation. *The EMBO Journal* **18**: 5592-600.

Rozen S, Skaletsky H. **1999**. Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* **132**: 365-86.

Sarikaya-Bayram Ö, Palmer JM, Keller N, Braus GH, Bayram O. **2015**. One Juliet and four Romeos: VeA and its methyltransferases. *Frontiers in Microbiology* **6**: 1.

Shimizu K, Keller NP. **2001**. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans. *Genetics* **157**: 591-600.

Shwab EK, Bok JW, Tribus M, Galehr J, Graessle S, Keller NP. **2007**. Histone deacetylase activity regulates chemical diversity in Aspergillus. *Eukaryotic Cell* **6**: 1656-64.

Trojer P, Brandtner EM, Brosch G, Loidl P, Galehr J, Linzmaier R, Haas H, Mair K, Tribus M, Graessle S. **2003**. Histone deacetylases in fungi: novel members, new facts. *Nucleic Acids Research* **31**: 3971-81.

Tsitsigiannis DI, Keller NP. **2006**. Oxylipins act as determinants of natural product biosynthesis and seed colonization in Aspergillus nidulans. *Molecular Microbiology* **59**: 882-92.

Van den Ackerveken GF, Dunn RM, Cozijnsen AJ, Vossen JP, Van den Broek HW, De Wit PJ. **1994**. Nitrogen limitation induces expression of the avirulence gene avr9 in the tomato pathogen Cladosporium fulvum. *Molecular & General Genetics : MGG* **243**: 277-85.

Wiemann P, Brown DW, Kleigrewe K, Bok JW, Keller NP, Humpf H, Tudzynski B. **2010**. FfVel1 and FfLae1, components of a velvet-like complex in Fusarium fujikuroi, affect differentiation, secondary metabolism and virulence. *Molecular Microbiology* **77**: 972-94.

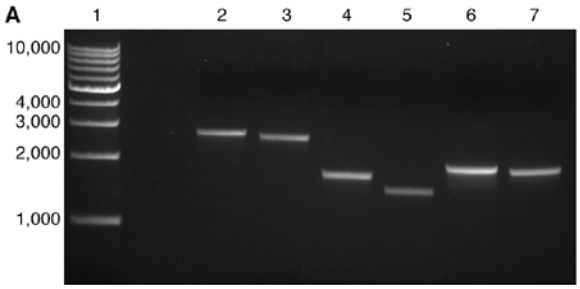
Wu D, Oide S, Zhang N, Choi MY, Turgeon BG. **2012**. ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen Cochliobolus heterostrophus. *PLOS Pathogens* **8**: e1002542.

Yu J, Keller N. **2005**. Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology* **43**: 437-58.

de Wit PJGM, van der Burgt A, Ökmen B, Stergiopoulos I, Abd-Elsalam KA, Aerts AL, Bahkali AH, Beenen HG, Chettri P, Cox MP, *et al.* 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.

Ökmen B, Collemare J, Griffiths S, van der Burgt A, Cox R, de Wit PJGM. 2014. Functional analysis of the conserved transcriptional regulator CfWor1 in Cladosporium fulvum reveals diverse roles in the virulence of plant pathogenic fungi. *Molecular Microbiology* **92**: 10-27.

Supplementary figures



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C

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Figure S1. Validation of global regulators predicted gene models.
(A) PCR on gDNA (Lane 2, 4 and 6) and cDNA (Lane 3, 5 and 7) isolated from *Cladosporium fulvum* grown in PDB liquid medium. Lane 1: 1kb DNA step ladder (bp; Pramega); Lane 2 and 3: *Clafu192224 (CfhdaA;* two introns of 50 and 59 bp); Lane 4 and 5 :*Clafu186126 (CflaeA;* three introns of 106, 58 and 71 bp); Lane 6 and 7: *Clafu188542 (CfveA;* single intron of 64 bp). Sequencing all fragments confirmed or determined intron positions. Image levels were adjusted for vizualisation purpose.
(B) Revised protein sequence of Clafu186126
(C) Revised protein sequence of Clafu188542.

A

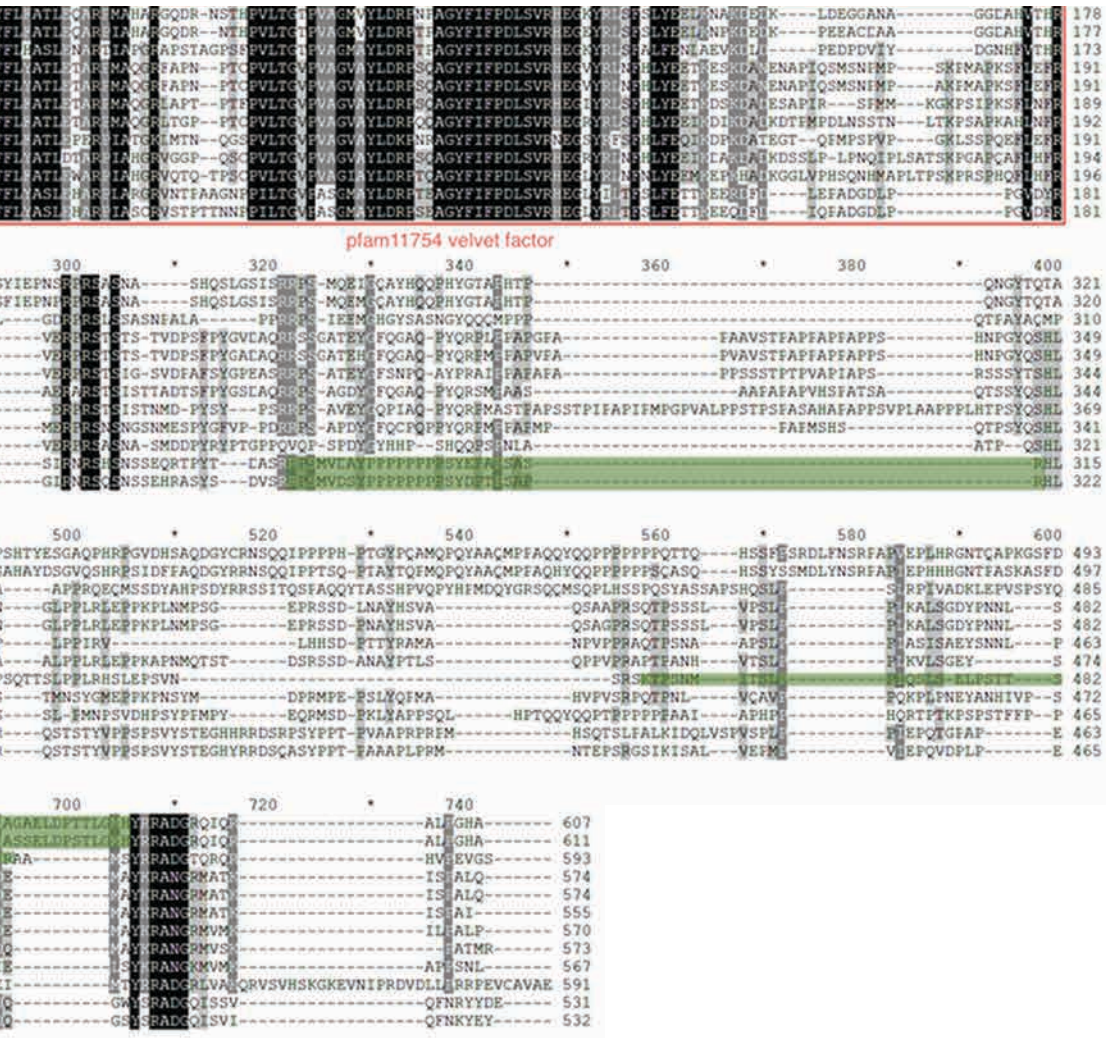
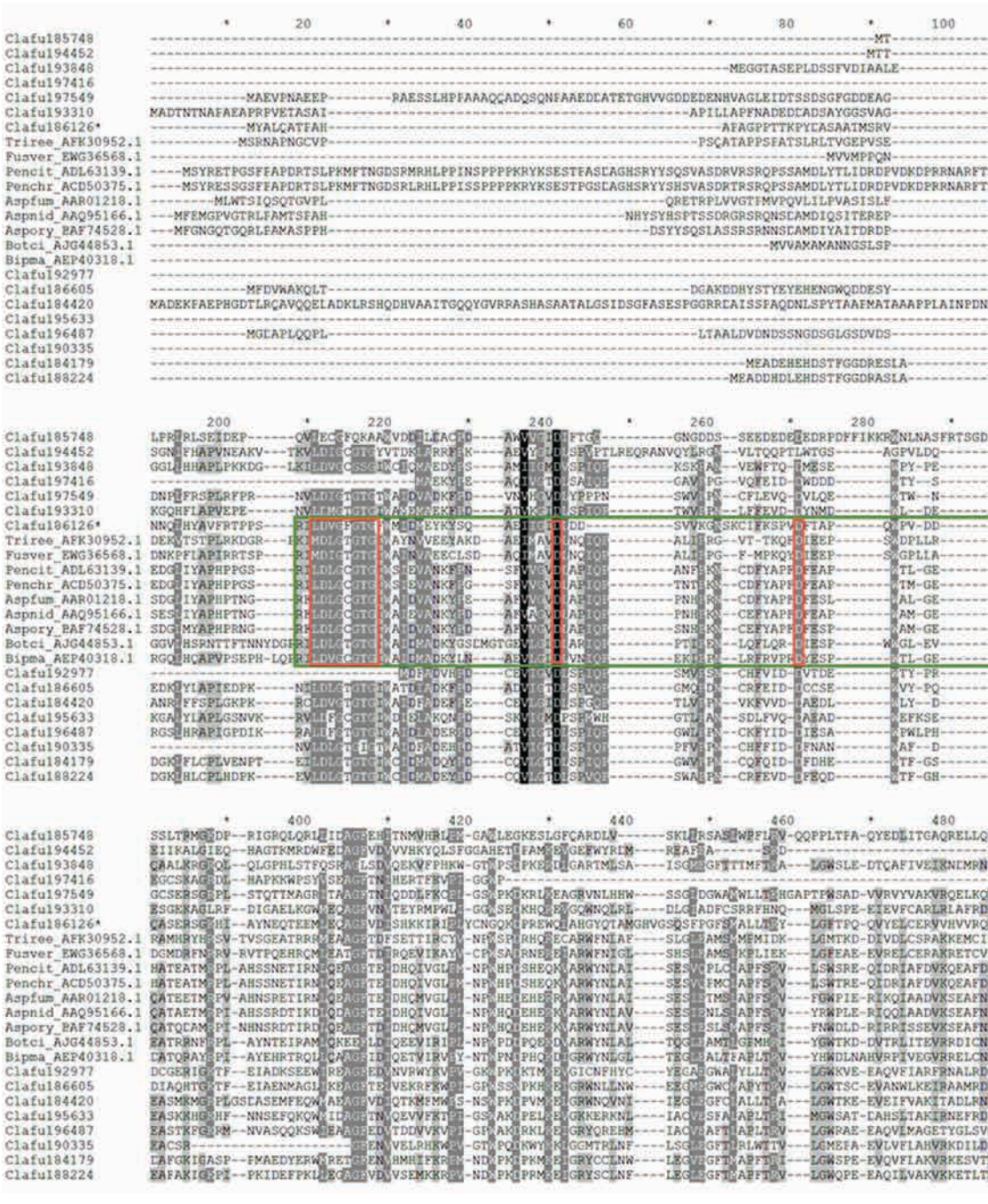


Figure S2. Identification of orthologues of global regulators in *Cladosporium fulvum*.
(A) Protein alignment of VeA orthologues. The conserved domain pfam11754 is indicated in red according to the NCBI Conserved Domains database(Marchler-Bauer *et al.,*2015).A putative Nuclear Localization Signal (NLS) identified in *Aspergillus nidulans* is indicated in blue. Putative PEST regions enriched in praline, glutamic acid, serine and threonine are shaded in green according to epestfind (emboss.bioinformatics.nl) . All predictions are consistent with previous reports (Kim *et al.,*2002; Chettri *et al.,*2012).

B



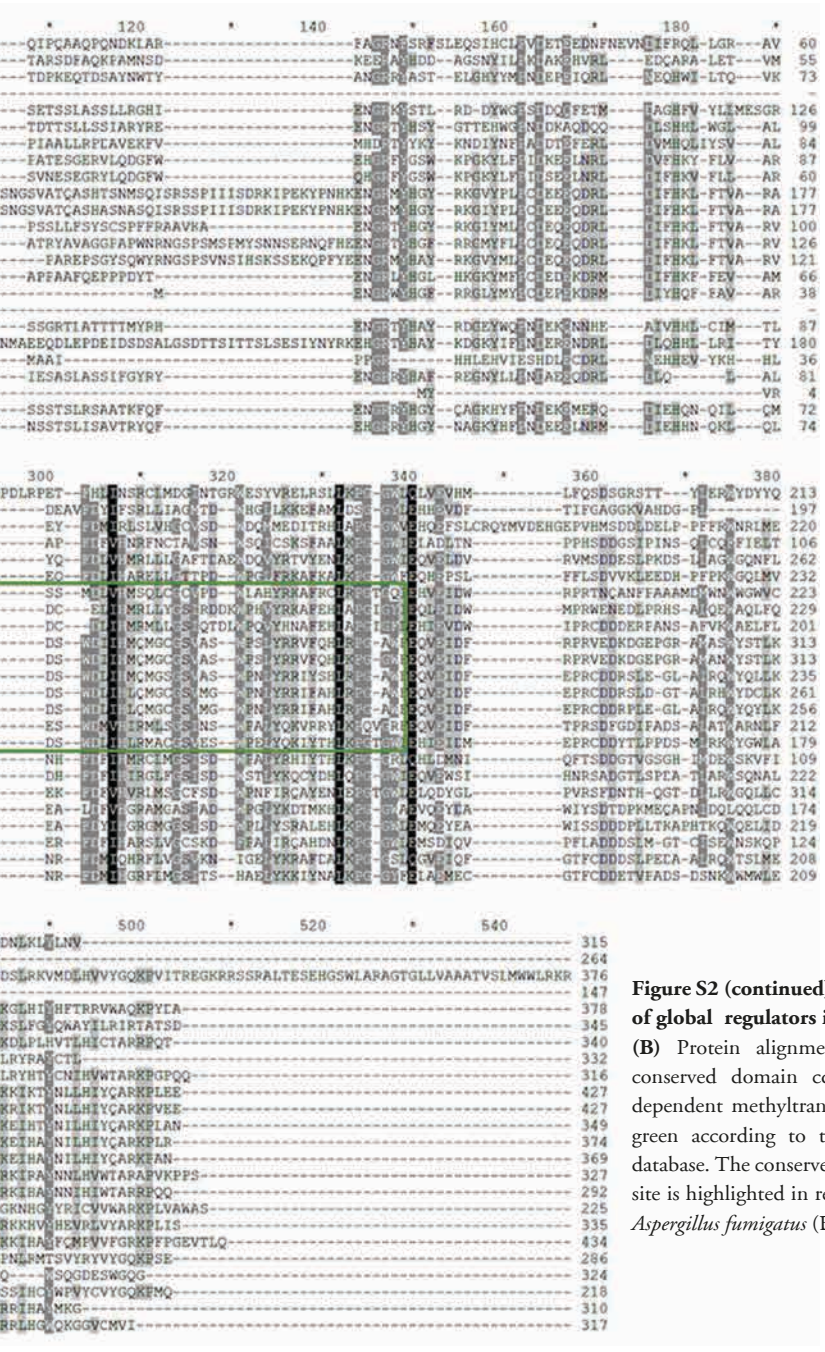
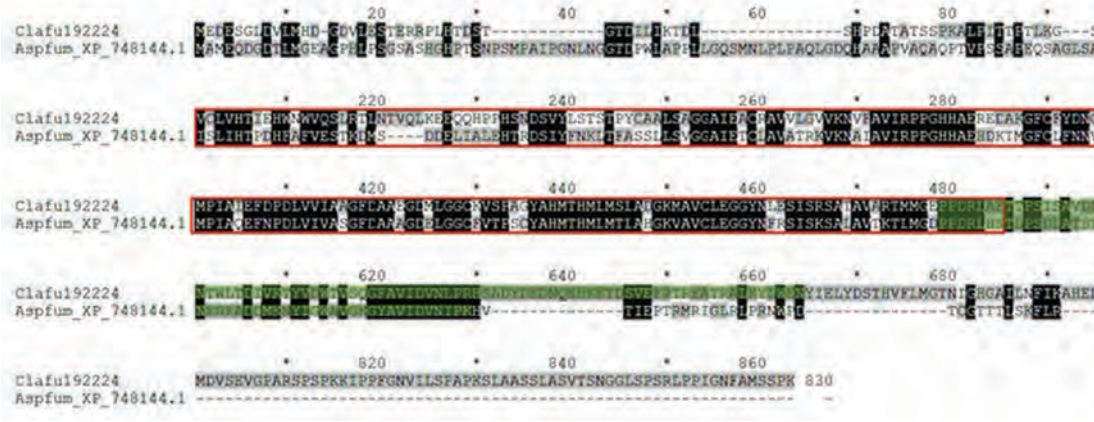


Figure S2 (continued). Identification of orthologues of global regulators in *Cladosporium fulvum*.
(B) Protein alignment of LaeA orthologues. The conserved domain cd02440 (S-adenosylmethionine-dependent methyltransferases , class I) is indicated in green according to the NCBI Conserved Domains database. The conserved S-adenosylmethionine binding site is highlighted in red as reported in *A. nidulans* and *Aspergillus fumigatus* (Bok and Keller, 2004).

C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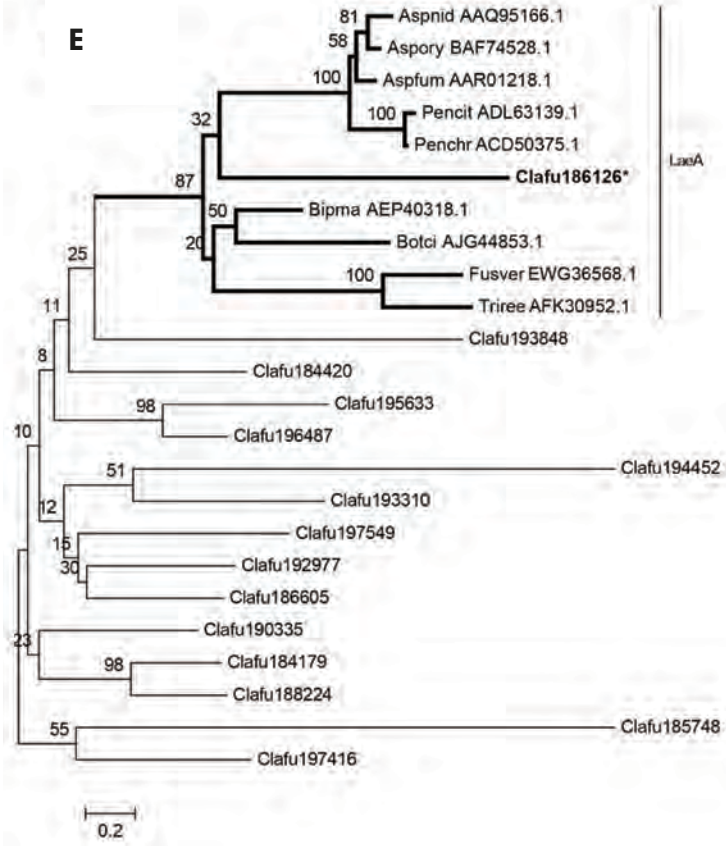
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LaeA
Clafu193848
Clafu184420
Clafu195633
Clafu196487
Clafu194452
Clafu193310
Clafu197549
Clafu192977
Clafu186605
Clafu190335
Clafu184179
Clafu188224
Clafu185748
Clafu197416
81
58
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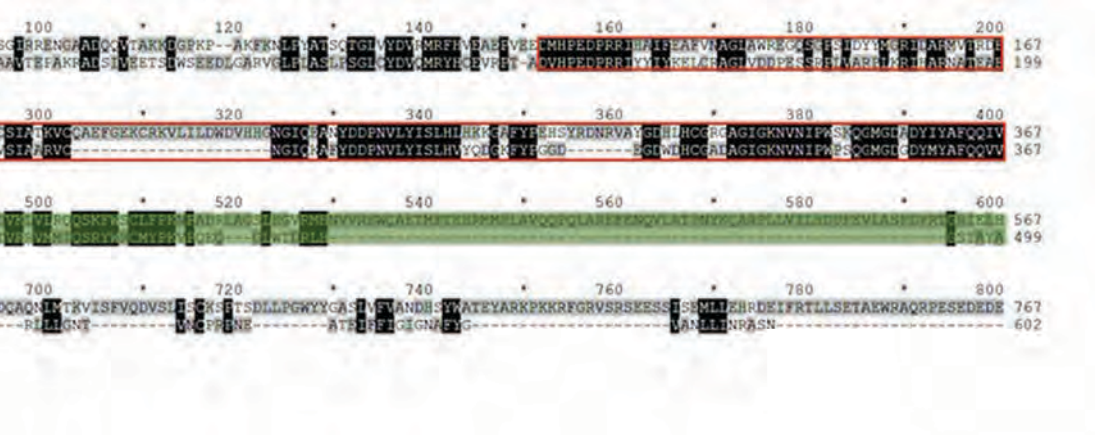
15
30
23
98
55
0.2



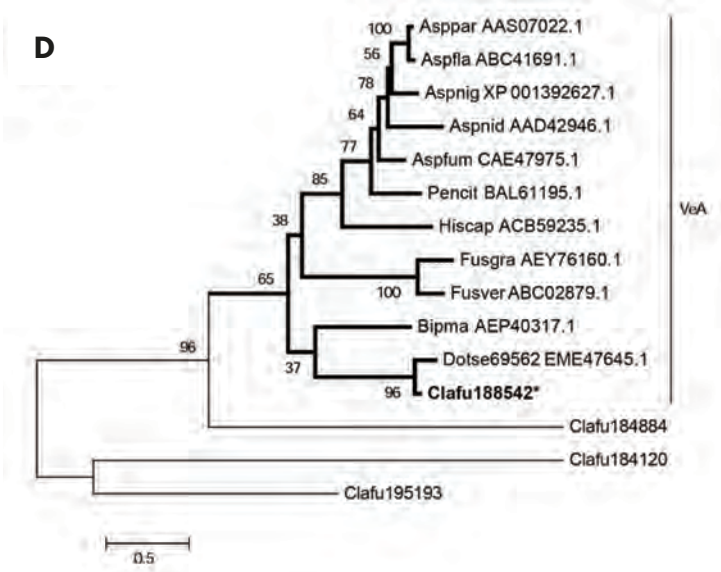


Figure S2 (continued). Identification of orthologues of global regulators in *Cladosporium fulvum*.
(C) Protein alignment of HdaA orthologues. The conserved domains cl17011(arginase-like and histone-like hydrolases)and cl10738 (Arb2 domain) are indicated in red and green, respectfully, according to the NCBI Conserved Domains database.
Minimum evolution phylogenetic tree of **(D)** VeA and **(E)** LaeA homologues built with MEGA 5.2 (100 bootstraps, Jones-Taylor-Thornton (JTT) substitution model, estimated (default parameters) gamma parameter for sites rates of 1.6046 and 1.7436, respectively, site coverage cutoff of 95%; Tamura *et al.*, 2011). Stars indicate corrected gene models.

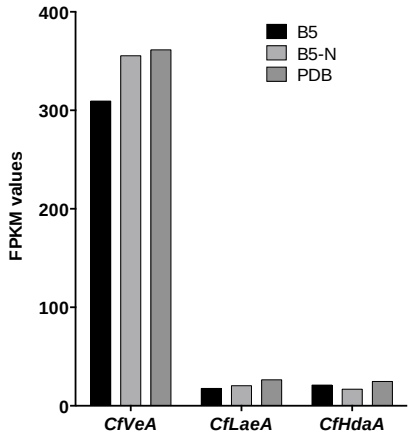


Figure S3. *In vitro* expression of global regulator genes *CfVeA*, *CfLaeA* and *CfHdaA*.
Cladosporium fulvum wild type strain was grown on PDB for 7 days prior to cultivation in fresh PDB, B5 or B5-N (nitrogen starvation) media for 2 additional days. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values as determined with Cufflinks were previously obtained (Mesarich et al., 2014).

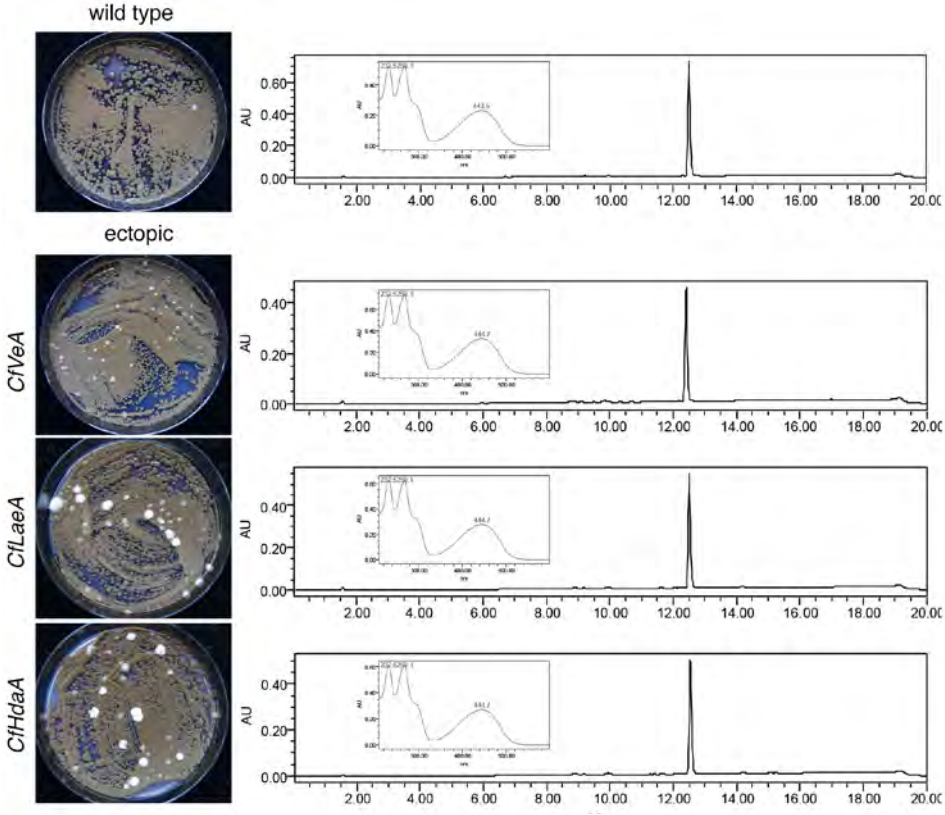


Figure S5. Phenotype and cladofulvin production in ectopic transformants.
On the left, wild type and ectopic transformants were grown on PDA medium for seven days. They do not show any defect compared to wild type. Secondary metabolites were extracted from the plates and only cladofulvin was detected as shown on the right with diode array chromatograms (254 nm) and UV spectra.

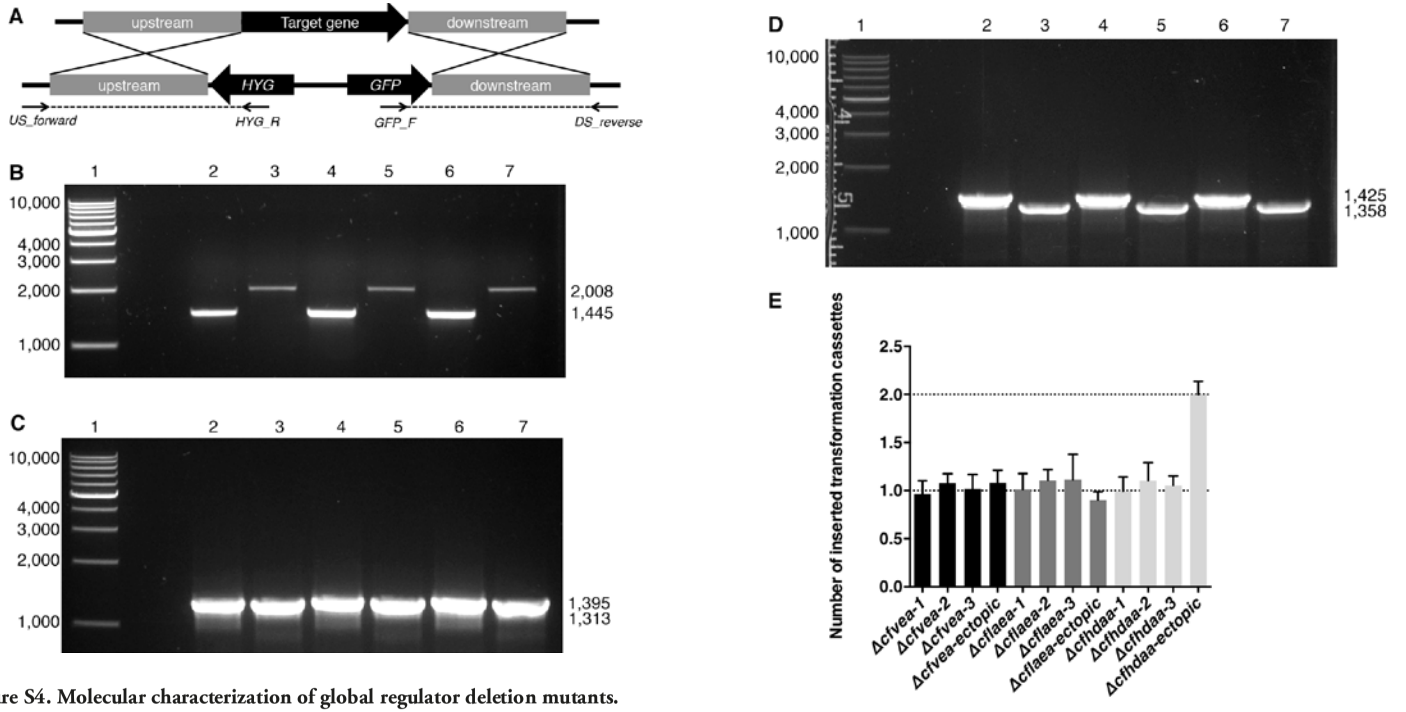


Figure S4. Molecular characterization of global regulator deletion mutants.

(A) Chart representing the targeted gene replacement of global regulator genes by a deletion cassette containing the hygromycin resistance (*HYG*) and green fluorescent protein (*GFP*) genes through homologous recombination. Oligonucleotides used to screen for mutants (recombination sites) are indicated.

PCR amplification of the upstream and downstream recombination sites for **(B)** *Δcfvea*, **(C)** *Δcflaea* and **(D)** *Δcfhdaa* deletion mutants. Lane 1 : 1Kb DNA Step ladder (bp); Lane 2 and 3: upstream and downstream recombination sites for mutant 1; Lane 4 and 5: upstream and downstream recombination sites for mutant 2 ; Lane 6 and 7: upstream and downstream recombinat ion sites for mutant 3. Expected amplicon sizes are indicated on the right (bp). Image level were adjusted for vizualisation purpose.

(E) Insert copy number was determined by quantitative PCR using genomic DNA of each strain. The *HYG* gene was used to measure the number of insertion events and the actin gene was used as a single copy reference gene. Ct values were normalized to the *tubulin* gene according to the $2^{-\Delta\Delta Ct}$ method. All transformants carry a single deletion cassette , except the *Δcfhdaa* ectopic transformant that carries two copies. However, this transformant did not show any difference compared to wild type and the two other ectopic transformants.

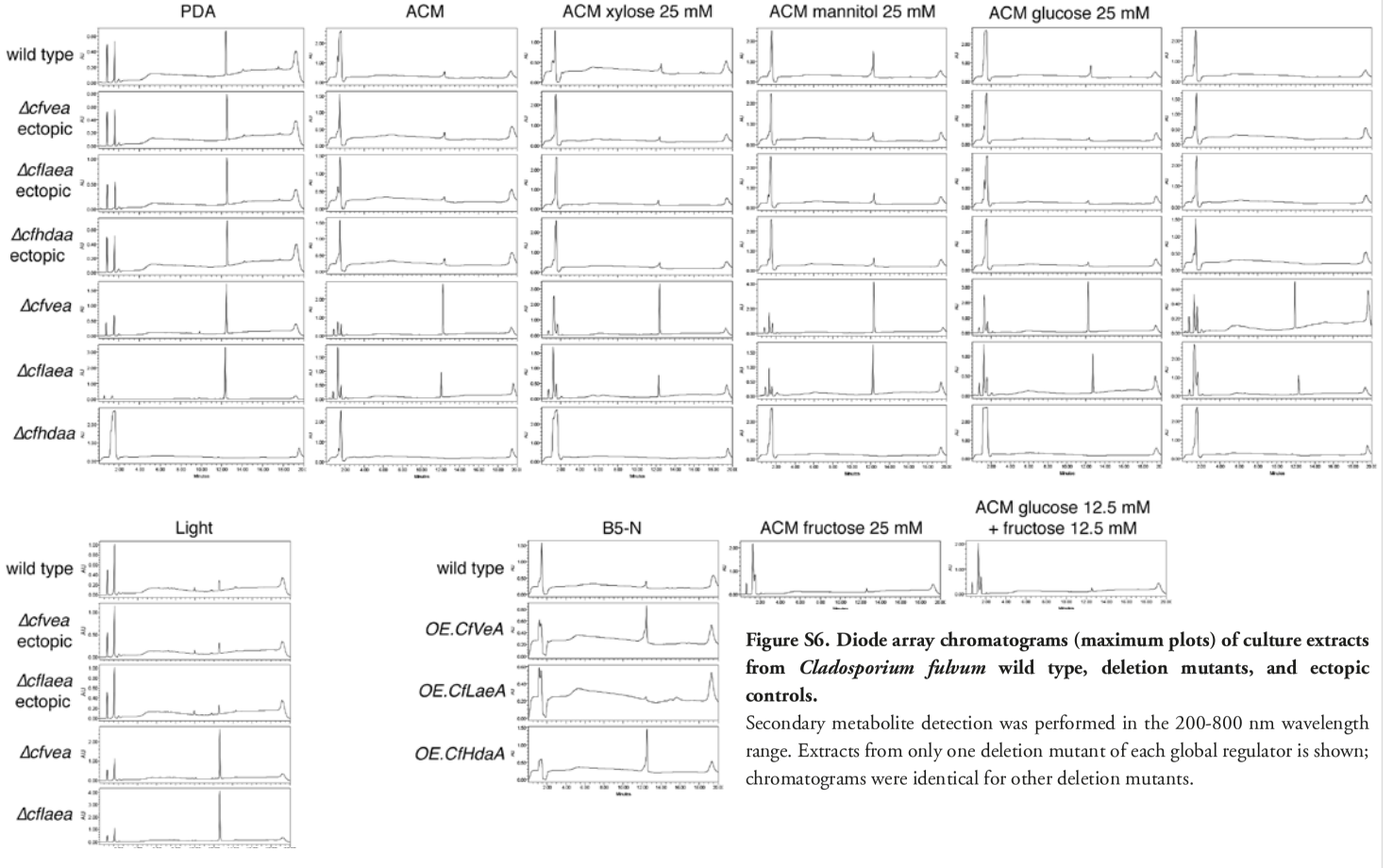


Figure S6. Diode array chromatograms (maximum plots) of culture extracts from *Cladosporium fulvum* wild type, deletion mutants, and ectopic controls.

Secondary metabolite detection was performed in the 200-800 nm wavelength range. Extracts from only one deletion mutant of each global regulator is shown; chromatograms were identical for other deletion mutants.

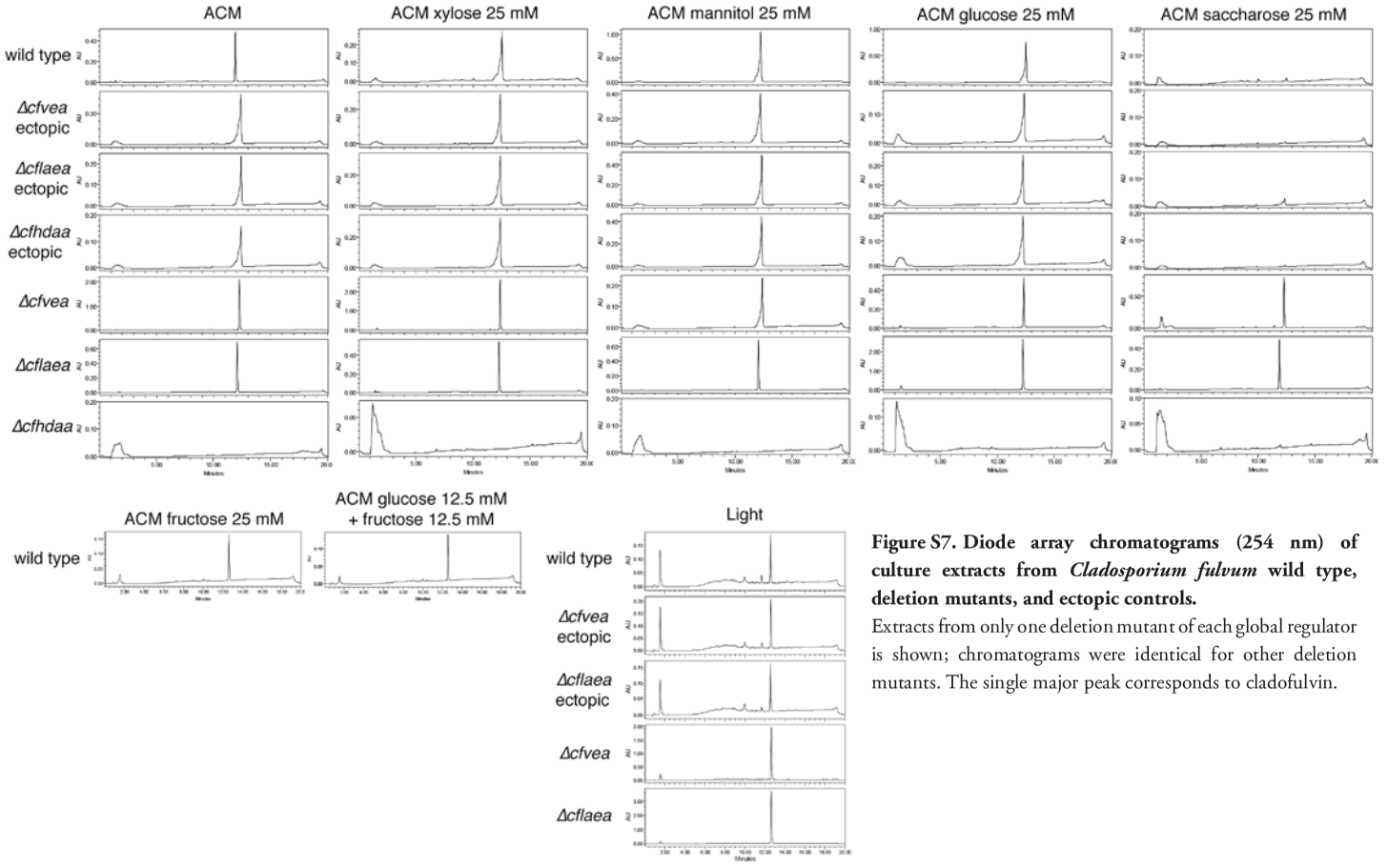


Figure S7. Diode array chromatograms (254 nm) of culture extracts from *Cladosporium fulvum* wild type, deletion mutants, and ectopic controls.
Extracts from only one deletion mutant of each global regulator is shown; chromatograms were identical for other deletion mutants. The single major peak corresponds to cladofulvin.

Table S1. Oligonucleotides used in this study.

Name	Sequence (5' to 3')
Construction of deletion cassettes	
CfHdaA_US_F	*GGGGACAACTTTGTATAGAAAAGTTG*ATTACGCTTGGCCTGTGAGC
CfHdaA_US_R	*GGGGACTGCTTTTTTGTACAAACTTG*CAAGTTCTGAAGTGAGCGAAAG
CfHdaA_DS_F	*GGGGACAGCTTTCTTGTACAAAGTGG*CGCGCTGGAGCGCTTTTGCTTCC
CfHdaA_DS_R	*GGGGACAACTTTGTATAATAAAGTTG*CCGCCCACTAGCTGCTGGTA
CfLaeA_US_F	*GGGGACAACTTTGTATAGAAAAGTTG*CCGAATGAGCGCGACCTCCG
CfLaeA_US_R	*GGGGACTGCTTTTTTGTACAAACTTG*CCTCGTCGCGACGAGAGCGGGGACG
CfLaeA_DS_F	*GGGGACAGCTTTCTTGTACAAAGTGG*GTTGCCTCAAACATTATACAGC
CfLaeA_DS_R	*GGGGACAACTTTGTATAATAAAGTTG*CTCCCCATTCTATGCGCTTT
CfVeA_US_F	*GGGGACAACTTTGTATAGAAAAGTTG*CGGTGGCTGTGATGAGCTGA
CfVeA_US_R	*GGGGACTGCTTTTTTGTACAAACTTG*GATGAAACTGGCTACCAGCAGTG
CfVeA_DS_F	*GGGGACAGCTTTCTTGTACAAAGTGG*TCGTCGAAGCAAAGGCCGACG
CfVeA_DS_R	*GGGGACAACTTTGTATAATAAAGTTG*TCTGCCTTCAACTACGTCTACATCATCATCTC
Construction of over-expression cassettes	
AscI_Avr9_Promoter_Forward	CATGTAGCGGCGCGCCGTGTGACTTTCCGTGGCCTAGAAG
PacI_Avr9_Promoter_Reverse	GGCTACATGTTAATTAAGATAGATACGTAAGTCGATAGAC
PacI_CfHdaA_F	GCTACATGTTAATTAAATGTTAAGCGCGATGTGATCTAGATCGTATAGCGGCC
NotI_CfHdaA_R	CATGTAGCGCGGCCGCTCATAATATCGCAGCGTTTCAGGCTACCATGAAGCTATTGAGC
PacI_CfLaeA_F	GCTACATGTTAATTAAATGTGAAGAGTTAGAAGTGCTGAGGCTGTG
NotI_CfLaeA_R	CATGTAGCGCGGCCGCCTAAATTCGGTATTGTGACCTGTGGACC
PacI_CfVeA_F	GCTACATGTTAATTAAATGAACCACGACACTCTGCTCCCC
NotI_CfVeA_R	CATGTAGCGCGGCCGCTTACTTTCCGGGCTTGTATTCGCC
Verification of transformants	
HdaA_Ver_F	ATGTTAAGCGCGATGTGATCTAGATCGTATAGCGGCC
HdaA_Ver_R	TCATAATATCGCAGCGTTTCAGGCTACC
LaeA_Ver_F	ATGTGAAGAGTTAGAAGTGCTGAGGCTGT
LaeA_Ver_R	CGCAGCCGCATAATTGTGACATGT
VeA_Ver_F	CAGGCTGGTGCCATCTTCGATCTCAATG
VeA_Ver_R	CTGAGGACGTTCTAGGACGAACATATG
Hyg_Ver_R	GCACTCGTCCGAGGGCAAAGGAATAG
Gfp_Ver_F	CGGGATCACTCACGGCATGGACGAG
RT-PCR	
VeA_F	ATGAACCACGACACTCTGCTCCCC
VeA_R	CTACGCATGACCAGGCAAAGCACG
HdaA_F	CTGCATAACTTGAGCTGTCGGTATGAGCAG
HdaA_R	TCATTTGGGAGATGACATCGCAAAATTC
LaeA_F	ATGTGAAGAGTTAGAAGTGCTGAGGCTGTG
LaeA_R	CTAAATTCGGTATTGTGACCTGTGGACC
qrtPCR	
qHdaA_F	AGAGCGAGGATGAGGATGAA
qHdaA_R	TCCATTGCTCGTCACAGAAG
qLaeA_F	GCATGGCACTACTCACCAGA
qLaeA_R	TCGGTATTGTGACCTGTGGA
qVeA_F	GAGAACCGAAATCTGGTGGA
qVeA_R	CGAGATGGACCCAAGTGATT

Annealing temperature for all oligonucleotide pairs is 60°C

Sequences in italics correspond to Gateway recombination sequences and underline sequences are restriction sites.

Table S2. Amino acid identity of *Cladosporium fulvum* orthologues to characterized global regulators (deduced from protein alignments shown in Figure S2).

Global regulator	Fungal species	Percentage of amino acid identity	Accession number	Reference
CfVeA (188542)	*Dothistroma septosporum*	84.31	EME47645.1	Chettri *et al.,* 2012
	Bipolaris maydis	43.30	AEP40317.1	Wu *et al.*, 2012
	Aspergillus flavus	38.18	ABC41691.1	Duran *et al.*, 2007
	Aspergillus parasiticus	37.84	AAS07022.1	Calvo *et al.,* 2004
	Aspergillus niger	39.26	XP_001392627.1	Wang *et al.*, 2015
	Aspergillus fumigatus	38.47	CAE47975.1	Krappmann *et al.*, 2005
	Aspergillus nidulans	36.83	AAD42946.1	Kim *et al.*, 2002
	Penicillium citrinum	39.12	BAL61195.1	Baba *et al.,* 2012
	Histoplasma capsulatum	41.02	ACB59235.1	Laskowski-Peak *et al.*, 2012
	Fusarium verticllioides	37.59	ABC02879.1	Li *et al.*, 2006
	Fusarium graminearum	38.64	AEY76160.1	Merhej *et al.*, 2012
CfLaeA (186126)	*Bipolaris maydis*	34.67	AEP40318.1	Wu *et al.*, 2012
	Botrytis cinerea	29.36	AJG44853.1	Schumacher*et al.,* 2015
	Aspergillus oryzae	30.28	BAF74528.1	Oda *et al.,* 2011
	Aspergillus nidulans	29.47	AAQ95166.1	Bok and Keller, 2004
	Aspergillus fumigatus	29.72	AAR01218.1	Bok and Keller, 2004
	Penicillium chrysogenum	30.41	ACD50375.1	Kosalková *et al.*, 2009
	Penicillium citrinum	29.81	ADL63139.1	Xing *et al.*, 2010
	Fusarium verticillioides	29.41	EWG36568.1	Butchko *et al.*, 2012
	Trichoderma reesei	28.66	AFK30952.1	Karimi-Aghcheh *et al.,* 2013
CfHdaA (192224)	*Aspergillus fumigatus*	38.37	XP_748144.1	Lee *et al.*, 2009

Supplementary references

Krappmann S., Bayram O., Braus G.H., 2005. Deletion and allelic exchange of the Aspergillus fumigatus veA locus via a novel recyclable marker module. Eukaryot Cell. 4:1298-307

Laskowski-Peak M.C., Calvo A.M., Rohrssen J., Smulian A.G., 2012. VEA1 is required for cleistothecial formation and virulence in Histoplasma capsulatum. Fungal Genet Biol. 49:838-46. doi:10.1016/j.fgb.2012.07.001

Li S., Myung K., Guse D., Donkin B., Proctor R.H., Grayburn W.S., Calvo A.M., 2006. FvVE1 regulates filamentous growth, the ratio of microconidia to macroconidia and cell wall formation in Fusarium verticillioides. Mol Microbiol. 62:1418-32

Marchler-Bauer A., Derbyshire M.K., Gonzales N.R., Lu S., Chitsaz F., Geer L.Y., Geer R.C., He J., Gwadz M., Hurwitz D.I., Lanczycki C.J., Lu F., Marchler G.H., Song J.S., Thanki N., Wang Z., Yamashita R.A., Zhang D., Zheng C., Bryant S.H., 2015. CDD: NCBI's conserved domain database. Nucleic Acids Res. 43(Database issue):D222-6. doi:10.1093/nar/gku1221

Merhej J., Urban M., Dufresne M., Hammond-Kosack K.E., Richard-Forget F., Barreau C., 2012. The velvet gene, FgVe1, affects fungal development and positively regulates trichothecene biosynthesis and pathogenicity in Fusarium graminearum. Mol Plant Pathol. 13:363-74. doi:10.1111/j.1364-3703.2011.00755.x

Oda K., Kobayashi A., Ohashi S., Sano M., 2011. Aspergillus oryzae laeA regulates kojic acid synthesis genes. Biosci Biotechnol Biochem. 75:1832-4

Schumacher J., Simon A., Cohrs K.C., Traeger S., Porquier A., Dalmais B., Viaud M., Tudzynski B., 2015. The VELVET complex in the gray mold fungus Botrytis cinerea: impact of BcLAE1 on differentiation, secondary metabolism and virulence. Mol Plant Microbe Interact. [Epub ahead of print]

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 28:2731-9. doi:10.1093/molbev/msr121

Wang F., Dijksterhuis J., Wyatt T., Wösten H.A., Bleichrodt R.J., 2015. VeA of Aspergillus niger increases spore dispersing capacity by impacting conidiophore architecture. Antonie Van Leeuwenhoek. 107:187-99. doi:10.1007/s10482-014-0316-z

Xing W., Deng C., Hu C.H., 2010. Molecular cloning and characterization of the global regulator LaeA in Penicillium citrinum. Biotechnol Lett. 32:1733-7. doi:10.1007/s10529-010-0375-9

Chapter 4

Elucidation of the cladofulvin biosynthetic pathway in *Cladosporium fulvum* reveals a cytochrome P450 monooxygenase required for dimerization of a monomeric anthraquinone

Scott Griffiths[1]
Carl H. Mesarich[1,5]
Benedetta Saccomanno[1]
Abraham Vaisberg[2]
Pierre J.G.M. de Wit[1,*]
Russell Cox[3,4,§]
Jérôme Collemare[1,6,§,*]

In preparation

1 Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands
2 Departamento de Ciencias Celulares y Moleculares y Laboratorios de Investigacion y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Perú
3 Institut für Organische Chemie, Leibniz Universität Hannover, Hannover, Germany
4 School of Chemistry, University of Bristol, Bristol, BS8 1TS, United Kingdom
5 Bioprotection Technologies, The New Zealand Institute for Plant & Food Research Limited, Auckland, New Zealand
6 UMR1345 IRHS-INRA, 42 rue Georges Morel, 49071 Beaucouzé Cedex, France
§ These authors contributed equally to this work
* Corresponding authors: j.collemare@gmail.com; pierre.dewit@wur.nl

Abstract

Anthraquinones are a large family of secondary metabolites (SMs) that are extensively studied for their diverse biological activities, which are strongly affected by dimerization. However, only very few anthraquinone biosynthetic pathways have been elucidated. Furthermore, no enzymes responsible for anthraquinone dimerization have been identified. We have studied the gene cluster that controls the biosynthetic pathway of cladofulvin, an asymmetrical homodimer of nataloe-emodin produced by the fungus *Cladosporium fulvum*. Upon release of atrochrysone carboxylic acid from the polyketide synthase ClaG, by the β–lactamase ClaF, the decarboxylase ClaH yields emodin, which is then converted to chrysophanol hydroquinone by the reductase ClaC and the dehydratase ClaB. We demonstrate that the predicted cytochrome P450 ClaM catalyzes the dimerization of nataloe-emodin to cladofulvin. Remarkably, dimerization dramatically increases cytotoxicity against tumour cell lines. This is the first report of an enzyme that catalyzes the dimerization of an anthraquinone, a step towards the engineering of novel dimeric anthraquinones.

4.1 Introduction

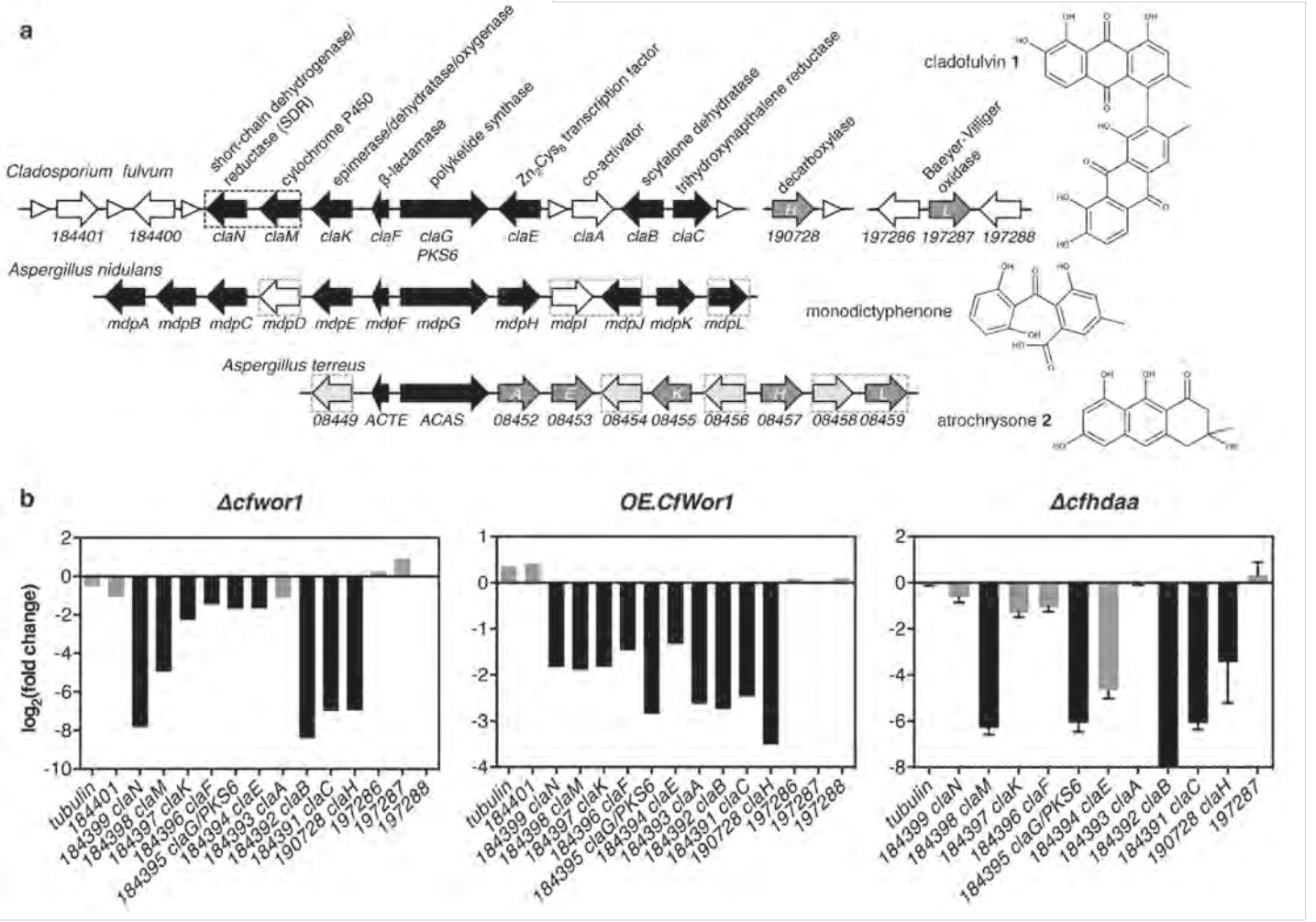
Naturally occurring anthraquinones (Singh & Chauhan, 2006; Dave & Ledwani, 2012) and structurally related xanthones (Peres & Nagem, 1997; Peres *et al.*, 2000; Negi *et al.*, 2013) are particularly interesting families of secondary metabolites (SMs) that have long been exploited for their medicinal qualities. Emodin is one of the most intensively studied anthraquinones due to its ubiquity and potent anti-cancer, anti-diabetic, anti-infective, anti-inflammatory, and cathartic properties, as well as its cardio- hepato- and neuro-protective qualities (Izhaki, 2002). Anthraquinones and xanthones contain an aromatic core that serves as a scaffold for the attachment of diverse functional groups, resulting in a wide variety of molecules with distinct biological and biochemical characteristics. This diversity is further increased by their assembly into homo- and hetero-dimers (Negi *et al.*, 2013; Hussain *et al.*, 2015) with activities that are distinct from those exhibited by their respective monomeric components (Xia *et al.*, 2014). Despite the large number of identified anthraquinones and xanthones, and their potential applications, very few of their biosynthetic pathways are known. Such knowledge is of great interest to chemists and biologists alike, particularly those searching for novel bioactive compounds or the enzymes that produce them.

Bacteria, fungi, lichens and plants produce anthraquinones and xanthones, but the best-characterised biosynthetic pathways are fungal, including those that produce the emodin-derived monodictyphenone in *Aspergillus nidulans* (Chiang *et al.*, 2010; Simpson, 2012) and the highly carcinogenic aflatoxin in *Aspergillus parasiticus* (Brown *et al.*, 1996). The biosynthesis of aflatoxin begins with the production of a raw polyketide by the core polyketide synthase (PKS) PksA, followed by a large number of modifications that are catalyzed by discrete tailoring/decorating enzymes (Yu *et al.*, 1995). Genes that encode the enzymes required for aflatoxin biosynthesis are arranged in a cluster, which is conserved across a number of other fungal species. Notably, changes in the complement of tailoring genes in this conserved gene cluster in other fungal species has been shown to drive the production of different anthraquinones, such as sterigmatocystin in *A. nidulans* (Brown *et al.*, 1996) and dothistromin in *Dothistroma septosporum* (Bradshaw *et al.*, 2002; Schwelm & Bradshaw, 2010). A large gene cluster is also involved in the production of monodictyphenone in *A. nidulans*, which consists of the core PKS gene *mdpG* and at least seven tailoring genes (Chiang *et al.*, 2010; Simpson, 2012). In this example, it has been shown that these genes also belong to a three-loci super-cluster that produces prenyl xanthones (Sanchez *et al.*, 2011; Simpson, 2012; Inglis *et al.*, 2013). Together, these examples illustrate how the diversification of tailoring genes can lead to the modification of conserved core biosynthetic pathways, yielding structurally related, yet functionally distinct compounds. Many anthraquinone and xanthone homo- and heterodimers have been isolated from natural producers (Wezeman *et al.*, 2014; Hussain *et al.*, 2015) or from genetically modified *Aspergillus* species expressing genes related to monodictyphenone and atrochrysone biosynthesis (Awakawa *et al.*, 2009; Chiang *et al.*, 2010). Although dimerization of anthraquinone through aryl-aryl

bond formation is assumed to be catalyzed enzymatically (Wezeman *et al.*, 2014), so far no responsible enzymes have been identified.

The bi-anthraquinone cladofulvin **1** is a homo-dimer composed of two nataloe-emodin moieties linked by an aryl-aryl bond, and is the sole detectable SM produced by the fungal plant pathogen *Cladosporium fulvum* during growth on artificial media (Agosti *et al.*, 1962; Collemare *et al.*, 2014). Yet, no biological activities or functions have been determined so far (Collemare *et al.*, 2014). From the 23 predicted core SM genes identified in the genome of *C. fulvum* (de Wit *et al.*, 2012), only the *PKS6* gene is consistently and highly expressed during cladofulvin production (Collemare *et al.*, 2014). Extensive synteny was reported between the *PKS6* locus and the monodictyphenone gene cluster in *A. nidulans*, but with clear signs of divergence (Fig. 1a) (Chiang *et al.*, 2010; Collemare *et al.*, 2014). Notably, the *PKS6* locus does not contain homologues of *mdpH*, *mdpJ*, or *mdpL*, genes that are essential for the production of monodictyphenone (Chiang *et al.*, 2010; Collemare *et al.*, 2014)**.** Conversely, the *PKS6* locus contains two genes that are not present in the monodictyphenone gene cluster, genes that encode a predicted cytochrome P450 monooxygenase and a predicted short chain dehydrogenase/ reductase (SDR), respectively (Collemare *et al.*, 2014). A homologous gene cluster appears to be present in *Aspergillus terreus* (Fig. 1a and Table S1), which contains genes that encode enzymes for the production of atrochrysone **2** (Lim *et al.*, 2012). To conform to the naming convention used to describe the monodictyphenone gene cluster, we henceforth use *cla* as the prefix to denote cladofulvin genes from *C. fulvum*, with a suffix that corresponds to their cognate homologue in *A. nidulans* (Table S1; Chiang *et al.*, 2010; Collemare *et al.*, 2014).

Figure 1. Definition of the cladofulvin gene cluster in *Cladosporium fulvum*.
(a) Organisation of the *claG* (*PKS6*) gene locus in the genome of *C. fulvum*, predicted to be involved in the biosynthesis of cladofulvin. Organization of characterized homologous gene clusters in *Aspergillus nidulans* involved in the biosynthesis of monodictyphenone (Chiang *et al.*, 2010) and in *Aspergillus terreus* involved in the biosynthesis of atrochrysone (Awakawa *et al.*, 2009). Black arrows represent co-regulated genes in each given species and/or shown to be involved in the production of the corresponding metabolite. Dark grey arrows represent genes potentially belonging to the depicted gene clusters; letters indicate the homologous monodictyphenone gene in *A. nidulans*. Light grey arrows indicate genes with functions that are not present in other gene clusters. White arrows indicate genes that are not co-regulated in a given species. The dashed-black square indicates genes specific to *C. fulvum* and dashed-grey squares indicate genes in *A nidulans* and *A. terreus* for which no close homologue could be found in the genome of *C. fulvum*. White triangles indicate repetitive elements. Above the arrows is written the functional annotation of genes from *C. fulvum*. See Table S1 for gene accession numbers. (b) Differential expression of genes at the *claG* (*PKS6*) locus in the Δ*cfwor1* deletion mutant compared to wild type as determined by RNA-seq (Ökmen *et al.*, 2014). (c) Differential expression of genes at the *claG* (*PKS6*) locus in the *OE.CfWor1* transformant compared to wild type as determined by RNA-seq (Ökmen *et al.*, 2014). (d) Differential expression of genes at the *claG* (*PKS6*) locus in the Δ*cfhdaa* deletion mutant compared to wild type as determined by RT-qrtPCR. Error bars represent standard deviation of three biological repeats. Black and grey bars show significant and non-significant fold change, respectively, according to Cuffdiff analysis of three biological repeats for (b) and (c). Statistics in (d) were performed on $2^{-\Delta Ct}$ values using a two-way ANOVA with a *post-hoc* Sidak multi-comparison test at the significance level 0.05.


a
short-chain dehydrogenase/ reductase (SDR)
cytochrome P450
epimerase/dehydratase/oxygenase
β-lactamase
polyketide synthase
Zn2Cys6 transcription factor
co-activator
scytalone dehydratase
trihydroxynaphthalene reductase
decarboxylase
Baeyer-Villiger oxidase
Cladosporium fulvum
184401 184400 claN claM claK claF claG PKS6 claE claA claB claC 190728 197286 197287 197288
cladofulvin 1
Aspergillus nidulans
mdpA mdpB mdpC mdpD mdpE mdpF mdpG mdpH mdpI mdpJ mdpK mdpL
monodictyphenone
Aspergillus terreus
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In this study we aimed to elucidate the cladofulvin biosynthetic pathway and identify the enzyme(s) involved in the dimerization of nataloe-emodin to cladofulvin. By using *C. fulvum* deletion mutants, and heterologous expression of presumed cladofulvin biosynthetic genes in *Aspergillus oryzae*, we propose a complete route to cladofulvin biosynthesis, including the final enzymatic dimerization step. We also report how the dimerization of nataloe-emodin increases the ability of this compound to inhibit the growth of mammalian tumour cell-lines.

4.2 Results

4.2.1 Nine genes at the *claG* locus and *claH* are co-regulated during cladofulvin production and define the putative cladofulvin gene cluster

We first wanted to ascertain which genes define the predicted cladofulvin gene cluster. For this purpose, we compared the expression of the *cla* genes predicted to be involved in cladofulvin **1** production in wild-type *C. fulvum* and in genetically modified strains of this fungus in which cladofulvin **1** production is abolished. *C. fulvum* deletion mutants of the global transcriptional regulator Wor1, and the over-expression *OE.CfWor1* transformant do not produce cladofulvin **1**, consistent with down-regulation of the *PKS6* gene in both genetic backgrounds (Ökmen *et al.*, 2014). Similarly, deletion of the histone deacetylase gene, *CfHdaA,* in *C. fulvum* blocked cladofulvin production (chapter 3). RNA-seq data from the Δ*cfwor1* deletion mutants showed that relative to wild type, with the exception of *claA*, all the predicted *cla* genes are significantly down regulated, whilst the β-*tubulin* control gene and *clafu184401* border gene are not (Fig. 1b). In the *OE.CfWor1* transformant, only genes from the predicted cladofulvin gene cluster, including *claA*, are significantly down regulated at the *claG* locus (Fig. 1b). Similarly in the Δ*cfhdaa* deletion mutants, *claB*, *claC*, *claG* and *claM* are significantly down regulated compared to wild type, with the other genes also showing a clear trend for down-regulation (Fig. 1b).

In addition to genes at the *claG* locus (Collemare *et al.*, 2014), the *clafu190728* and *clafu197287* genes were included because their products bear high similarity to MdpH and MdpL, respectively, in *A. nidulans* (Table S1b). The putative *claH* gene (*clafu190728*) is significantly down regulated in all three genetic backgrounds compared to wild type, but the expression of *clafu197287* was unchanged (Fig. 1b). In conclusion, these results confirm the predicted gene cluster to be at the *claG* locus, with the exception of the putative regulatory gene *claA*. Although not located at the same locus, *claH* is co-regulated and likely belongs to the same biosynthetic pathway as the *claG* gene cluster.

4.2.2 Biosynthesis of cladofulvin depends on the megasynthase ClaG

To validate the hypothesis that the non-reducing polyketide synthase (nrPKS; Cox, 2007) ClaG is responsible for cladofulvin **1** production, we replaced the *claG* gene in *C. fulvum* wild type

via homologous recombination by a deletion cassette that contains the hygromycin resistance marker gene *HYG*. Two confirmed independent deletion mutants and an ectopic transformant were selected for further analysis (Fig. S1). Both Δ*clag* deletion mutants appeared to be greyer than the ectopic transformant and wild type, but otherwise showed no obvious phenotypic abnormalities (Fig. 2a).

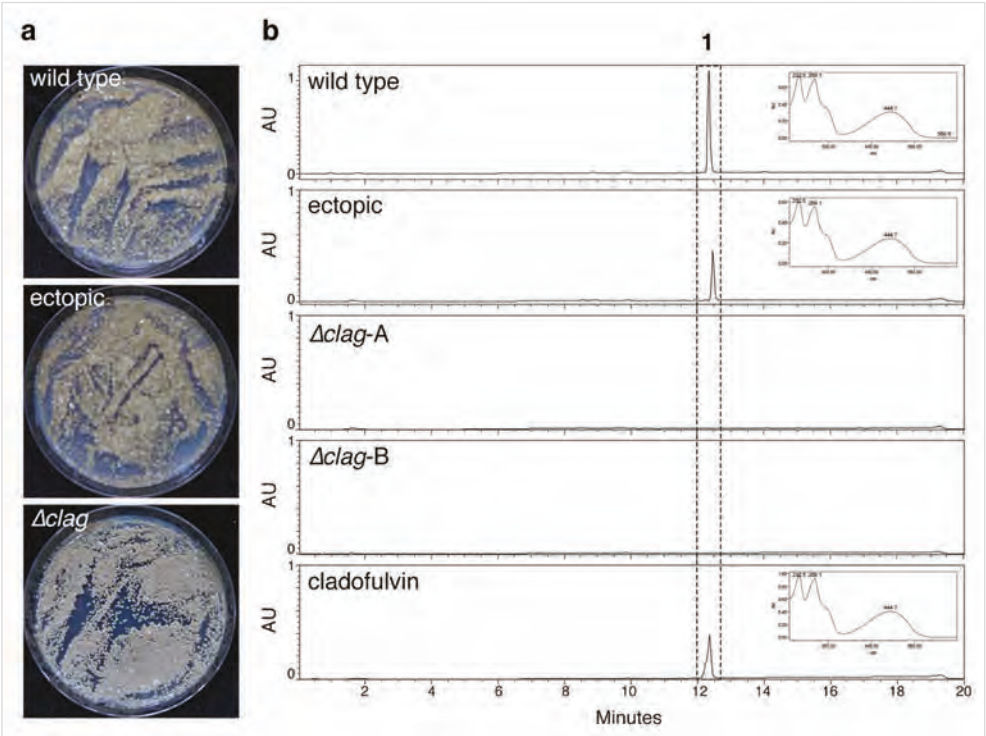

Figure 2. The megasynthase ClaG is required for cladofulvin production.
(a) Deletion of *claG* altered the pigmentation of *Cladosporium fulvum* during growth on potato-dextrose agar (PDA).
(b) Diode array chromatograms (254 nm) of ethyl acetate extracts from wild type, ectopic transformant and deletion mutant strains showed the absence of cladofulvin **1** (RT = 12.3 min) in Δ*clag* deletion mutants. Pure cladofulvin **1** (0.5 mg) was used as a standard. The UV spectrum of detected compounds is shown on the right.

Ethyl acetate extracts from cultures of two independent Δ*clag* deletion mutants, wild type and ectopic transformant were analysed by HPLC and LC-MS, alongside purified cladofulvin **1** as a standard (Collemare *et al.*, 2014). Cladofulvin **1** (retention time (RT) = 12.3 min) was detected in the controls but not in the Δ*clag* deletion mutants (Fig. 2b), confirming that *claG* indeed encodes the responsible PKS. All other co-regulated genes identified above might therefore play a role in the regulation or production of cladofulvin **1** in *C. fulvum*.

4.2.3 Determination of the first cladofulvin biosynthetic steps identified three enzymes required for emodin biosynthesis

To characterise the early steps of cladofulvin **1** biosynthesis, we heterologously expressed selected *cla* genes in *A. oryzae* M-2-3. Despite the high number of SM genes encoded in the genome of *A. oryzae* (Machida *et al.*, 2005), this fungal strain has a silent SM profile and a proven track record in expressing heterologous SM genes (Halo *et al.*, 2008; Awakawa *et al.*, 2009; Pahirulzaman *et al.*, 2012).

The nrPKSs MdpG, ACAS, EncA, AptA, and AdaA, involved in the first step of monodictyphenone, atrochrysone **2**, endocrocin **3**, asperthecin and TAN-1612 biosynthesis, respectively, do not contain any canonical thiolesterase (TE) or Claisen cyclase (CLC) domains (Szewczyk *et al.*, 2008; Awakawa *et al.*, 2009; Chiang *et al.*, 2010; Li *et al.*, 2011; Lim *et al.*, 2012). Instead, the metallo-β-lactamase-type thiolesterase (MβL-TE) MdpF, ACTE, EncB, AptB, and AdaB, respectively, are required for closure and release of each nascent polyketide. ClaG is orthologous to these enzymes (Collemare *et al.*, 2014); Fig. S2) and does not contain a TE or CLC domain; and ClaF is a predicted orthologous MβL-TE (Collemare *et al.*, 2014); Fig. S2). Consistent with our expectations, *A. oryzae* transformants expressing *claG* alone did not produce any detectable polyketide (Fig. 3a). In contrast, *A. oryzae* transformants co-expressing *claG* and *claF* genes produced several new compounds, **2-9**, bearing UV signatures diagnostic of aromatic polyketides (Fig. 3b and Fig. S3). Product **2** (RT = 5.1 min; UV max = 225, 271, 320, 397 nm; *m/z* (ES^-) 273 $[M-H]^-$) was identified as atrochrysone by comparing its UV and MS data to those published (Awakawa *et al.*, 2009). Product **3** (RT = 5.5 min; UV max = 224, 287, 442 nm; *m/z* (ES^-) 313 $[M-H]^-$) was identified as endocrocin by comparing its UV and MS spectra to those published (Awakawa *et al.*, 2009; Chiang *et al.*, 2010).

Products **4** (RT = 8 min; UV max = 226, 351 nm; *m/z* (ES^-) 255 $[M-H]^-$) and **5** (RT = 8.1 min; UV max = 224, 288, 440 nm; *m/z* (ES^-) 269 $[M-H]^-$) were identified as emodin anthrone and emodin, respectively, by comparing their UV and MS data to those published (Awakawa *et al.*, 2009; Chiang *et al.*, 2010). Products **6** (RT = 5.6 min; UV max = 224, 361 nm; *m/z* (ES^-) 597 $[M-H]^-$) and **7** (RT = 5.8 min; UV max = 224, 359; *m/z* (ES^-) 597 $[M-H]^-$) are likely diastereoisomers of homodimers of endocrocin anthrone as previously reported when ACAS and ACTE enzymes were co-expressed in *A. oryzae* (Awakawa *et al.*, 2009). Also as reported in this latter study, products **8** (RT = 6.9 min; UV max 255, 361 nm; *m/z* (ES^-) 553 $[M-H]^-$) and **9** (RT = 7 min; UV max 255, 361 nm; *m/z* (ES^-) 553 $[M-H]^-$) are likely diastereoisomers of heterodimers made of emodin anthrone and endocrocin anthrone**.** Evaporative Light Scattering Detector (ELSD) chromatogram showed that ClaG and ClaF produced atrochrysone **2** as the major product in *A. oryzae* (Fig. S4). Detection of atrochrysone **2** and endocrocin **3** suggests that ClaG and ClaF released atrochrysone carboxylic acid as an unstable intermediate as previously suggested in biosynthetic pathways for atrochrysone and monodictyphenone (Awakawa *et al.*, 2009; Chiang *et al.*, 2010). In addition, detection of homodimers and heterodimers of endocrocin anthrone suggests that this compound served as an intermediate between atrochrysone carboxylic acid and endocrocin **3**.

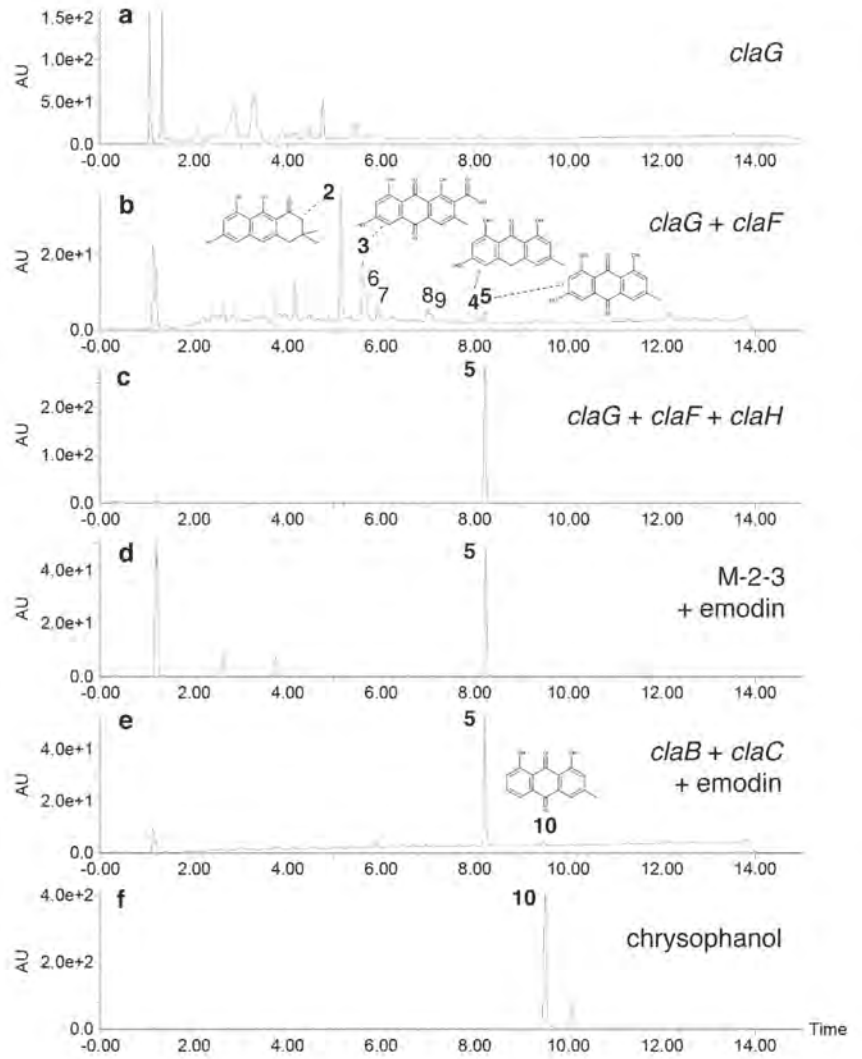

Figure 3. Identification of intermediates in the cladofulvin biosynthetic pathway using heterologous expression of candidate biosynthetic *cla* genes in *Aspergillus oryzae*.

A. oryzae M-2-3 was transformed with expression vectors carrying the polyketide synthase *claG*, β-lactamase thiolesterase *claF,* putative decarboxylase *claH*, scytalone dehydratase *claB,* and trihydroxynaphthalene reductase *claC* genes. Ethyl acetate extracts were analysed by LC-MS. Diode array chromatograms are shown.

(a) The transformants carrying *pTAex3GS-claG* yielded no polyketides.

(b) The transformants carrying *pTAYAGSargPage-claF::claG* produced eight compounds including atrochrysone **2**, endocrocin **3**, emodin anthrone **4**, emodin **5**, homodimers of endocrocin anthrone **6** and **7**, and heterodimers of endocrocin anthrone and emodin anthrone **8** and **9**.

(c) The transformants carrying *pTAYAGSargPage-claF::claG::claH* produced emodin **5** only.

(d) Cultures of *A. oryzae* M-2-3 supplemented with emodin **5** produced no new compound.

(e) Cultures of the transformants carrying *pTAYAGSargPage-claB::claC* supplemented with emodin **5** produced chrysophanol **10**.

(f) Pure chrysophanol **10** (Sigma-Aldrich) was used as a standard.

In *A. nidulans*, biosynthesis of monodictyphenone involves atrochrysone **2** and emodin **5** as intermediate compounds, which requires decarboxylation of atrochrysone carboxylic acid (Chiang *et al.*, 2010). It was hypothesized that *mdpH* is responsible for this post-PKS step because only endocrocin accumulated in Δ*mdph* deletion mutants (Chiang *et al.*, 2010). In contrast, deletion of any other tailoring gene resulted in the production of emodin and other emodin-related shunt products, which lack a carboxyl group (Chiang *et al.*, 2010). In the *C. fulvum* genome, *claH* is a gene that is co-regulated with the other cladofulvin genes (Fig. 1) and encodes a predicted protein bearing 64% identity to the N-terminal region of MdpH (Table S1 and Fig. S5). To address the role of ClaH in cladofulvin biosynthesis, we co-expressed *claH* with *claF* and *claG* in *A. oryzae.* The resulting transformants produced only emodin **5** as shown by comparing its RT, UV and MS spectra to a standard (Fig. 3c and 3d). This suggests that *claH* and *mdpH* are functional decarboxylase homologues, and emodin is the second stable intermediate in cladofulvin biosynthesis.

4.2.4 ClaC and ClaB are responsible for the conversion of emodin to chrysophanol

Simpson (2012) proposed that during monodictyphenone biosynthesis, emodin **5** is converted to chrysophanol **10** by reduction and dehydration, catalyzed successively by the trihydroxynaphthalene reductase MdpC and scytalone dehydratase MdpB. It was subsequently shown that MdpC is indeed capable of reducing emodin hydroquinone to 3-hydroxy-3,4-dihydroanthracen-1(2H)-one (Schätzle *et al.*, 2012). To test the involvement of *claC* and *claB* in converting emodin **5** to chrysophanol **10**, both genes were co-expressed in *A. oryzae*. Induced cultures of these transformants, in addition to untransformed *A. oryzae*, were supplemented with emodin **5** (10 mg·L^{-1}) and left for 8 h prior to ethyl acetate extraction. LC-MS examination of organic extracts from the untransformed *A. oryzae* contained emodin **5** only (Fig. 3d). Extracts from transformants co-expressing *claB* and *claC* contained not only emodin **5** but also the new product **10** (RT = 9.3 min; UV max = 225, 258, 428 nm; *m/z* (ES^+) 255 $[M\text{-}H]^+$), which was identified as chrysophanol by comparing its RT, UV and MS spectra to an authentic standard (Fig. 3e and 3f). These results show that ClaC and ClaB convert emodin **5** to chrysophanol **10**, presumably *via* sequential reduction and dehydratation.

4.2.5 The cytochrome P450 ClaM is responsible for C5-C7′ dimerization of nataloe-emodin

Targeted deletion of *claM* was performed in *C. fulvum* in order to determine the role of this cytochrome P450 in cladofulvin **1** biosynthesis. A gene cassette containing the *HYG* gene was used to replace *claM* by homologous recombination. Two confirmed independent deletion mutants and an ectopic transformant were selected for further analysis (Fig. S1). The Δ*clam* deletion mutants were more grey-brown in colour compared to wild-type and ectopic controls (Fig. 4a). A dark brown compound accumulated in the cytoplasm of fungal cells and diffused into the agar (Fig. 4a and 4b). LC-MS analysis of ethyl acetate extracts confirmed that cladofulvin **1**

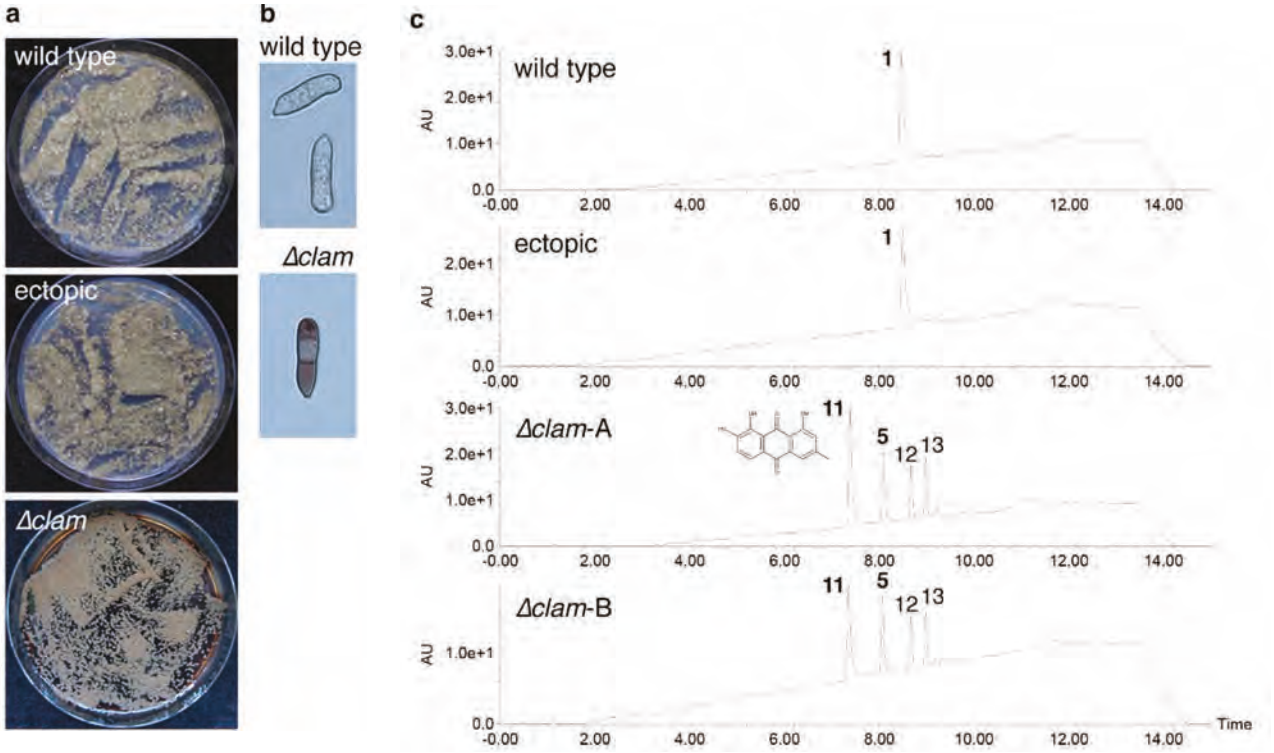

Figure 4. Accumulation of nataloe-emodin in *Cladosporium fulvum* Δ*clam* deletion mutants.
(a) The deletion of *claM* altered the pigmentation of *C. fulvum* during growth on potato-dextrose agar (PDA).
(b) Spores of Δ*clam* deletion mutants accumulate a pigment in the cytoplasm compared to wild type.
(c) Diode array chromatograms of ethyl acetate extracts from *C. fulvum* wild type, ectopic transformant and Δ*clam* deletion mutants. Wild type and ectopic transformant produced only cladofulvin **1**. Δclam deletion mutants produced emodin **5** and nataloe-emodin **11**, Compounds **12** and **13** are shunt products of emodin **5**.

was present in the controls, but not in the Δ*clam* deletion mutants (Fig. 4c). The Δ*clam* deletion mutants produced several new peaks and the major species were isolated by mass-directed HPLC and examined by NMR. The first-eluted compound **11** (RT = 7.3 min; UV max = 231, 259, 430 min; m/z (ES^-) 269 $[M-H]^-$) was purified and comparison of its ^1H NMR data with a literature standard proved it to be nataloe-emodin (Table S2), a SM previously unknown in fungi. The next-eluted product is emodin **5,** also identified by ^1H NMR (Fig. 4c). Later-eluted products **12** and **13** were shown by High-Resolution Mass Spectrometry (HRMS) to be reduced and aminated emodin shunt isomers, but full structural elucidation was prevented by their rapid degradation. These results confirm that nataloe-emodin **11** is indeed the immediate precursor to cladofulvin, and, most importantly, that its dimerization is catalyzed by the cytochrome P450 ClaM.

4.2.6 Cytotoxicity of cladofulvin and its precursors

Nataloe-emodin **11** potently inhibits the growth of immortalised mammalian cell-lines (Aponte *et al.*, 2008), but no biological activities have been reported for cladofulvin **1** so far (Collemare *et al.*, 2014). To test if it exhibits a similar cytotoxic activity, cladofulvin **1** was applied to diverse nataloe-emodin-sensitive mammalian cell-lines, including non-tumorigenic BALB/c mouse embryo cells (BALB/3T3), human lung large cell carcinoma (H460), human amelanotic melanoma (M-14), human prostate carcinoma (DU145), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HuTu80 and HT-29), and human chronic myelogenous leukaemia (K562) (Aponte *et al.*, 2008). In addition, emodin **5** and chrysophanol **10** were also tested on the same cell lines. Emodin **5** inhibited the growth of cell-lines with significantly lower potency than nataloe-emodin **11** (multiple t-tests corrected for multiple comparisons with the Holm-Sidak method; α = 0.025; P-values ranging from $7.5e^{-6}$ (H460) to 0.012 (3T3)), except for M-14 and K562 cell lines against which emodin 5 showed higher (P-value = 0.005) and equal (P-value = 0.121) inhibition, respectively (Table 1). In contrast, chrysophanol **10** weakly inhibited the growth of HuTu-80 and K562 cells only (GI_{50} 47 and 132 μg mL^{-1}, respectively; P-values = 5.7e-7 and 0.001 when compared to emodin) (Table 1). These results suggest that the C3 or C2 hydroxyl group in emodin **5** and nataloe-emodin **11**, respectively, is essential for the cytotoxic activity of these compounds, yet the C2 position appears to provide significantly higher potency. Strikingly, compared to nataloe-emodin **11**, cladofulvin **1** was significantly more active towards each target cell-line, ranging from a 24-fold difference for HT-29 to a 320-fold increase for K562 (P-values ranging from 1.2e-10 (MCF-7) to 0.001 (K562); Table 1). These results show that dimerization of nataloe-emodin enhanced dramatically its cytotoxicity and might provide clues about a biological function in *C. fulvum*.

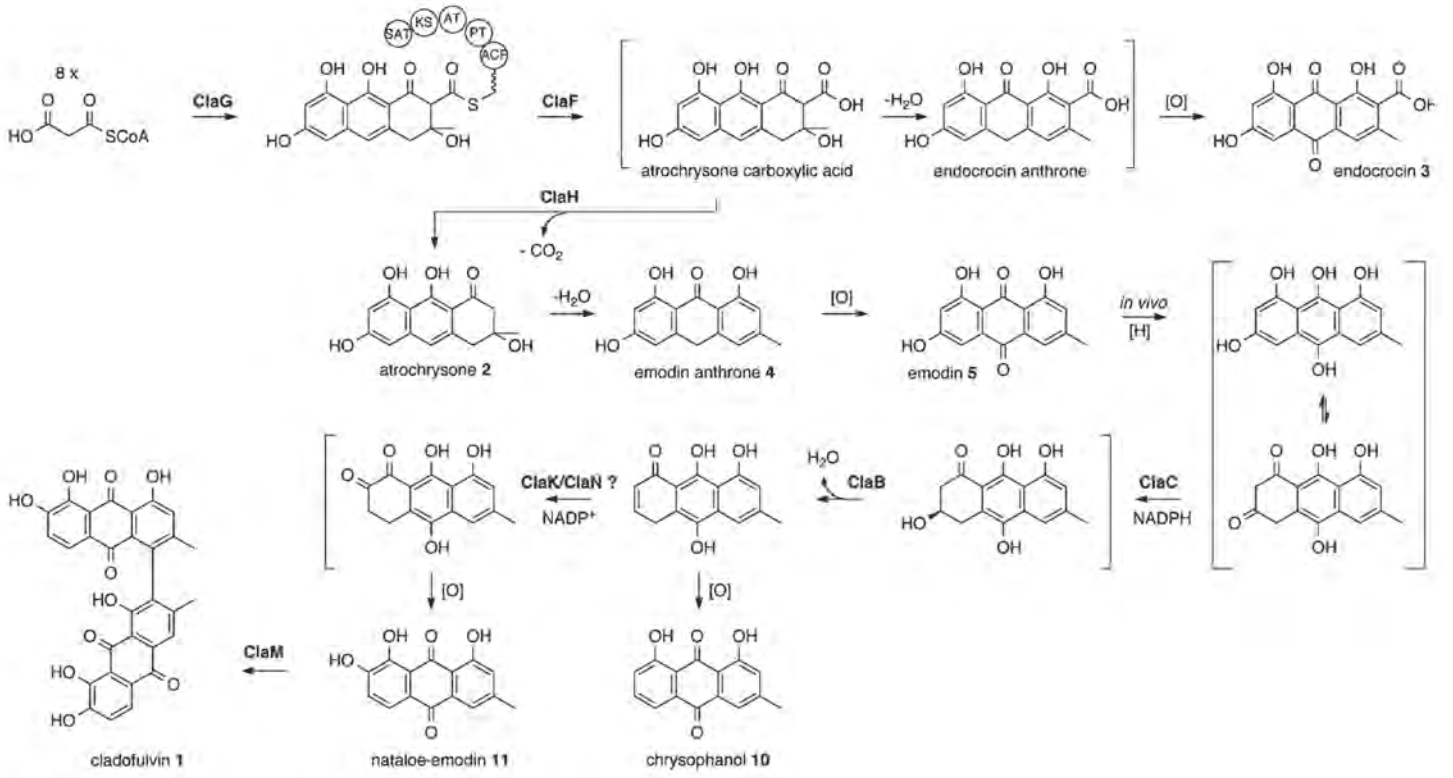

Figure 5. Proposed route for cladofulvin biosynthesis and mechanism of nataloe-emodin coupling

Proposed cladofulvin **1** biosynthetic pathway. The formation of endocrocin **3** and emodin **5** might occur spontaneously. All oxidation ([O]) steps could be spontaneous in air. Conversion of chrysophanol **10** to nataloe-emodin **11** is the only step without support and might involve ClaN and ClaK. Compounds in between brackets were not identified in this study. SAT, starter unit ACP transacylase; KS, keto synthase; AT, acyl transferase; PT, product template; ACP, acyl carrier protein.

Table 1. Toxicity of cladofulvin and its precursors against animal cell lines.
GI_{50} (μg mL^{-1}) of each compound against different cell lines was determined. Numbers in between brackets indicate the standard deviation of at least three technical replicates.

	3T3	H460	HuTu80	DU145	MCF-7	M-14	HT-29	K562
emodin	15.329 (0.193)	9.928 (0.182)	7.831 (0.308)	13.973 (0.831)	8.178 (0.138)	11.802 (0.072)	17.007 (0.293)	8.094 (0.197)
chrysophanol[a]	>200	>200	46.972 (1.153)	>200	>200	>200	>200	132.042 (26.754)
nataloe-emodin[b]	10.345 (1.975)	4.800 (0.236)	Na	7.928 (0.608)	7.324 (0.087)	13.640 (0.557)	6.545 (1.194)	12.499 (3.887)
cladofulvin	0.060 (0.002)	0.037 (0.001)	0.097 (0.005)	0.055 (0.002)	0.036 (0.001)	0.097 (0.003)	0.275 (0.015)	0.039 (0.001)

[a] The maximum concentration tested was 200 μg mL^{-1}
[b] Data published in (Aponte *et al.*, 2008)

4.3 Discussion

4.3.1 Atrochrysone carboxylic acid is the raw polyketide produced by ClaF and ClaG

Based on the metabolic profiles of *C. fulvum* deletion mutants and *A. oryzae* transformants expressing putative cladofulvin **1** biosynthetic genes, we propose a biosynthetic route to cladofulvin **1** production (Fig. 5). Consistent with the SM profile of *A. oryzae* expressing *ACAS* and *ACTE* (Awakawa *et al.*, 2009), the co-expression of *claF* and *claG* in the same host yielded the same compounds, apart from endocrocin anthrone. However, detection of endocrocin **3**, and homo- and heterodimers **6–9** of endocrocin anthrone suggest that this SM was unstable under our conditions. These data support the phylogenetic relationship between ClaF-ClaG and ACAS-ACTE and confirm they are functional orthologues (Collemare *et al.*, 2014; Fig. S2). Spontaneous conversion of endocrocin **3** to emodin **5** is considered to be thermodynamically unfavourable (Awakawa *et al.*, 2009; Chiang *et al.*, 2010). Thus, to account for the co-detection of endocrocin **3**, emodin **5,** and atrochrysone **2**, the highly unstable compound atrochrysone carboxylic acid was proposed as the raw polyketide released from ACAS by ACTE through a hydrolysis mechanism (Awakawa *et al.*, 2009). We propose the same mechanism in the cladofulvin **1** biosynthetic pathway with atrochrysone carboxylic acid as the first unstable intermediate, which preferentially degrades to form atrochrysone **2** and endocrocin **3** (Fig. 5).

4.3.2 ClaH is a decarboxylase that yields emodin

LC-MS analysis of transformants co-expressing *claH*, *claG* and *claF* suggests that atrochrysone carboxylic acid is decarboxylated by ClaH to yield atrochrysone **2** (Fig. 5). Indeed, the sole presence of emodin **5** in these transformants suggests that ClaH significantly accelerates

decarboxylation of atrochrysone carboxylic acid, consistent with the absence of endocrocin **3**. When transformants expressing *claG* and *claF* only were grown for two additional days, endocrocin **3** became the major product produced instead of atrochrysone **2** (Fig. S4). This is consistent with the decarboxylase activity of ClaH and suggests that spontaneous decarboxylation of atrochrysone carboxylic acid is slower than its spontaneous dehydration. The trace amounts of emodin anthrone **4** and emodin **5** detected in *A. oryzae* transformants expressing *claF* and *claG* are consistent with successive spontaneous dehydration and oxidation of atrochrysone **2** and emodin anthrone **4**, respectively. The latter step yielding emodin **5** was indeed shown to occur spontaneously and may bypass the activity of anthrone oxidases (Awakawa *et al.*, 2009; Chiang *et al.*, 2010; Ehrlich *et al.*, 2010).

MdpH from *A. nidulans* shares homology with HypC and EncC that are involved in the production of aflatoxin and endocrocin **3**, respectively (Yu *et al.*, 2004; Lim *et al.*, 2012). HypC is an anthrone oxidase that converts norsolorinic acid anthrone to norsolorinic acid (Ehrlich *et al.*, 2010). EncC was also hypothesized to catalyze the oxidation of endocrocin anthrone to endocrocin **3** (Lim *et al.*, 2012). The three enzymes contain the conserved domain DUF1772, which is hypothesized to be involved in protein-protein interaction (Lim *et al.*, 2012). MdpH also contains the additional conserved domain EthD (Fig. S5), which was initially thought to be involved in ethyl tert-butyl ether degradation in *Rhodococcus ruber* (Chauvaux *et al.*, 2001). ClaH contains this EthD domain only and does not share any similarity with HypC or EncC (Fig. S5). The results of Chiang *et al.*, (2010) and our study suggest that MdpH and ClaH are decarboxylases. In contrast, HypC and EncC clearly do not have such activity as shown by the oxidation of norsolorinic acid anthrone (Ehrlich *et al.*, 2010) and by the absence of emodin **5** in *A. fumigatus* (Lim *et al.*, 2012), respectively. This suggests that the EthD domain is responsible for the observed decarboxylase activity. A gene encoding a protein that carries an EthD domain is present at the ACAS-ACTE locus (Table S1). It would be interesting to test whether this enzyme is also able to catalyze decarboxylation of atrochrysone carboxylic acid and would lead to accumulation of emodin **5** and the absence of atrochrysone **10**.

4.3.3 Conversion of emodin into nataloe-emodin involves chrysophanol hydroquinone as an intermediate

Heterologous expression of *claC* and *claB* in *A. oryzae* yielded chrysophanol **10** after the respective cultures were supplemented with emodin **5**. However, it was shown that the substrate of MdpC is emodin hydroquinone, which is expected to be the prevalent form *in vivo* (Schätzle *et al.*, 2012). Thus, we propose that ClaC also uses emodin hydroquinone as a substrate, which implies that the product of ClaB is chrysophanol hydroquinone (Fig. 5). We hypothesized that ClaK and ClaN might be involved in the next step towards the formation of nataloe-emodin **11**. However, co-expression of *claK* and *claN* in *A. oryzae* did not yield any new compound when cultures of the respective transformants were supplemented with chrysophanol **10**. This suggests that ClaK/

ClaN might oxidise the intermediate chrysophanol hydroquinone rather than chrysophanol **10**. In contrast to the monodictyphenone biosynthetic pathway proposed by Simpson (2012), our results suggest that chrysophanol is actually a shunt product in the cladofulvin biosynthetic pathway. Such a route between emodin **5** and nataloe-emodin **11** is in agreement with findings by Schätzle et al. (2012) and with the absence of chrysophanol **10** in Δ*clam* deletion mutants. Further investigations are needed to ascertain this part of the proposed route to cladofulvin **1**.

4.3.4 Dimerization of nataloe-emodin by the cytochrome P450 ClaM

Despite the large number of known dimeric anthranoids and xanthonoids occurring in nature, no enzymes that catalyze their formation have been previously reported (Wezeman *et al.*, 2014; Hussain *et al.*, 2015). A number of dimeric anthrones have been isolated from fungi (Awakawa *et al.*, 2009; Chiang *et al.*, 2010; Zheng *et al.*, 2012), with strong evidence that their formation is enzymatically catalyzed (Awakawa *et al.*, 2009). Our results show that ClaM is required for the production of the bi-anthraquinone cladofulvin **1** *via* the C5-C7' linkage of nataloe-emodin **11** (Fig. 5). However, cladofulvin **1** was not produced when *A. oryzae* transformants expressing *claM* were supplemented with nataloe-emodin **11**. Therefore, it cannot be excluded that the reduced form of nataloe-emodin **11** is the actual substrate for dimerization by ClaM.

The involvement of cytochrome P450s in the dimerization of naphthoquinones, coumarins and diketopiperazine alkaloid was reported in bacteria, in *A. niger*, and in *Aspergillus flavus,* respectively (Zhao *et al.*, 2005; Girol *et al.*, 2012) (Saruwatari *et al.*, 2014). In *Streptomyces coelicolor* A3(2), flaviolin is dimerized by a cytochrome P450 (Zhao *et al.*, 2005), and a similar enzyme in *Streptomyces griseus* dimerizes tetrahydroxynapthalene into 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (Funa *et al.*, 2005). In *A. niger*, the cytochrome P450 KtnC dimerizes dimethyl siderin into kotanin (Girol *et al.*, 2012). The cytochrome P450 DtpC in *A. flavus* is responsible for the concomitant pyrroloindole ring formation and homo- or hetero- dimerization of the diketopiperazine alkaloids ditryptophenaline, dibrevianamide F and tryptophenaline (Saruwatari *et al.*, 2014). Nataloe-emodin **11** dimers with alternative aryl-aryl bonds have not been observed in *C. fulvum*, nor have dimeric forms of emodin **5**. This suggests that ClaM might be highly selective for both substrate and type of linkage it catalyzes. In contrast, *Alternaria* species produce a large variety of alterporriol compounds, which are heterodimers of macrosporin and altersolanol exhibiting various couplings, including C5-C5', C1-C7', C7-C5' and C2-C2' bonds (Debbab *et al.*, 2009; Zheng *et al.*, 2012). Homodimers of macrosporin or altersolanol were also isolated and show C4-C6' and C4-C8' bonds (Zheng *et al.*, 2012). The diversity of dimers for a given anthraquinone likely depends on both substrates and specificity of enzymes involved in dimerization. The former will determine the positions at which radicals or cations can be generated (Zhao *et al.*, 2005). Combined with substrate specificity of cytochrome P450s, diverse aryl-aryl bond formations will be catalyzed. The production of so many different alterporriols by *Alternaria* species may be due to multiple dimerizing enzymes, or fewer, but less-selective enzymes that catalyze multiple reactions.

Dimerization, and the type of bond that links monomers, profoundly affects biological activities of SMs. As we showed for nataloe-emodin **11** and cladofulvin **1**, dimerization can strengthen an existing activity. The potential utility of cytochrome P450s is considerable, as a biocatalyst for the bio- or semi-synthetic conversion of natural monomers into novel dimeric compounds. The identification of ClaM ought to accelerate the *in silico* discovery of functionally similar enzymes in other fungal species and further investigation will determine their usefulness in designing new compounds with interesting increased existing or new biological activities.

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References

Agosti G, Birkinshaw JH, Chaplen P. 1962. Studies in the biochemistry of micro-organisms. 112. Anthraquinone pigments of strains of Cladosporium fulvum Cooke. *The Biochemical Journal* **85**: 528-30.

Aponte JC, Vaisberg AJ, Rojas R, Caviedes L, Lewis WH, Lamas G, Sarasara C, Gilman RH, Hammond GB. 2008. Isolation of Cytotoxic Metabolites from Targeted Peruvian Amazonian Medicinal Plants. *Journal of Natural Products* **71**: 102-5.

Awakawa T, Yokota K, Funa N, Doi F, Mori N, Watanabe H, Horinouchi S. 2009. Physically discrete beta-lactamase-type thioesterase catalyzes product release in atrochrysone synthesis by iterative type I polyketide synthase. *Chemistry & Biology* **16**: 613-23.

Boyd MR, Paull KD. 1995. Some practical considerations and applications of the national cancer institute in vitro anticancer drug discovery screen. *Drug Development Research* **34**: 91-109.

Bradshaw RE, Bhatnagar D, Ganley RJ, Gillman CJ, Monahan BJ, Seconi JM. 2002. Dothistroma pini, a forest pathogen, contains homologs of aflatoxin biosynthetic pathway genes. *Applied and Environmental Microbiology* **68**: 2885-92.

Brown DW, Yu JH, Kelkar HS, Fernandes M, Nesbitt TC, Keller NP, Adams TH, Leonard TJ. 1996. Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in Aspergillus nidulans. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 1418-22.

Chauvaux S, Chevalier F, Le Dantec C, Fayolle F, Miras I, Kunst F, Beguin P. 2001. Cloning of a genetically unstable cytochrome P-450 gene cluster involved in degradation of the pollutant ethyl tert-butyl ether by Rhodococcus ruber. *Journal of Bacteriology* **183**: 6551-7.

Chiang Y, Szewczyk E, Davidson AD, Entwistle R, Keller NP, Wang CCC, Oakley BR. 2010. Characterization of the Aspergillus nidulans monodictyphenone gene cluster. *Applied and Environmental Microbiology* **76**: 2067-74.

Collemare J, Griffiths S, Iida Y, Jashni MK, Battaglia E, Cox RJ, de Wit PJGM. 2014. Secondary Metabolism and Biotrophic Lifestyle in the Tomato Pathogen Cladosporium fulvum. *PloS One* **9**: e85877.

Cox RJ. 2007. Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. *Organic Biomolecular Chemistry.* **5**: 2010-26.

Dave H, Ledwani L. 2012. A review on anthraquinones isolated from Cassia species and their applications. *Indian Journal of Natural Products and Resources* **3**: 291-319.

Debbab A, Aly AH, Edrada-Ebel R, Wray V, Müller WEG, Totzke F, Zirrgiebel U, Schächtele C, Kubbutat MHG, Lin WH, *et al.* 2009. Bioactive metabolites from the endophytic fungus Stemphylium globuliferum isolated from Mentha pulegium. *Journal of Natural Products* **72**: 626-31.

Ehrlich KC, Li P, Scharfenstein L, Chang P. 2010. HypC, the anthrone oxidase involved in aflatoxin biosynthesis. *Applied and Environmental Microbiology* **76**: 3374-7.

Funa N, Funabashi M, Ohnishi Y, Horinouchi S. 2005. Biosynthesis of hexahydroxyperylenequinone melanin via oxidative aryl coupling by cytochrome P-450 in Streptomyces griseus. *Journal of Bacteriology* **187**: 8149-55.

Girol CG, Fisch KM, Heinekamp T, Günther S, Hüttel W, Piel J, Brakhage AA, Müller M. 2012. Regio- and stereoselective oxidative phenol coupling in Aspergillus niger. *Angewandte Chemie International Edition* **51**: 9788-91.

Halo LM, Marshall JW, Yakasai AA, Song Z, Butts CP, Crump MP, Heneghan M, Bailey AM, Simpson TJ, Lazarus CM, *et al.* 2008. Authentic heterologous expression of the tenellin iterative polyketide synthase nonribosomal peptide synthetase requires coexpression with an enoyl reductase. *ChemBioChem* **9**: 585-94.

Hussain H, Al-Harrasi A, Green IR, Hassan Z, Ahmed I. 2015. Recent Advances in the Chemistry and Biology of Natural Dimeric Quinones. *Studies in Natural Products Chemistry* **46**: 1-567.

Inglis DO, Binkley J, Skrzypek MS, Arnaud MB, Cerqueira GC, Shah P, Wymore F, Wortman JR, Sherlock G. 2013. Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of Aspergillus nidulans, A. fumigatus, A. niger and A. oryzae. *BMC Microbiology* **13**: 91.

Izhaki I. 2002. Emodin - a secondary metabolite with multiple ecological functions in higher plants. *New Phytologist* **155**: 205-217.

Li Y, Chooi Y, Sheng Y, Valentine JS, Tang Y. 2011. Comparative characterization of fungal anthracenone and naphthacenedione biosynthetic pathways reveals an α-hydroxylation-dependent Claisen-like cyclization catalyzed by a dimanganese thioesterase. *Journal of the American Chbemical Society* **133**: 15773-85.

Lim FY, Hou Y, Chen Y, Oh J, Lee I, Bugni TS, Keller NP. 2012. Genome-based cluster deletion reveals an endocrocin biosynthetic pathway in Aspergillus fumigatus. *Applied and Environmental Microbiology* **78**: 4117-25.

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, *et al.* 2005. Genome sequencing and analysis of Aspergillus oryzae. *Nature* **438**: 1157-61.

Negi JS, Bisht VK, Singh P, Rawat MSM, Joshi GP. 2013. Naturally Occurring Xanthones: Chemistry and Biology. *Journal of Applied Chemistry* **2013**: 1.

Pahirulzaman KAK, Williams K, Lazarus CM. 2012. A toolkit for heterologous expression of metabolic pathways in Aspergillus oryzae. *Methods in Enzymology* **517**: 241-60.

Peres V, Nagem TJ. 1997. Trioxygenated naturally occurring xanthones. *Phytochemistry* **44**: 191-214.

Peres V, Nagem TJ, de Oliveira FF. 2000. Tetraoxygenated naturally occurring xanthones. *Phytochemistry* **55**: 683-710.

Sanchez JF, Entwistle R, Hung J, Yaegashi J, Jain S, Chiang Y, Wang CC, Oakley BR. 2011. Genome-based deletion analysis reveals the prenyl xanthone biosynthesis pathway in Aspergillus nidulans. *Journal of the American Chemical Society* **133**: 4010-7.

Saruwatari T, Yagishita F, Mino T, Noguchi H, Hotta K, Watanabe K. 2014. Cytochrome P450 as dimerization catalyst in diketopiperazine alkaloid biosynthesis. *ChemBioChem* **15**: 656-9.

Schwelm A, Bradshaw RE. 2010. Genetics of dothistromin biosynthesis of Dothistroma septosporum: an update. *Toxins* **2**: 2680-98.

Schätzle MA, Husain SM, Ferlaino S, Müller M. 2012. Tautomers of Anthrahydroquinones: Enzymatic Reduction and Implications for Chrysophanol, Monodictyphenone, and Related Xanthone Biosyntheses. *Journal of the American Chemical Society* **134**: 14742-45.

Simpson TJ. 2012. Genetic and biosynthetic studies of the fungal prenylated xanthone shamixanthone and related metabolites in Aspergillus spp. revisited. *ChemBioChem* **13**: 1680-1688.

Singh R, Chauhan SMS. 2006. 9,10-Anthraquinones and other biologically active compounds from the genus Rubia. *Chemistry & Biodiversity* **1**: 1241-64.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *Journal of the National Cancer Institute* **82**: 1107-12.

Szewczyk E, Chiang YM, Oakley CE, Davidson AD, Wang CCC, Oakley BR. 2008. Identification and Characterization of the Asperthecin Gene Cluster of Aspergillus nidulans. *Applied and Environmental Microbiology* **74**: 7607-7612.

Vichai V, Kirtikara K. 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols* **1**: 1112-6.

Wezeman T, Bräse S, Masters K. 2014. Xanthone dimers: a compound family which is both common and privileged. *Natural. Product Reports.* **32**: 6-28.

Yu J, Chang PK, Cary JW, Wright M, Bhatnagar D, Cleveland TE, Payne GA, Linz JE. 1995. Comparative mapping of aflatoxin pathway gene clusters in Aspergillus parasiticus and Aspergillus flavus. *Applied and Environmental Microbiology* **61**: 2365-71.

Zheng C, Shao C, Guo Z, Chen J, Deng D, Yang K, Chen Y, Fu X, She Z, Lin Y, *et al.* 2012. Bioactive hydroanthraquinones and anthraquinone dimers from a soft coral-derived Alternaria sp. fungus. *Journal of Natural Products* **75**: 189-97.

Zhao B, Guengerich FP, Bellamine A, Lamb DC, Izumikawa M, Lei L, Podust LM, Sundaramoorthy M, Kalaitzis JA, Reddy LM, *et al.* 2005. Binding of two flaviolin substrate molecules, oxidative coupling, and crystal structure of Streptomyces coelicolor A3(2) cytochrome P450 158A2. *Journal of Biological Chemistry* **280**: 11599-607.

de Wit PJGM, van der Burgt A, Ökmen B, Stergiopoulos I, Abd-Elsalam KA, Aerts AL, Bahkali AH, Beenen HG, Chettri P, Cox MP, *et al.* 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.

Ökmen B, Collemare J, Griffiths S, van der Burgt A, Cox R, de Wit PJGM. 2014. Functional analysis of the conserved transcriptional regulator CfWor1 in Cladosporium fulvum reveals diverse roles in the virulence of plant pathogenic fungi. *Molecular Microbiology* **92**: 10-27.

Xia G, Li J, Li H, Long Y, Lin S, Lu Y, He L, Lin Y, Liu L, She Z. 2014. Alterporriol-type dimers from the mangrove endophytic fungus, Alternaria sp. (SK11), and their MptpB inhibitions. *Marine Drugs* **12**: 2953-69.

Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW. 2004. Clustered Pathway Genes in Aflatoxin Biosynthesis. *Applied and Environmental Microbiology* **70**: 1253-62.

Ökmen B, Etalo DW, Joosten MHAJ, Bouwmeester HJ, de Vos RCH, Collemare J, de Wit PJGM. 2013. Detoxification of α-tomatine by Cladosporium fulvum is required for full virulence on tomato. *New Phytologist* **198**: 1203-14.

Supplementary Items

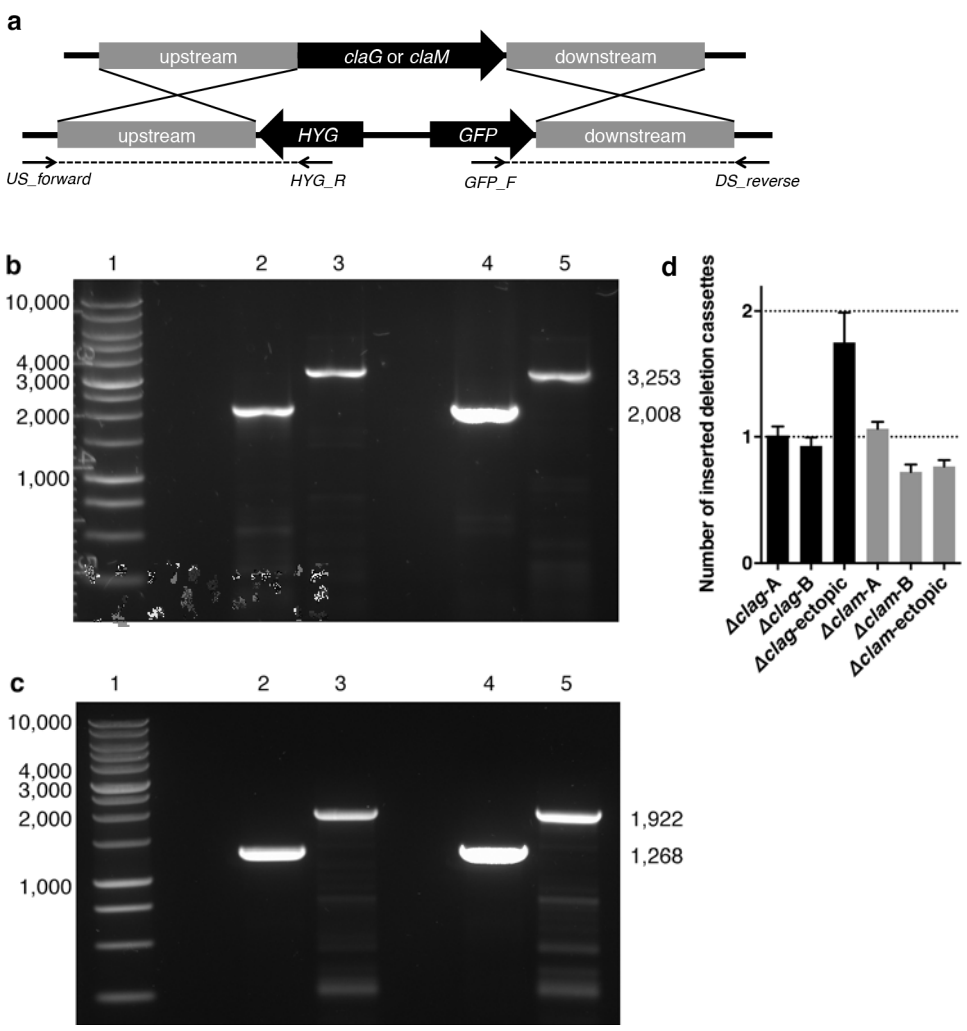


Figure S1. Molecular characterization of *Δclag* and *Δclam* deletion mutants.
(a) Chart representing the targeted gene replacement of *claG* and *claM* by a deletion cassette containing the hygromycine resistance (*HYG*) and green fluorescent protein (*GFP*) genes through homologous recombination. Oligonucleotides used to screen for mutants (recombination sites) are indicated below. PCR amplification of the upstream and downstream recombination sites for **(b)** *Δclag* and **(c)** *Δclam* deletion mutants. Lane 1: 1 Kb DNA ladder (bp); Lane 2 and 3: upstream and downstream recombination sites for mutant A; Lane 4 and 5: upstream and downstream recombination sites for mutant B. Expected amplicon sizes are indicated on the right (bp).
(d) Insert copy number was determined by quantitative PCR using genomic DNA of each strain. The *HYG* gene was used to measure the number of insertion events and the actin gene was used as a single copy reference gene. Ct values were normalized to the *tubulin* gene according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All transformants carry a single deletion cassette apart from the *Δclag*-ectopic transformant that carries two copies. However, this transformant does not show any phenotype different from wild type under all tested conditions.

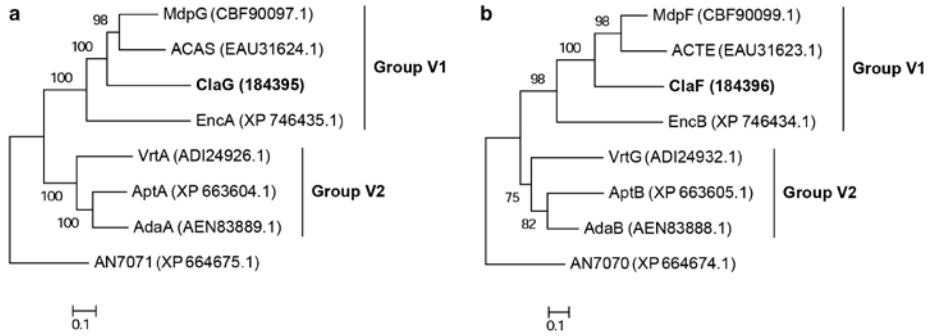


Figure S2. Phylogenetic analysis of ClaG and ClaF.

(a) Phylogenetic tree of characterized ClaG homologues. AN7071 serves to root the tree (Li *et al.*, 2011).

(b) Phylogenetic tree of characterized ClaF homologues. AN7070 serves to root the tree (Li *et al.*, 2011).

Both types of enzymes were classified in two groups, V1 and V2 (Li *et al.*, 2011).

Protein sequences were aligned using Muscle (Edgar, 2004) and poorly aligned positions were removed using Gblocks (Castresana, 2000). A Maximum Likelihood phylogeny reconstruction was performed using MEGAS (Tamura *et al.*, 2011) (JTT model for amino acid substitution, rates distributed in four gamma categories, using all sites and 100 bootstrap replications).

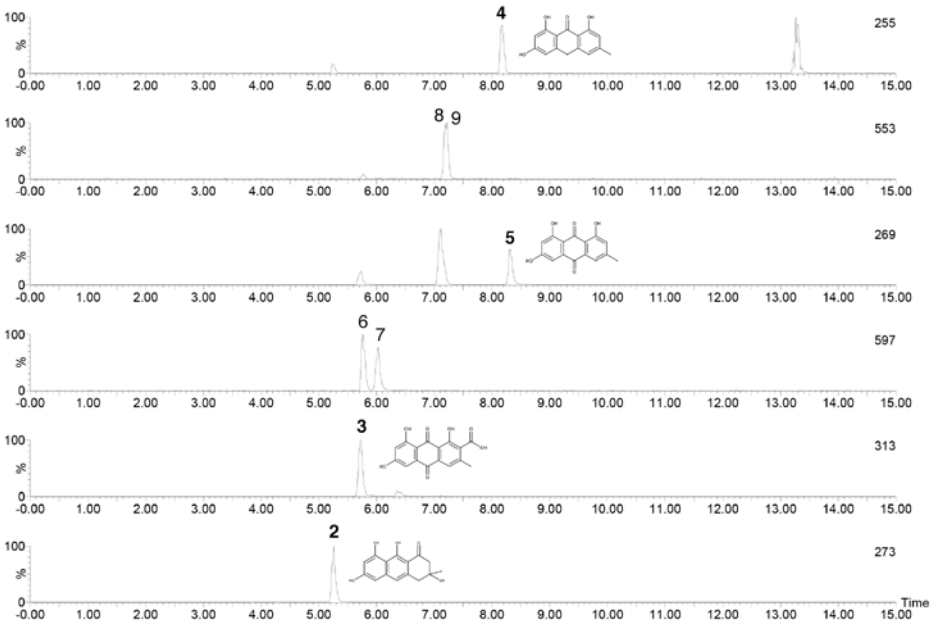


Figure S3. LC-MS analysis of *Aspergillus oryzae* transformant expressing *claG* and *claF*.

EiC (ES^-) for m/z (indicated on the right) of expected compounds produced by this transformant. According to metabolites identified by Awakawa *et al.,* (2009): atrochrysone **2** *m/z* 273 $[M\text{-}HJ^-$; endocrocin **3** *m/z* 313 $[M\text{-}HJ^-$; emodin anthrone **4** *m/z* 255 $[M\text{-}HJ^-$; emodin **5** *m/z* 269 $[M\text{-}HJ^-$; diastereomers of endocrocin anthrone homodimers 6 and 7 *m/z* 597 $[M\text{-}H]^-$; diastereomers of emodin anthrone and endocrocin anthrone heterodimers 8 and 9 *m/z* 553 $[M\text{-}H]^-$. Endocrocin anthrone (*m/z* 299 $[M\text{-}H]^-$) could not be clearly identified.

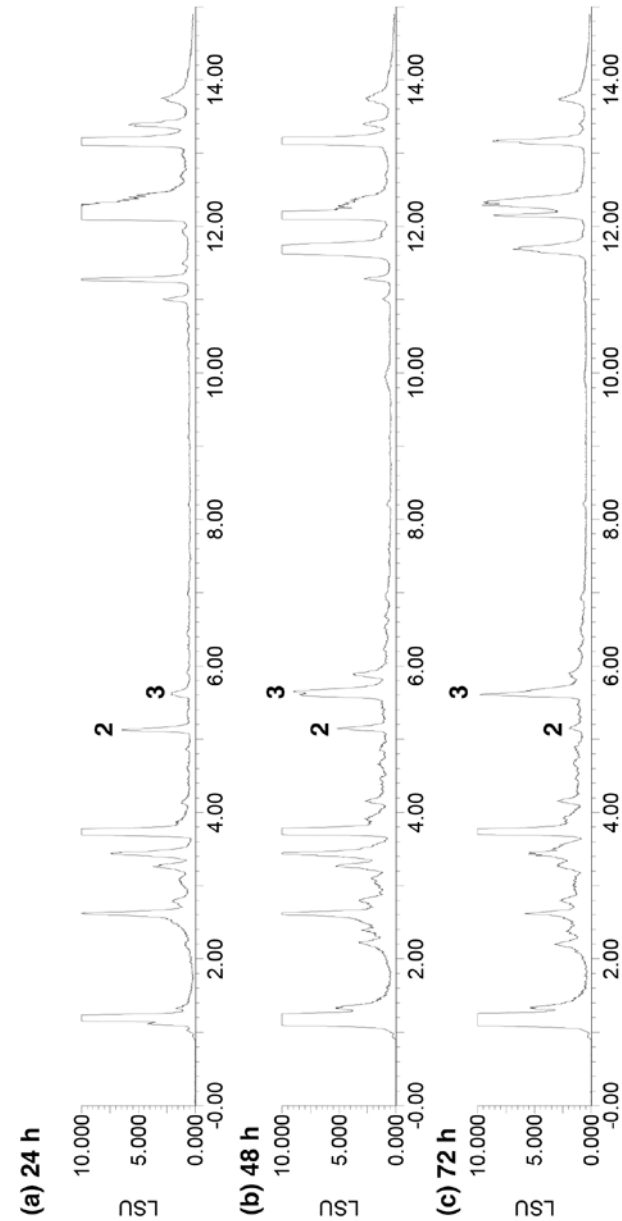


Figure S4. ELSD analysis of the *Aspergillus oryzae* transformant that expresses *claG* and *claF*.
Secondary metabolites were extracted using ethyl acetate (**a**) 24 h, (**b**) 48 h and (**c**) 72 h after induction of the expression of *claG* and *claF*. At 24 h, the major product related to cladofulvin biosynthesis is atrochrysone **2**, but at 72 h, the major product is endocrocin **3**.

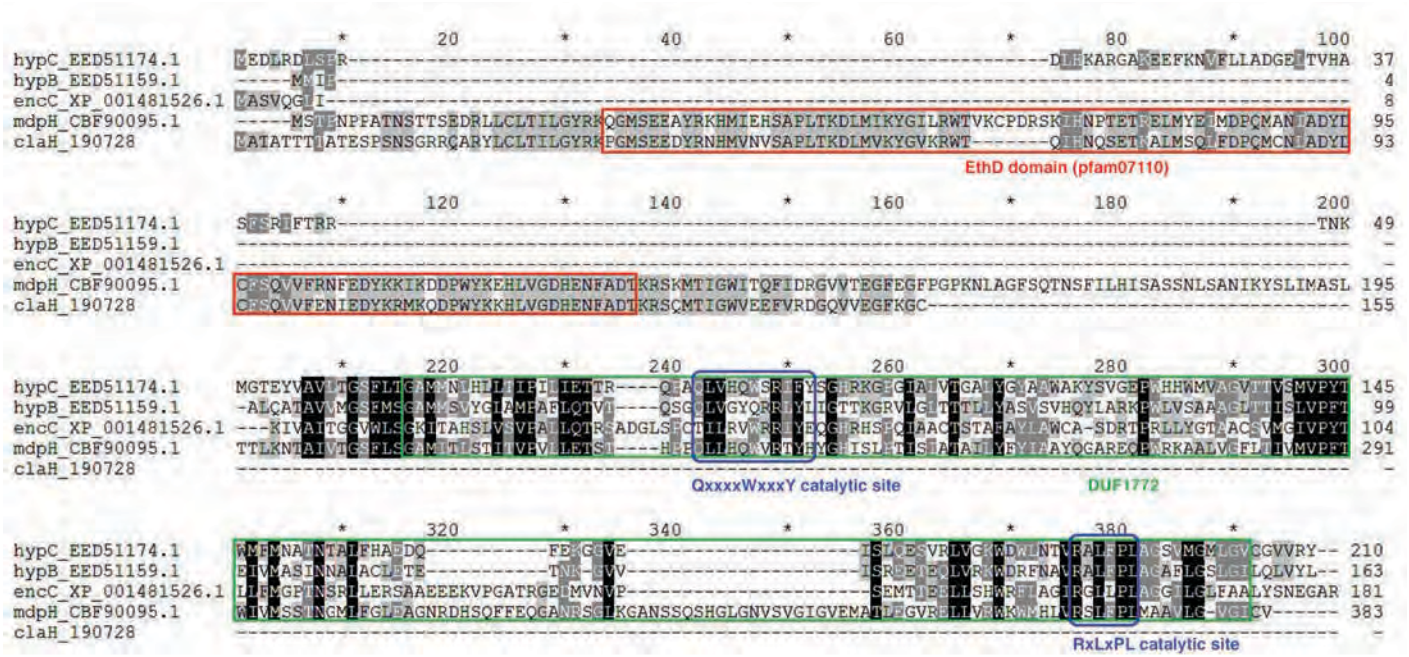


Figure S5. Alignment of ClaH and MdpH homologues.

Protein sequences were aligned with Muscle (Edgar, 2004). Conserved domains are indicated according to the NCBI Conserved Domains database (Marchler-Bauer *et al.*, 2015) and previous reports (Ehrlich, 2009; Lim *et al.*, 2012).

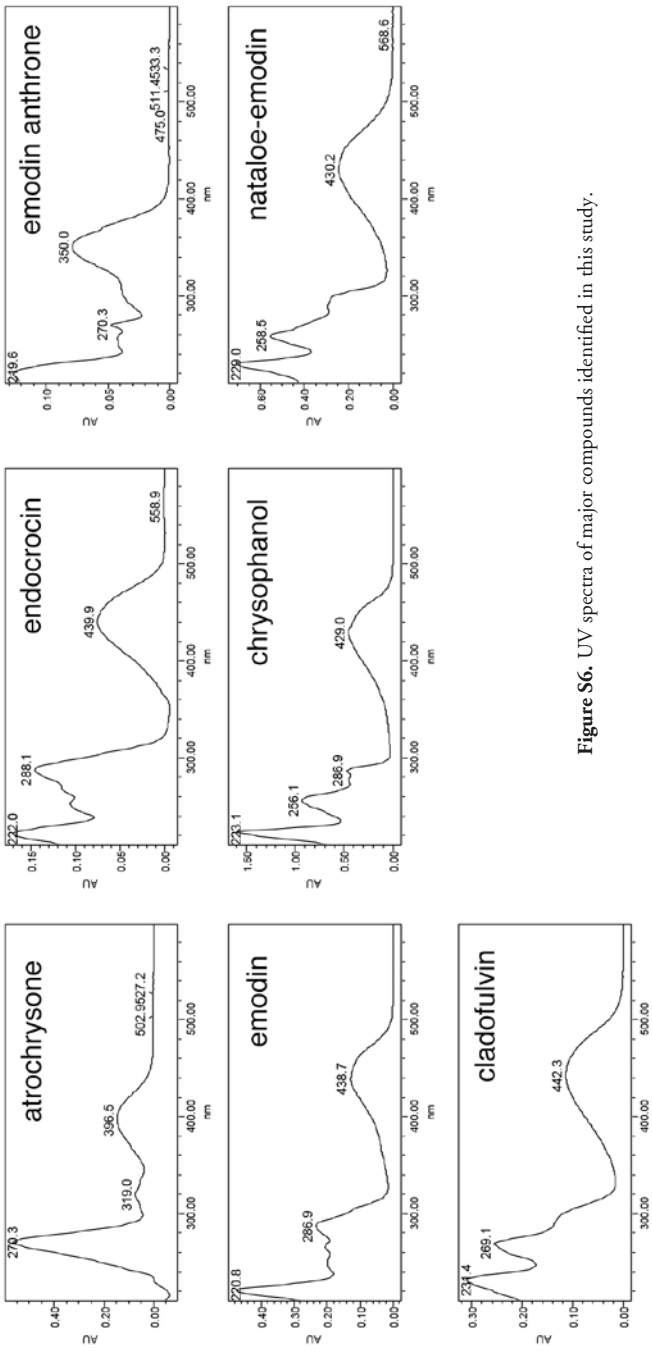


Figure S6. UV spectra of major compounds identified in this study.

Table S1a. Accession numbers of genes in Figure 1.

Fungal species	Designation	Predicted function	Gene number	GenBank accession number
Aspergillus nidulans	*mdpA*	regulatory gene	AN10021.4	CBF90109.1
	mdpB	scytalone dehydratase	AN10049.4	CBF90107.1
	mdpC	trihydroxynaphthalene reductase	AN0146.4	CBF90105.1
	mdpD	monooxygenase	AN0147.4	CBF90103.1
	mdpE	transcription factor	AN0148.4	CBF90101.1
	mdpF	β-lactamase	AN0149.4	CBF90099.1
	mdpG	polyketide synthase	AN0150.4	CBF90097.1
	mdpH	decarboxylase	AN10022.4	CBF90095.1
	mdpI	acyl-CoA synthase	AN10035.4	CBF90094.1
	mdpJ	glutathione S transferase	AN10038.4	CBF90092.1
	mdpK	oxidoreductase	AN10044.4	CBF90090.1
	mdpL	Baeyer–Villiger oxidase	AN10023.4	CBF90088.1
Aspergillus terreus	none	O-methyltransferase	ATEG_08449	XP_001217070.1
	ACTE	β-lactamase	ATEG_08450	XP_001217071.1
	ACAS	polyketide synthase	ATEG_08451	XP_001217072.1
	none	regulatory gene	ATEG_08452	XP_001217073.1
	none	transcription factor	ATEG_08453	XP_001217075.1
	none	glutathione S transferase mdpJ-like	ATEG_08454	XP_001217593.1
	none	oxidoreductase	ATEG_08455	XP_001217594.1
	none	methyltransferase	ATEG_08456	XP_001217595.1
	none	decarboxylase	ATEG_08457	XP_001217596.1
	none	multicopper oxidase	ATEG_08458	XP_001217597.1
	none	Baeyer–Villiger oxidase	ATEG_08459	XP_001217598.1

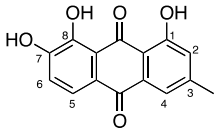
Table S1b. Accession numbers of genes in Figure 1.

Fungal species	Designation	Predicted function	Protein ID[a]	Identity and similarity to *A. nidulans* homologues (%)[b]
Cladosporium fulvum	*claA*	regulatory gene	184393	37 / 53 (91)
	claB	scytalone dehydratase	184392	56 / 74 (93)
	claC	trihydroxynaphthalene reductase	184391	71 / 88 (97)
	claE	transcription factor	184394	27 / 48 (71)
	claF	β-lactamase	184396	58 / 71 (93)
	claG (PKS6)	polyketide synthase	184395	56 / 70 (99)
	claH	decarboxylase	190728	64 / 76 (95)
	claK	oxidoreductase	184397	55 / 74 (96)
	none	Baeyer–Villiger oxidase	197287	38 / 56 (94)
	claM	P450 monooxygese	184398	-
	claN	oxidoreductase/dehydrogese	184399	-
	none	predicted protein	184400	-
	none	predicted protein	184401	-
	none	telomere reverse transcriptase	197286	-
	none	MFS transporter	197288	-

[a] Protein IDs for *C. fulvum* are from the Joint Genome Institute (genome.jgi.doe.gov)

[b] query cover is indicated in between brackets (%) (determined using bl2seq of the NCBI website (blast.ncbi.nlm.nih.gov)

Table S2. ^{1}H NMR data for nataloe-emodin.



Position	δ_H 500 MHz $CDCl_3$	Literature [a] data δ_H 400 MHz $CDCl_3$
Me	2.49, 3H, brs	2.45, 3H, s
7-OH	6.30, 1H, brs	6.20, 1H, brs
2	7.10, 1H, dq, *J* = 1.7, *J* = 0.6 Hz.	7.05, 1H, d, *J* = 1.8
6	7.28, 1H, d, *J* = 8.3 Hz	7.25, 1H, d, *J* = 8.5
4	7.69, 1H, brd, *J* = 1.7 Hz	7.65, 1H, d, J = 1.8 Hz
5	7.86, 1H, d, *J* = 8.3 Hz	7.82, 1H, d, J = 8.5 Hz
1-OH	11.93, 1H, s	11.88, 1H, s
8-OH	12.33, 1H, brs	12.28, 1H, s

[a] Alemayehu G., Abegaz B., Snatzkea G., Duddeck H. (1993) Bianthrones from *Senna longiracemosa*. Phytochemistry. 32(5):1273–1277.

Table S3. Oligonucleotides used in this study.

	Name	Sequence (5' to 3')	Expected fragment size (bp)
Construction of deletion cassettes			
1	*claG_US_F*	*GGGACAACTTTGTATAGAAAAGTTG*GCGAACGTCTGTGCATGGTAG	1282
2	*claG_US_R*	*GGGGACTGCTTTTTTGTACAAACTTG*CAGAAACCGGAAGAGCAATC	
3	*claM_DS_F*	*GGGGACAGCTTTCTTGTACAAAGTGG*CCTGGTAGCTGGTGCTTTGT	1253
4	*claM_DS_R*	*GGGGACAACTTTGTATAATAAAGTTG*GCTTGACGATCACTCCCTGT	
Construction of plasmids for heterologous expression			
5	*pEYA2::claG*	TAATGCCAACTTTGTACAAAAAAGCAGGCTATGACGACGAATCATTTGGAGAACAAGG	5580
6	*claG::pEYA2*	TAATGCCAACTTTGTACAAGAAAGCTGGGTTCAGGCATAAGTCTCCTCCAACCAGTTG	
7	*Padh::ClaF_F*	CTTTCTTTCAACACAAGATCCCAAAGTCAAAATGGACGACAAAGGTGGATATCGACAGATC	1063
8	*ClaF_R::Term_ClaF*	TCACTCTTGAGTGTACAAGGGATACGTTATCTATGCGGCGACCAGTATCTCCCGAG	
9	*ClaF_Term_F*	ATAACGTATCCCTTGTACACTCAAGAGTGATTTGCTTTC	1030
10	*ClaF_Term::Vector*	TTTCACACCGCATACGTCAAAGCAACCATATCCCACCGCCCTTCCGGATCATCCGC	
11	*ClaF_Term_F*	ATAACGTATCCCTTGTACACTCAAGAGTGATTTGCTTTC	1030
12	*ClaF_Term::Pgpda*	ACGTATTTCAGTGTCGAAAGATCCACTAGATCCCACCGCCCTTCCGGATCATCCG	
13	*Pgpda::ClaH_F*	AACAGCTACCCCGCTTGAGCAGACATCACCATGGCAACAGCAACAACAACAACCG	528
14	*ClaH_R::Term_ClaH*	CCAGTAGCTAGCTATGGCAGCACTGCTTCTCTAGCATCCTTTGAAGCCTTCCACGAC	
15	*ClaH_Term_F*	AGAAGCAGTGCTGCCATAGCTAGCTACTG	530
16	*ClaH_Termin::Vector*	TTTCACACCGCATACGTCAAAGCAACCATAACCGGCAACAGTCACACAATCTATGC	
17	*Padh::ClaC*	TTTCTTTCAACACAAGATCCCAAAGTCAAAATGGCTGTTGTGAATGGCAACTACATTC	1546
18	*ClaC::Pgpda*	ACGTATTTCAGTGTCGAAAGATCCACTAGAGCCGTATTGGCTCTAACTAAGCGATTTC	
19	*Pgpda::ClaB*	AACAGCTACCCCGCTTGAGCAGACATCACCATGACTCGGCAAGCAGTTCCAAAGC	549
20	*ClaB::Term_ClaB*	CTTTCAGCTTTCGTAGTTGATGGAATCTATTCAATGCTCGAAAATCTTGTCACAGTC	
21	*ClaB_Term*	ATAGATTCCATCAACTACGAAAGCTGAAAGG	1030
22	*ClaB_Term:Vector*	TTTCACACCGCATACGTCAAAGCAACCATATACGCAGACGGCAAGCCGGTC	
23	*Padh::ClaN*	TTTCTTTCAACACAAGATCCCAAAGTCAAAATGTCATACACCTGGCTCATAACCCG	807
24	*ClaN_Rev*	CTAATCGCAGCCACTCGCCAGAC	

25	*ClaN::ClaN_Term*	GCTAGCTGTCTGGCGAGTGGCTGCGATTAGCTGAATCTATAGGGGTGGGGCTCG	560
26	*ClaN_Term_R::PgpdA*	ACGTATTTCAGTGTCGAAAGATCCACTAGACATGTAATCTTCATTGATCGAGACATCGC	
27	*PgpdA::claK*	AACAGCTACCCCGCTTGAGCAGACATCACCATGGACAGCAAGGTCACAACATATGC	1873
28	*ClaK+Term::Vector*	TTTCACACCGCATACGTCAAAGCAACCATATGAAACTTCTCATCTCGCGGCG	
29	*PgpdA::claM_F*	AACAGCTACCCCGCTTGAGCAGACATCACCATGAATTCAACAGCGGCCAACGTGAAG	1617
30	*ClaM_R::Term_claM*	CGTACGCTTTGACGCAACTATCTGAGCCATTCACTCCCTTGGAATGAGCTTGACGA	
31	*ClaM_Term_F*	ATGGCTCAGATAGTTGCGTCAAAGCGTA	1000
32	*ClaM_Term::Vector*	TTTCACACCGCATACGTCAAAGCAACCATAGCGGCTGGACCAACTATCCATACGCC	

Verification of transformants

claG_Ver_F	GTTGAGCCTCGACGATGTCTTCGTG	See Figure S1
claG_Ver_R	GGGTACGGGGTCCGACGGGTAAG	
claM_Ver_F	CCGTCTCCGACCTGATACTGATCACC	
claM_Ver_R	CTGCTTGCATCAAGAACACACGAGC	
Hyg_Ver_R	GCACTCGTCCGAGGGCAAAGGAATAG	
Gfp_Ver_F	CGGGATCACTCACGGCATGGACGAG	

qrtPCR	**Forward**	**Reverse**	**Efficiency**
claA	GTGGTGGATTTCAGCCTGTT	TCATCGACGATTGTGGTGTT	1.83
claB	CAGTTCCAAAGCCTGCCTAC	AGGAAGTGTGGACTGGATGC	1.84
claC	AGCTTCGGTCATCTCAAGGA	CGAGTGTCGAGGAACACTGA	1.87
claE	TCTGTCTAGACGGCGAGGAT	CTTCGAAGATCCGTTTCGAG	1.82
claF	GGACCTGGAGCATCACATCT	GGATGGTGTCACCCGTAAAC	1.86
claG	CTGCATATCGGAGCAGTGAA	TTGCGTTTCTTGAAGTCGTG	1.97
claH	CCACTGACCAAGGACCTCAT	CATGGATCCTGCTTCATCCT	1.99
clafu197287	AGATCCGGCGTGAATACAAC	TTCCTGCCAGCTTGACTTTT	1.89
claM	ATCGGACACTGGACCTTCTG	CACACCCAAAGGCGTAAGAT	1.83
claN	ATGCTCTTCGCGGAGTCTTA	CCGGTTATGTCGGACGTACT	1.85

Annealing temperature for all oligonucleotide pairs is 60°C
Sequences in italics correspond to Gateway recombination sequences

SI Materials and Methods

Materials used to construct deletion and heterologous expression vectors

All PCRs were performed using Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) according to the manufacturer's protocol. Gel purifications were performed using a Zymoclean™ Gel DNA Recovery Kit (Baseclear). *Escherichia coli* plasmid extractions were performed using a Zyppy™ Plasmid Miniprep Kit (Baseclear). Yeast plasmid extractions were performed using a Zymoprep™ Yeast Plasmid Miniprep II (Baseclear). Restriction digests were performed using Fast Digest enzymes (Fermentas). Sequencing was done by Macrogen (Amsterdam). Primers used and their respective amplicon sizes are listed in Table S3.

Construction of vectors for targeted gene deletion

Gene replacement constructs for Δ*clag* and Δ*clam* were prepared using the MultiSite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) according to the manufacturer's instructions. The upstream (US) and downstream (DS) regions flanking *claG* and *claM* were amplified using primers given in Table S3 and gel-extracted. Purified US fragments were cloned into *pDONR P4-P1R* using the BP reaction, yielding *pP4-P1R_US_claG* and *pP4-P1R_US_claM*. Purified DS fragments were cloned into *pDONR P2-P3* using the BP reaction, yielding *pP2R-P3_DS_claG* and *pP2R-P3_DS_claM*. To generate the final mutagenizing vectors, the plasmid pairs carrying each respective US and DS regions for a given gene were used in an LR reaction with *p221_GFP_HYG* (*pDONR* containing a cassette with *GFP* and *HYG* resistance marker genes) (Ökmen *et al.*, 2013) and the destination vector *pDEST R4-R3*. The LR reaction was catalyzed by the LR clonase II plus enzyme mix (Invitrogen), and 1 µL from each reaction was used to transform electrocompetent *E. coli* DH5α. Positive clones were grown, and their plasmids were extracted for verification by restriction digest. One correct plasmid was chosen from each reaction and named *pDest43-*Δ*clag* or *pDest43-*Δ*clam*.

Agrobacterium tumefaciens-mediated transformation of *Cladosporium fulvum* and secondary metabolite extraction

Plasmids for targeted gene deletion were introduced into *C. fulvum* 0WU using the *A. tumefaciens* transformation method as described in (Ökmen *et al.*, 2013). Transformants were selected on PDA containing hygromycin (100 µg mL^{-1}). Genomic DNA of each strain was isolated using a Zymo Research Genomic DNA Clean & Concentrator™ (Baseclear), according to the manufacturer's recommendations. PCR and quantitative real-time PCR were performed to screen for double crossovers and measure the number of inserted deletion cassettes, respectively (Fig. S1; Table S3). *C. fulvum* wild type, ectopic transformants and deletion mutants were grown on PDA plates for two weeks in the dark at 22°C. Secondary metabolites were extracted from the whole plate, with the agar placed into two 50 mL tubes. Twenty-five mL of ethyl acetate was added to each sample,

and the tubes were placed on a rotary shaker for 4 h in a fume hood at room temperature. The organic extracts originating from the same samples were combined, passed through a Schleicher & Schuell 595½ folded paper and recovered into a clean glass tube. The extracts were concentrated to approximately 1 mL using a nitrogen flow, transferred to a microcentrifuge tube and centrifuged at 14,000 x *g* for 10 min. The ethyl acetate phase was recovered to a clean glass tube and then dried under a nitrogen flow. The residue was dissolved in 1 mL acetonitrile (ACN) and transferred to a 2 mL micro-centrifuge tube. The samples were centrifuged at 14,000 x *g* for 10 min then transferred to a 1 mL clear glass shell vial (Waters) for analysis by UV-HPLC and LC-MS. The cladofulvin standard was previously purified for NMR analyses using a mass-directed chromatography system (Collemare *et al.*, 2014).

Construction of vectors for heterologous expression in *Aspergillus oryzae*

Assembly and expression vectors were constructed by homologous recombination in *Saccharomyces cerevisiae* BMA 64 according to a published method with modifications (see below) (Pahirulzaman *et al.*, 2012).

claG was amplified from *C. fulvum* cDNA by PCR using primers 5 + 6 (Table S3). The *pEYA2* plasmid (kindly provided by Dr. C. Lazarus) was linearized with *Not*I and all fragments were gel purified using a Zymoclean™ Gel DNA Recovery Kit (Baseclear). *S. cerevisiae* was co-transformed with 300 ng of the *claG* amplicon and 300 ng linearized *pEYA2*, and then plated on synthetic dropout medium (SDM) without uracil (see below). Plasmids were extracted from positive transformants and 1 µL was used to transform electrocompetent *E. coli* DH5α. Positive clones were grown, and their plasmids were extracted for verification by restriction digest. One correct plasmid was chosen for sequencing and named *pEYA2-claG*.

Plasmid *pTAex3GS-claG* was generated by LR reaction using 50 ng of *pEYA2-claG* and 50 ng of *pTAex3GS*. The LR reaction was performed as described above and 1 µL was used to transform electrocompetent *E. coli* DH5α. Positive clones were grown, and their plasmids were extracted for verification by restriction digest. In this plasmid, the starch-inducible *amyB* promoter controls the expression of *claG*.

To construct the plasmid *pTAYAGSargPage-claF*::*claG, claF* was amplified from *C. fulvum* cDNA by PCR using primers 7 + 8 (Table S3) and the terminator region downstream of *claF* was amplified from *C. fulvum* gDNA by PCR using primers 9 + 10 (Table S3). To construct the plasmid *pTAYAGSargPage-claF*::*claG*::*claH*, *claH* was amplified from *C. fulvum* cDNA by PCR using primers 13 + 14 (Table S3). The terminator region downstream of *claF* and *claH* were amplified from *C. fulvum* gDNA by PCR using primers 11 + 12 and 15 + 16, respectively (Table S3). The *pTAYAGSargPage* plasmid (kindly provided by Dr. C. Lazarus) was restricted with *Asc*I and all fragments were gel purified using a Zymoclean™ Gel DNA Recovery Kit (Baseclear). For each construct, *S. cerevisiae* was co-transformed with 300 ng of restricted plasmid plus equal amounts of each amplicon (*claF* and terminator or *claF*, *claH* and respective terminators, respectively) and

plated on SDM without uracil (see below). Plasmids were extracted from positive transformants and 1 µL was used to transform electrocompetent *E. coli* DB3.1. Positive clones were grown, and their plasmids were extracted for verification by restriction digest. One correct plasmid was chosen for sequencing and named *pTAYAGSargPage-claF* or *pTAYAGSargPage-claF::claH*. The addition of *claG* in each plasmid was catalyzed by an LR reaction using 50 ng of the respective plasmid and 50 ng of *pEYA2-claG* as described above. Selection and amplification of correct plasmids was performed as above, and they were named *pTAYAGSargPage-claF*::*claG* or *pTAYAGSargPage-claF*::*claG::claH*. In these plasmids, the constitutive promoters from the alcohol dehydrogenase (*Padh*) and from the glyceraldehyde-3-phosphate dehydrogenase (*Pgpda*) genes control the expression of *claF* and *claH*, respectively. As previously, the starch-inducible *amyB* promoter controls the expression of *claG*.

The same method was employed to construct the plasmids *pTAYAGSargPage-claB*::*claC*, *pTAYAGSargPage-claK*::*claN* and *pTAYAGSargPage-claF*::*claM*. *claC*, *claK* and their respective terminator region were amplified from *C. fulvum* gDNA by PCR using primers 17 + 18 and 27 + 28, respectively (Table S3). *claB* and *claM* were amplified from *C. fulvum* cDNA by PCR using primers 19 + 20 and 29 + 30, respectively (Table S3). Their downstream terminator regions were amplified from *C. fulvum* gDNA by PCR using primer pairs 21 + 22 and 31 + 32, respectively (Table S3). *claN* was amplified from *C. fulvum* cDNA by PCR using primers 23 + 24 and its downstream terminator region was amplified from *C. fulvum* gDNA by PCR using primers 25 + 26 (Table S3). All fragments were gel purified before cloning as described above. In the resulting plasmids, the constitutive promoter from the alcohol dehydrogenase (*Padh*) control the expression of *claC, claN* and *claF*; and the constitutive promoter from the glyceraldehyde-3-phosphate dehydrogenase (*Pgpda*) genes control the expression of *claB, claK* and *claM*, respectively. All final vectors contain the *arginine* gene for selection of fungal transformants.

Homologous recombination in *Saccharomyces cerevisiae*

A single colony of *S. cerevisiae* BMA 64 was picked and used to inoculate 3 mL yeast peptone D-glucose (YPD; 10 g L^{-1} yeast extract (Difco), tryptone 20 g L^{-1} glucose 20 g L^{-1}) medium in a 50 mL centrifuge tube. The culture was grown overnight at 28-30°C and agitated at 230 rpm. Two mL from the overnight culture was used to inoculate 50 mL YPD medium in a 250 mL Erlenmeyer flask, which was incubated for 5 h at 28-30°C and agitated at 230 rpm. The culture was transferred to a 50 mL centrifuge tube and centrifuged at 4,000 x *g* for 10 min at 20°C. The supernatant was discarded and the pellet was thoroughly resuspended in 25 mL sterile distilled water (SDW). The sample was centrifuged at 4, 000 x *g* for 10 min at room temperature and the supernatant was discarded. The pellet was resuspended in 300 µL SDW. In a microcentrifuge tube the following were added: 50 µL salmon sperm DNA (2 µg μL^{-1}, denatured at 95°C and snap cooled on ice), linearized vector (300 ng), 8-20 µl PCR product(s) (100-300 ng per amplicon), 50 µL *S. cerevisiae* cells, 32 µL 1M lithium acetate and 240 µL PEG 4000 (50% w/v; Prolabo). The sample was mixed by vortexing and incubated at 30°C for 30 min followed by heat shock at 45°C for 15 min. The samples were centrifuged at 2,000 x *g* for 2 min at room temperature and the supernatant was

removed using a pipette. The cells were resuspended in 200 µL SDW and plated on SDM (1.7 g L^{-1} yeast nitrogen base without amino acids (Fluka) 5 g L^{-1} ammonium sulphate, 5 g L^{-1} casein hydrolysate (Fluka), 20 mg L^{-1} adenine (Sigma-Aldrich), 20 g L^{-1} agar and 20 g L^{-1} separately autoclaved glucose). The plates were incubated for 3 – 7 days at 30°C until colonies appeared.

PEG-mediated transformation of *Aspergillus oryzae*

A. oryzae M-2-3 was grown on DPY agar plates at 30°C for 3 – 5 days. A 1 cm^2 agar plug was cut and transferred to a 500 mL baffled Nalgene flask (Sigma Aldrich) containing 100 mL DPY (dextrin 20 g L^{-1}, polypeptone 10 g L^{-1} , yeast extract 5 g L^{-1}, KH_2PO_4 5 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1}) medium. The flask was incubated at 30°C for 48 h and agitated at 280 rpm. Mycelia were collected by filtration through sterile Miracloth and excess DPY was removed by lightly compressing the biomass with a spatula. Approximately 25 mL of mycelia were transferred to a 50 mL tube and centrifuged for 10 min at 4,000 x *g*. The supernatant was discarded and the mycelia were washed with 0.9 M NaCl. The sample was centrifuged for 10 min at 4,000 x *g* and the supernatant was discarded. Twenty mL filter sterilized protoplasting solution (lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich) 20 g L^{-1}, Driselase from *Basidiomycetes* (Sigma-Aldrich) 10 g L^{-1} in 0.8 M NaCl) was added and the sample was shaken vigorously to resuspend. The tube was incubated at room temperature with gentle mixing on a rotator. After 5 h the sample was gently drawn into a Corning Costar 25 mL sterile plastic pipette (Sigma-Aldrich) in order to release protoplasts from the mycelia. The protoplasts were filtered through sterile Micracloth and recovered into a 50 mL centrifuge tube. The filtrate was centrifuged for 5 min at 700 x *g* and the supernatant was removed. The pellet containing protoplasts was resuspended in 20 mL Solution 1 (0.9 M NaCl, 10 mM $CaCl_2$ and 50 mM Tris-HCl pH 7.5) by gentle pipetting with a Corning Costar 25 mL sterile plastic pipette. The protoplasts were centrifuged for 5 min at 700 x *g* and the supernatant was removed. The pellet was resuspended in 1 mL Solution 1 by gentle pipetting with a standard 1 mL pipette tip and 0.2 mL Solution 2 (60% polyethyleneglycol 3350 (Sigma-Aldrich), 10 mM $CaCl_2$ and 50 mM Tris-HCl pH 7.5 in H_20) was added. The sample was hand mixed by gentle rotation and 0.2 mL was dispensed into 50 mL centrifuge tubes. Between 5 – 10 µg circular plasmid DNA was added (maximum volume of 20 µL) to the protoplasts, followed by 1 mL Solution 2. Approximately 10 mL Czapek-Dox agar (Oxoid) containing sorbitol (1 M) at a temperature of less than 45°C was added to each transformation mixture and immediately poured onto plates containing 25 mL Czapek-Dox agar (1 M sorbitol). The plates were incubated at 30°C for between 2 – 5 days. Transformants containing the arginine gene were picked using sterile toothpicks and transferred to Czapek-Dox agar without sorbitol. The plates were incubated at 30°C for 48 h followed by two further rounds of selection. The genuine transformants were stored at room temperature until required.

Heterologous expression in *Aspergillus oryzae* and secondary metabolite extraction

Selected *A. oryzae* transformants were transferred to DPY agar plates for multiplication. Although the *amyB* promoter is induced by starch, this repression is not tight, and SMs were obtained in high amounts from DPY agar medium when incubated at room temperature. *A. oryzae* transformants containing *claG*, *claF::claG* or *claF::claG::claH* were therefore incubated at 30°C until their growth on DPY agar plates was confluent and then transferred to room temperature for 24 h to 72 h. The cultures were freeze-dried and then homogenised with a pestle and mortar. The homogenate was resuspended in water, acidified to pH4 using HCl, and then twice extracted with ethyl acetate. The ethyl acetate phase was recovered and dried using a nitrogen flow.

Untransformed *A. oryzae* and transformants containing *claB::claC*, *claK::claN* or *claM* were grown for 48 h in a baffled flask containing 100 mL DPY medium, rotating at 280 rpm, at 30°C, in the dark. The cultures were then left standing at room temperature for 24 h. Approximately 50 mL of biomass was transferred to a centrifuge tube. For the respective transformants, emodin (HPLC grade ≥97%, Sigma), chrysophanol (HPLC grade ≥98%, Sigma) or nataloe-emodin (purified in this study for NMR analysis, see below) dissolved in dimethylsulphoxide, was added to a final concentration of 25 µg ml^{-1} and the samples were left standing at 20°C for 8 h. The samples were freeze-dried, homogenised and extracted as above.

Secondary metabolite analysis using UV-HPLC and LC-MS

Prior to analysis, the samples in ACN were centrifuged at 20,000 x *g* for 5 min in a microcentrifuge tube and then transferred to a 1 mL clear glass shell vial (WAT025054c). HPLC analysis with a Waters Symmetry reverse phase (RP) 5µ, C_{18}, 100 Å column (WAT046980) was carried out on a Waters 600S system. The sample was eluted with a variable gradient of solvents (A) H_2O and (B) CH_3CN (both containing 0.1% trifluoroacetic acid) at a flow rate of 1 mL min^{-1}. The following gradient was used: 0 min, A (95%); 10 min, A (10%); 12 min, A (10%), 15 min, A (0%), 16 min, A (95%), 20 min, A (95%). UV spectra were obtained using a 996-photodiode array (PDA) detector and analysed with the Waters Empower software.

LC-MS data were obtained using a Waters LC-MS system composed of a Waters 2767 autosampler, Waters 2545 pump system, a Phenomenex Kinetex column (2.6 µ, C_{18}, 100 Å, 4.6 × 100 mm) equipped with a Phenomenex Security Guard precolumn (Luna C_5 300 Å) eluted at 1 mL/min. Detection was by Waters 2998 Diode Array detector between 200 and 400 nm; Waters 2424 ELSD and Waters SQD-2 mass detector operating simultaneously in ES+ and ES- modes between 100 *m/z* and 650 *m/z*. Solvents were: A, HPLC grade H_2O containing 0.05% formic acid; B, HPLC grade MeOH containing 0.045% formic acid; and C, HPLC grade CH_3CN containing 0.045% formic acid). Gradients were as follows: Kinetex/CH_3CN: 0 min, 10% C; 10 min, 90% C; 12 min, 90% C; 13 min, 10% C; 15 min, 10% C. Samples were generally diluted to 1 mg mL^{-1} and 10 µL injected (10 µg). Traces were obtained and visualizes using Mass Lynx 4.1.

UV spectra of all major compounds identified in this study are shown in Fig. S6.

Semi-Preparative LC-MS and compound purification.

Purification of compounds was achieved using a Waters mass-directed autopurification system comprising of a Waters 2767 autosampler, Waters 2545 pump system, a Phenomenex Kinetex Axia column (5µ, C_{18}, 100 Å, 21.2 × 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna C_5 300 Å) eluted at 20 mL/min at ambient temperature. Solvent A, HPLC grade H_2O + 0.05% formic acid; Solvent B, HPLC grade CH_3CN + 0.045% formic acid. The post-column flow was split (100:1) and the minority flow was made up with HPLC grade MeOH + 0.045% formic acid to 1 $mL{\cdot}min^{-1}$ for simultaneous analysis by diode array (Waters 2998), evaporative light scattering (Waters 2424) and ESI mass spectrometry in positive and negative modes (Waters SQD-2). Detected peaks were collected into glass test tubes. Combined tubes were evaporated under a flow of dry N_2 gas, weighed, and residues dissolved directly in NMR solvent for NMR analysis.

Tumor cell cytotoxicity bioassays

Cytotoxicity of the different compounds were evaluated in various tumor cell lines and/or normal cell lines using the sulforhodamine B (SRB) assay method (Skehan *et al.*, 1990; Boyd & Paull, 1995; Vichai & Kirtikara, 2006). Cell lines tested include BALB/3T3 (Non-tumorogenic, BALB/c mouse embryo cells), H460 (human lung large cell carcinoma), HuTu 80 (human adenocarcinoma of the dudodenum), M-14 (human amelanotic melanoma), DU145 (human prostate carcinoma), MCF-7 (human breast adenocarcinoma), HT-29 (human colon adenocarcinoma), and K562 (human chronic myelogenous leukemia). To determine the cytotoxicity of the extracts, cells were plated into 96-well tissue culture plates and in their corresponding growth medium at approximately 10% confluency and incubated at 37°C in a 5% CO_2 and 95% air humidified atmosphere for 24 h to allow cells to attach. A plate containing each of these cells was fixed *in situ* with trichloroacetic acid (TCA) in order to obtain the cell values at zero time before adding the compounds. The rest of the plates containing the different cell lines received serial dilutions of emodin, chrysophanol or cladofulvin and further incubated at 37°C in a 5% CO_2 and 95% air humidified atmosphere for 48 h. The assay was terminated by the addition of cold TCA. TCA treated plates were incubated at 4 °C for 1 h, washed five times with tap water to remove TCA and then air-dried. Background optical densities were measured in wells incubated with growth medium without cells. TCA-fixed cells were stained for 20 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. At the end of the staining period unbound dye was removed by washing four times with 1% acetic acid. After air-drying, bound dye was solubilized with 10 mM Tris base (pH 10.5) and the absorbance was read on an automated plate reader at a wavelength of 510 nm. The GI_{50} value is defined as the concentration of test sample resulting in a 50% reduction of absorbance as compared with untreated controls that received a serial dilution of the solvent in which the test samples were dissolved, and determined by linear regression analysis. For K562 cells, which grow in suspension, instead of fixing and staining with SRB, cells were counted using a Coulter Counter.

Chapter 5

Activation of a repressed secondary metabolite gene cluster in the fungus *Cladosporium fulvum* prevents biotrophic parasitism on tomato

Scott Griffiths[1]
Carl H. Mesarich[1,3]
Elysa Overdijk[1]
Benedetta Saccomanno[1]
Pierre J.G.M. de Wit1[*]
Jérôme Collemare[1,2*]

In preparation

1 Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands
2 UMR1345 IRHS-INRA, 42 rue Georges Morel, 49071 Beaucouzé Cedex, France
3 Present address: Bioprotection Technologies, The New Zealand Institute for Plant & Food Research Limited, Auckland, New Zealand
* Corresponding authors: j.collemare@gmail.com; pierre.dewit@wur.nl

Abstract

Fungal biotrophy is associated with a reduced capacity to produce potentially toxic secondary metabolites (SMs). However, the genome of the biotrophic plant pathogenic fungus *Cladosporium fulvum* contains many SM biosynthetic gene clusters. Of these though, only the gene cluster responsible for the production of the pigment cladofulvin is not silent. It was suggested that down regulation of this SM gene cluster during parasitism of tomato allowed biotrophy without gene loss.

To address this hypothesis, we analysed the biotrophic behaviour and virulence of *C. fulvum* mutants that cannot produce or overproduce cladofulvin.

Disease symptoms caused by *C. fulvum* mutants unable to produce cladofulvin were indistinguishable from wild type. In contrast, a *C. fulvum* transformant overproducing cladofulvin during tomato colonization showed enhanced fungal growth and caused strong desiccation of tomato leaves, but did not differentiate conidia when relative humidity was low. Biological activity assays suggested that cladofulvin does not play a role in fungal virulence, but instead protects conidia against abiotic stresses encountered outside its host such as UV light and cold.

This study demonstrates that repression of cladofulvin production is required for biotrophic growth of *C. fulvum* in tomato, while its production is important for its survival outside its host.

Key words: biotrophy, cladofulvin, abiotic stress resistance, regulation of secondary metabolism, virulence, *Fulvia fulva*

5.1 Introduction

Secondary metabolites (SMs) are very potent compounds that are more studied for their biological activities, which can be both useful and harmful to mankind (antibiotics, immune-suppressors, anti-cholesterolemic drugs, mycotoxins, etc.), than for their intrinsic function(s) in the organisms that produce them. However, studies on SMs produced by bacteria, fungi, and plants have revealed functions in their ecological niches associated with competition and survival (Demain & Fang, 2000). Accordingly, the enormous chemical diversity of SMs is thought to result from adaptations of SM producers to different ecological niches (Osbourn, 2010). Filamentous fungi are considered to be good SM producers and sequenced fungal genomes have revealed that Ascomycota have a particularly high potential for SM production (Collemare *et al.*, 2008; Ohm *et al.*, 2012). For instance, it was estimated that the genomes of just four *Aspergillus* species, *A. fumigatus*, *A. nidulans, A. niger,* and *A. oryzae*, collectively have the capacity to produce 226 different SMs (Inglis *et al.*, 2013).

Hemi-biotrophic and necrotrophic fungal plant pathogens are well-known producers of SMs. During colonization of their respective hosts, these plant pathogens deploy SMs as virulence factors that destroy host tissue and promote disease. Cercosporin and elsinochromes, for instance, are light-activated generators of toxic reactive oxygen species (ROS) produced during infections by the fungal plant pathogens *Cercospora spp.* and *Elsinoë spp.*, respectively (Lousberg *et al.*, 1969; Daub & Hangarter, 1983). Mutants unable to produce these SMs showed a significant reduction in virulence (Choquer *et al.*, 2005; Liao & Chung, 2008). The necrotrophic fungi *Cochliobolus spp*. and *Alternaria spp.* produce SMs that are host-specific toxins (Stergiopoulos *et al.*, 2012). These compounds play an important role in determining fungal host range, with loss of SM production resulting in a loss of virulence (Wolpert *et al.*, 2002). In contrast to these lifestyles, biotrophic fungal plant pathogens acquire nutrients from living host tissues. It is axiomatic that a biotroph should limit the production of SMs that are toxic towards its host. The non-obligate biotrophic maize pathogen *Ustilago maydis* contains only three polyketide synthase (*PKS*) genes, which are unlikely functional, and only three non-ribosomal peptide synthetase (*NRPS*) genes, of which two are required for the production of siderophores (Bölker *et al.*, 2008; Winterberg *et al.*, 2009). Strikingly, the obligate biotrophic pathogens *Blumeria graminis* f. sp. *hordei*, *Melampsora larici-populina*, *Puccinia graminis* f. sp. *tritici* and *Puccinia triticina* have a highly reduced SM gene complement (Spanu *et al.*, 2010; Duplessis *et al.*, 2011). The last three of these species do not have any *PKS* gene and carry only a single *NRPS* gene, while *B. graminis* f. sp. *hordei* carries one *PKS* and one *NRPS* gene. Similarly, the symbionts *Tuber melanosporum* and *Laccaria bicolor* possess only two *PKS* genes and one *NRPS* gene, and one *PKS* and one hybrid *PKS-NRPS* gene, respectively (Martin *et al.*, 2008; Martin *et al.*, 2010; Collemare & Lebrun, 2011). *L. bicolor* shows an expansion of a terpene cyclase gene family (TCs; 10 members), but it was suggested that this high number of TCs is related to the symbiotic interaction between *L. bicolor* and its hosts because the production of terpenes occurs more commonly in plants than in

fungi (Collemare & Lebrun, 2011). These observations suggested that loss of SM biosynthetic pathways is associated with a biotrophic lifestyle (Collemare & Lebrun, 2011).

Genome analysis of *Cladosporium fulvum*, the biotrophic fungal pathogen responsible for tomato leaf mold disease, suggested an alternative mechanism associated with biotrophy (Collemare *et al.*, 2014). Indeed, the *C. fulvum* genome contains fifteen predicted functional SM gene clusters, including two that are related to the biosynthesis of the aforementioned cercosporin (*PKS7*) and elsinochrome (*PKS1*) toxins (de Wit *et al.*, 2012). However, apart from the *claG* (*PKS6*) gene cluster that is involved in the biosynthesis of the bi-anthraquinone pigment cladofulvin (chapter 4), all other SM biosynthetic pathways remained cryptic under various conditions (Collemare et al., 2014). Because *claG* and *NPS9* are expressed during growth of *C. fulvum* on the host leaf surface, but repressed during colonization of the apoplast surrounding tomato mesophyll cells, we hypothesized that down-regulation and low expression levels of SM genes can be an alternative mechanism to irreversible gene losses observed in other biotrophs (Collemare *et al.*, 2014). This would be similar to the observation that many of the genes encoding predicted functional cell wall-degrading enzymes and carbohydrate-active enzymes of *C. fulvum* are not expressed or expressed at low levels during biotrophic growth (de Wit *et al.*, 2012).

The observed down-regulation of the *claG* gene cluster during tomato leaf colonization by *C. fulvum* represents a unique system to experimentally assess the association between biotrophy and repression of SM production. In the present study, we manipulated the *claG* gene cluster to either suppress or activate cladofulvin production during infection of tomato. We assessed the impact of these modifications on *C. fulvum* biotrophy and virulence. In addition, we suggest possible biological roles for cladofulvin in the protection of *C. fulvum* against abiotic stresses outside its host plant.

5

5.2 Materials and methods

5.2.1 Fungal strains employed in this study

Cladosporium fulvum 0WU (de Wit *et al.*, 2012) was the parental strain used to perform transformation. The *C. fulvum* Δ*clag*-A, Δ*clag*-B deletion mutants and ectopic insertion transformants were created during a previous study (chapter 4).

5.2.2 Generation of *OE.claE* and *OE.claE::GFP* transformants

The putative local regulator from the cladofulvin gene cluster, *claE*, was amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix (Life Technologies) from *C. fulvum* gDNA using the primer pair *PacI_claE_Forward* (GCTACAGTTAATTAAATGTCCCTGTCACGCAGCGTGGCTG) and *NotI_claE_Reverse* (CATGTAGCGCGGCCGCTCACAGATTCTTCAGACGATC)

(expected size: 1341 nt). The *claE* amplicon and pFBTS3, a plasmid that contains the inducible promoter of the *Avr9* gene (Van den Ackerveken *et al.*, 1994; chapter 3), were restricted using *Pac*I and *Not*I restriction enzymes (Fermentas Fast Digest), cleaned with Zymogen DNA Clean & Concentrator (Baseclear), and ligated using T4 DNA polymerase (Promega) to yield *pFBTS3-claE*. *Escherichia coli* DH5α cells were transformed using a standard heat-shock protocol and transformants were selected on LB-kanamycin agar (50 $\mu g.ml^{-1}$). Plasmids were extracted from transformants and screened by restriction digest analysis using *Pac*I and *Not*I. A plasmid bearing the correct restriction pattern was sent to Macrogen (Amsterdam, NL) for sequencing of the insert. *Agrobacterium tumefaciens* AGL1 was transformed with *pFBTS3-claE* by electroporation, and plated on LB-kanamycin agar (50 $\mu g.ml^{-1}$). One positive transformant was picked, verified and named AT-pFBTS3-claE. The plasmid was introduced into *C. fulvum* using *A. tumefaciens*-mediated transformation as previously described (Ökmen *et al.*, 2013), Transformants were selected on PDA medium supplemented with hygromycin (100 $\mu g.ml^{-1}$). Several transformants and wild-type *C. fulvum* were grown on potato-dextrose broth (PDB; Oxoid) and then transferred to Gambourg B5 medium without nitrogen (Van den Ackerveken *et al.*, 1994) in order to induce the *Avr9* promoter (Van den Ackerveken *et al.*, 1994). Total RNA was extracted as previously described (Ökmen et al., 2013) and cDNA synthesis was performed as described below. The induction of the cladofulvin biosynthetic cluster was confirmed by RT-qrtPCR using published primers (Collemare *et al.*, 2014; chapter 4). One transformant showing the expected strong induction of cladofulvin biosynthetic genes was selected and named *C. fulvum-OE.claE*.

Using the same methods, *A. tumefaciens* AGL1 was transformed with plasmid pRM254, which contains *GFP* and geneticin resistance genes (Mehrabi *et al.*, 2015) to yield AT-pRM254 strain. The plasmid was introduced into the *C. fulvum OE.claE* transformant as described above. Transformants were selected on potato-dextrose agar (PDA) medium supplemented with geneticin (100 $\mu g.ml^{-1}$). One transformant was picked and screened for *GFP* fluorescence.

5.2.3 Plant inoculation, determination of fungal growth and *in planta* gene expression

Inoculation of tomato with *C. fulvum* wild-type, deletion mutant and transformant strains was carried out according to a previously described method (Mesarich *et al.*, 2014). To determine fungal growth, the fourth composite leaf of infected tomato plants was harvested at 4, 8, and 12 days post-inoculation (dpi) and flash frozen in liquid nitrogen. Samples were ground to a fine powder in liquid nitrogen, and total RNA was extracted from 100 mg of material using a Zymogen Direct-zol™ RNA MiniPrep kit (Baseclear) according to the manufacturer's recommended protocol. cDNA synthesis was performed using 100 – 2,000 ng of total RNA and M-MLV reverse transcriptase (Promega), following the manufacturer's protocol. The remaining powder was retained for SM extraction and analysis. To assess *C. fulvum* growth during infection, the *actin* gene of this fungus was targeted by qrtPCR using the *Cf-actin_RT-qrtPCR_F/Cf-actin_RT-*

qrtPCR_R primer pair (Mesarich *et al.*, 2014). For sample calibration, the *Solanum lycopersicum* gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*RUBISCO*) was targeted using the *Sl-rubisco_qrtPCR_F/Sl-rubisco_qrtPCR_R* primer pair (Mesarich *et al.*, 2014). The same cDNA samples were used to measure the expression of cladofulvin biosynthetic genes by qrtPCR using previously reported methods and primers (Collemare *et al.*, 2014; chapter 4). Results were analyzed according to the $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2002) and are the average of three biological replicates.

5.2.4 Secondary metabolite isolation from plant leaf homogenates and UV-HPLC analysis

The powder that was retained for SM analysis was weighed and soaked in 20 mL acetone at room temperature (RT) for 24 h. The sample was passed through a Schleicher & Schuell 595½ folded paper filter and the supernatant was dried under a nitrogen flow at RT. Acetonitrile (ACN; 2 mL) was added and the sample left to soak at RT for 24 h. ACN was decanted into a 2 mL micro-centrifuge tube and stored at –20°C for subsequent analysis by UV-HPLC. Prior to analysis, samples in ACN were centrifuged at 20,000 x *g* for 5 min in a micro-centrifuge tube, then transferred to a 1 mL clear glass shell vial (WAT025054c). HPLC analysis with a Waters Symmetry reverse phase C18 column (WAT046980) was carried out on a Waters 600S system. The sample was eluted with a variable gradient of solvents (A) H_2O and (B) ACN (both containing 0.1% trifluoroacetic acid) at a flow rate of 1 mL min^{-1}. The following gradient was used: 0 min, A (95%); 10 min, A (10%); 12 min, A (10%), 15 min, A (0%), 16 min, A (95%), 20 min, A (95%). UV spectra were obtained using a 996-photodiode array detector. Metabolite concentrations were calculated by measuring the surface area of their respective peaks (254 nm) and divided by the response factor (RF) of cladofulvin (chapter 3). All reagents and solvents were of analytical and HPLC grades, respectively.

5.2.5 Microscopic examination of *GFP*-expressing strains

Imaging of infected tomato leaves was performed using a spinning disc confocal microscope (Nikon Ti microscope body, Yokogawa CSUX1 scanner, Photometrics Evolve camera, Metamorph software, 491 nm laser line; 60x oil 1.40NA objective). Z-stacks were acquired with an internal spacing of 0.5 µm. Screening of transformants and imaging of *C. fulvum-OE. claE::GFP* on wet tissue paper and colonies grown in PDB were performed using a Nikon 90i epifluorescence microscope (GFP fluorescence was visualized using a GFP-B filter cube (EX 460-500, DM 505, BA 510-560). All images were processed using Fiji software (Schindelin *et al.*, 2012).

5.2.6 Quantification of conidiation

Six-well culture plates containing PDA were inoculated with 2,000 conidia of wild-type *C. fulvum*, Δ*clag*-A, and an ectopic insertion transformant control, and incubated for 10 days in the dark at 22°C. Conidia were recovered by flooding each well with water and abrading the colony using an inoculating loop. The samples were centrifuged at 4,000 x *g* and then re-suspended in an equal volume of water for enumeration using a haemocytometer to give total conidia per well. The results are an average of three technical replicates.

5.2.7 Quantification of cladofulvin

When *C. fulvum* wild type is grown in PDB still liquid culture in six-well plates, mycelium is formed at the bottom and floating colonies develop on the surface. From each sample, submerged and floating colonies were recovered separately and transferred to micro-centrifuge tubes and freeze-dried. One mL ethyl acetate and 1 mL water was added and the samples were incubated at RT for 30 min on a rotary shaker. After centrifugation at 13,000 *x g* for 15 min, the organic phase was recovered, transferred to a clean glass vial and concentrated under a dry nitrogen flow. In a separate experiment, *C. fulvum* wild type was grown on PDA plates for 12 days in the dark at 22°C. Plates were transferred to 10°C, 22°C or 37°C for 72 h. Agar plugs containing entire colonies were transferred to a 50 mL tube. Twenty-five mL of ethyl acetate were added, and the tubes were incubated at RT for 30 min on a rotary shaker. After centrifugation at 4,000 x *g* for 15 min, the organic phase was recovered, transferred to a clean glass vial and concentrated under a dry nitrogen flow. All samples were re-suspended in 1 mL ACN for UV-HPLC analysis as described above.

5.2.8 Biological activity assays

Germination assay**:** 20 µL of a *C. fulvum* conidial suspension ($1x10^5.mL^{-1}$ in water) was transferred to a glass microscope slide and covered with a cover slip. The slides were placed in a humidity chamber and incubated at 20°C in the dark. Conidia and germlings were enumerated using a bright field microscope at 48 h post-incubation. Freeze-thawing assay**:** slides were prepared as above, and then subjected to three rounds of freeze-thaw cycling. One cycle consisted of 10 min at -20°C, followed by incubation at RT for 10 min. After treatment, slides were incubated for 48 h and processed as above. UV survival assay**:** slides were prepared as above, except treatment was applied prior to the addition of a cover slip. Uncovered slides were positioned 1 cm above a Bio-Rad ChemiDoc XRS UV transilluminator and irradiated for 3 min at 302 nm. Then a cover slip was added and the slides were processed as in the above described germination assay.

5.3 Results

5.3.1 The cladofulvin gene cluster is down regulated during biotrophic growth of *C. fulvum* on tomato

The *claG* gene cluster responsible for cladofulvin production is comprised of nine co-regulated genes (*claA, B, C, E, F, G, K, M* and *N*), which encode seven enzymes involved in its biosynthesis, a predicted transcription factor (*claE*) and a putative co-regulator (*claA*)(Collemare *et al.*, 2014; 4). In addition, although not located at the same genomic locus, a decarboxylase-encoding gene, *claH*, is also co-regulated and implicated in cladofulvin biosynthesis (chapter 4). *ClaG* encodes the PKS that produces the first precursor of cladofulvin biosynthesis (chapter 4). This gene is expressed during the early and late stages of infection, but not during colonisation of the apoplastic space surrounding the leaf mesophyll cells (Collemare *et al.*, 2014). We first wanted to confirm *in planta* down-regulation of the whole gene cluster. For this purpose, we measured the *in planta* expression of all genes from the cladofulvin biosynthetic pathway using RT-qrtPCR. By four days post-inoculation (dpi), conidia have germinated and the runner hyphae are exploring the leaf surface for open stomata (de Wit *et al.*, 2012; Collemare *et al.*, 2014). At this stage, all cladofulvin genes exhibited an expression level similar to or higher than the endogenous control gene *tubulin* (Fig. 1a). At eight dpi when the fungus is colonizing the apoplastic space surrounding mesophyll cells (de Wit *et al.*, 2012; Collemare *et al.*, 2014), all genes but *claA* and *claE* were significantly down regulated (Fig. 1a). Although not statistically significant, expression of *claA* and *claE* was reduced by 18- and 15-fold, respectively, when compared to their expression levels at four dpi. The expression level of most genes started to increase at 12 dpi compared to eight dpi, ranging from 1.2-fold for *claK* to 13-fold for *claA* (Fig. 1a). This stage corresponds to the onset of conidiophore formation and conidiation on the leaf surface (de Wit *et al.*, 2012; Collemare *et al.*, 2014). Although the gene cluster is expressed at four and 12 dpi, the amount of cladofulvin produced was below the detection threshold. Our results confirm that all ten genes of the cladofulvin gene cluster are co-regulated *in planta* and all are down regulated during biotrophic growth within tomato leaves.

5.3.2 Cladofulvin is not required for virulence of *C. fulvum* on tomato

Because the cladofulvin gene cluster is expressed prior to penetration of stomata and during conidiation on the leaf surface at the end of the infection cycle, we assessed whether cladofulvin might be a crucial factor required for growth outside the plant. Tomato plants were inoculated with the wild-type parental strain of *C. fulvum*, a pair of independent Δ*clag* deletion mutants that are deficient in cladofulvin production only, and an ectopic insertion transformant control. These genetically modified strains were previously obtained and characterized *in vitro* (chapter 4). The resulting disease symptoms were similar for all strains, with each producing visible signs of conidiation from 12 dpi onwards (Fig. 2a and Fig. 3a,b). *In planta* growth of Δ*clag* deletion

mutants was similar to wild-type *C. fulvum* and ectopic insertion transformant controls when measured at 12 dpi (Fig. 4). These results show that cladofulvin is not required for virulence of *C. fulvum*, and does not overtly affect fungal biomass production or conidiation.

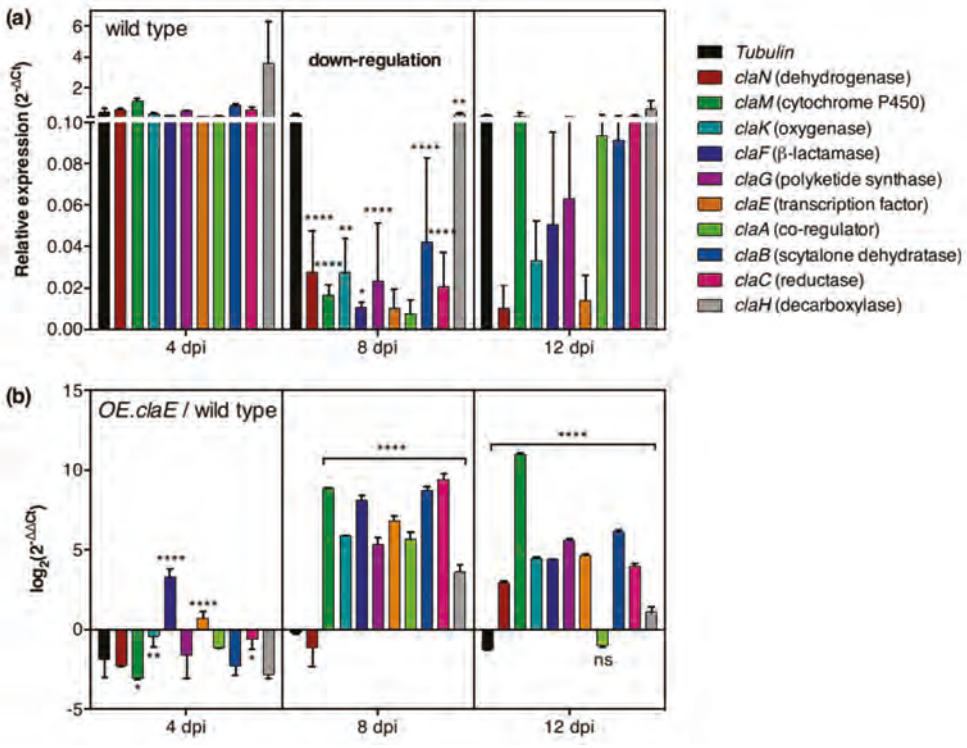


Figure 1. Relative expression of the cladofulvin gene cluster during infection of tomato by *Cladosporium fulvum*. Expression of the genes was measured by RT-qrtPCR at 4, 8 and 12 days post-inoculation (dpi) and normalized to expression of the *actin* gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Bars show the average of three independent biological repeats with standard deviation. **(a)** Relative gene expression during infection of Heinz tomato susceptible cultivar by wild-type *C. fulvum*. For each gene, each *in planta* time point was compared to the previous one using a 2-way ANOVA with *post-hoc* Fisher's LSD multiple comparisons test. **(b)** Expression of the cladofulvin gene cluster during infection of tomato by the *OE.claE* transformant is compared to *in planta* expression in wild type. For a given time point, each ratio was compared to the ratio for *tubulin* control using a 2-way ANOVA with *post-hoc* Dunnett's multiple comparisons test. Asterisks indicate significant differences (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001; ns: non-significant). The function of each gene according to chapter 4 is indicated in between brackets in the legend.

5.3.3 Induction of the cladofulvin gene cluster during colonization of tomato results in enhanced fungal growth and strong leaf desiccation

The specific down-regulation of the cladofulvin gene cluster during leaf colonization suggests that it might interfere with *C. fulvum* biotrophic growth. The presence of a conserved transcription

factor gene (*claE*) inside the cladofulvin gene cluster allowed us to specifically manipulate the expression of this gene cluster, a strategy that has proven successful to activate silent SM gene clusters in other fungi (Bergmann *et al.*, 2007; Chiang *et al.*, 2010; Cary *et al.*, 2015). For this purpose, we used the promoter of the effector gene, *Avr9*, which is highly induced *in planta* after the runner hyphae have penetrated the stomata of tomato leaves (Van den Ackerveken *et al.*, 1994; Collemare *et al.*, 2014; Mesarich *et al.*, 2014). By fusing the coding sequence of *claE* to the promoter region of *Avr9*, we aimed to induce cladofulvin biosynthesis during leaf colonization. During infection of tomato by the *OE.claE* transformant, the cladofulvin gene cluster is indeed strongly induced at 8 and 12 dpi, apart from *claN* and *claA* that show regular expression at 8 and 12 dpi, respectively (Fig. 1b). Consistent with gene cluster activation, cladofulvin could be detected as early as 4 dpi (0.011 ± 0.003 $\mu g.mg^{-1}$ leaf tissue; 0.014 ± 0.001 $\mu g.mg^{-1}$ leaf tissue at 8 dpi) and greatly accumulated by 12 dpi (0.268 ± 0.066 $\mu g.mg^{-1}$ leaf tissue). Remarkably, the *OE.claE* transformant triggered severe desiccation of the infected leaves by 12 dpi (Fig. 2b), with first necrotic spots appearing between 8 and 10 dpi. Also in contrast to control strains, no conidiation on the leaf surface could be observed (Fig. 2a,b). Thus, activating the cladofulvin gene cluster during colonization of tomato clearly prevented biotrophic growth.

The *OE.claE* transformant was transformed with a plasmid carrying *GFP* to visualise leaf colonization with fluorescence confocal microscopy. The *OE.claE::GFP* transformant also caused heavy desiccation of host tissue (Fig. 2b), but it did not show any microscopic difference with wild type at 4 and 8 dpi (Fig. 3c). At 12 dpi, fluorescence was detected in hyphae within the desiccated lesions (Fig. 3c), confirming that hyphae were alive and physiologically normal. The mesophyll tissues appeared heavily colonized, which was confirmed by a significantly higher fungal biomass at 12 dpi (Fig. 4). In contrast to all other controls, *OE.claE* and *OE.claE::GFP* transformants did not produce conidia on the leaf surface and no stromata were observed in the sub-stomatal chambers (Fig. 3c). To test if the absence of conidia was due to desiccation, the relative humidity in compartments where plants were infected, was increased to 100% at 21 dpi and leaves were observed after 5 days (Fig. 2c). Conidiophores and conidia of the *OE.claE::GFP* transformant were observed on leaves under high relative humidity whereas none was visible on control plants that did not experience the relative humidity increase. Fungal hyphae appear quiescent in desiccated leaves because nine weeks after the onset of necrosis, fluorescent conidiophores and conidia were observed on desiccated leaves after 72 h rehydration on PDA supplemented with geneticin or on tissue paper saturated with water (Fig. 2c).

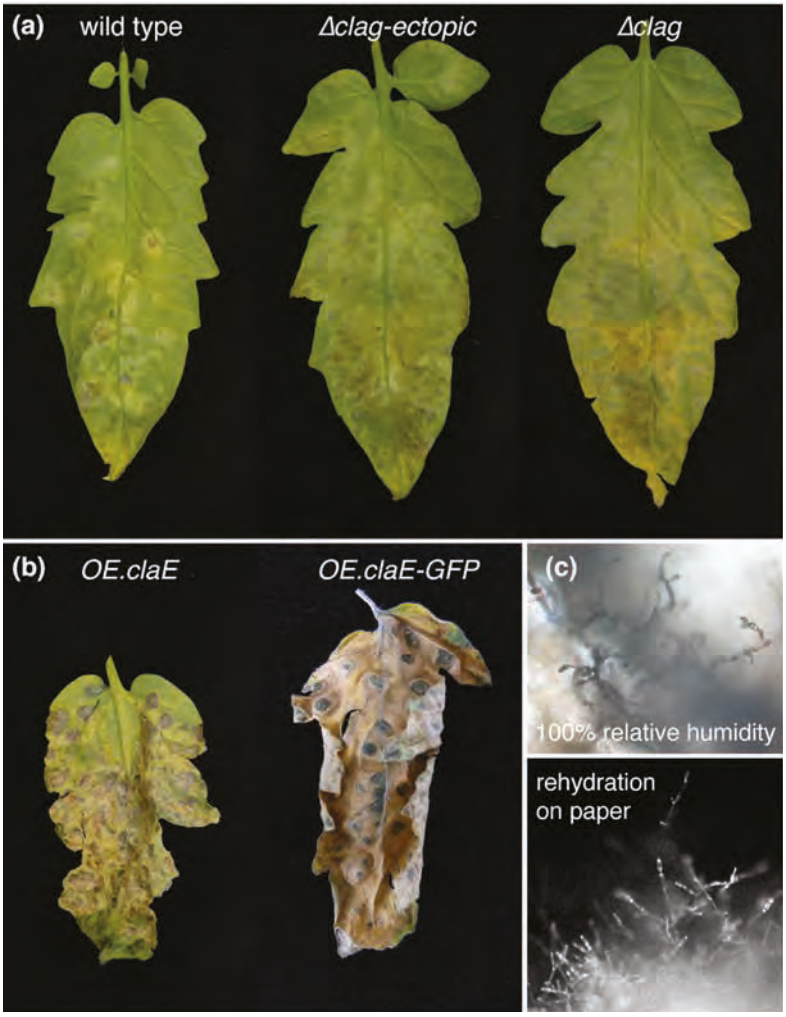


Figure 2. Effect of cladofulvin production on virulence and biotrophy of *Cladosporium fulvum*. Leaves of the susceptible Heinz tomato cultivar were inoculated with conidial suspensions of wild type and transformants of *C. fulvum*. Symptoms were monitored daily and representative pictures were taken at 16 days post-inoculation. **(a)** Leaves inoculated with wild type, ectopic insertion transformant and the Δ*clag* deletion mutant (not producing cladofulvin), respectively. **(b)** Leaf inoculated with the *OE.claE* and *OE.claE::GFP* transformants that over-express ClaE, the predicted local regulator of the cladofulvin gene cluster, specifically during host colonization. The Δ*clag* deletion mutants show typical leaf mould symptoms with conidiation on the leaf surface whereas the *OE.claE* and *OE.claE::GFP* transformants induce necrosis, but does not produce conidia. **(c)** 5 days after increasing relative humidity to 100% at 21 dpi in the compartment where plant were infected, and 72 h after rehydration of infected necrotic leaves on a wet tissue paper, the *OE.claE::GFP* transformant differentiated GFP fluorescent conidia.

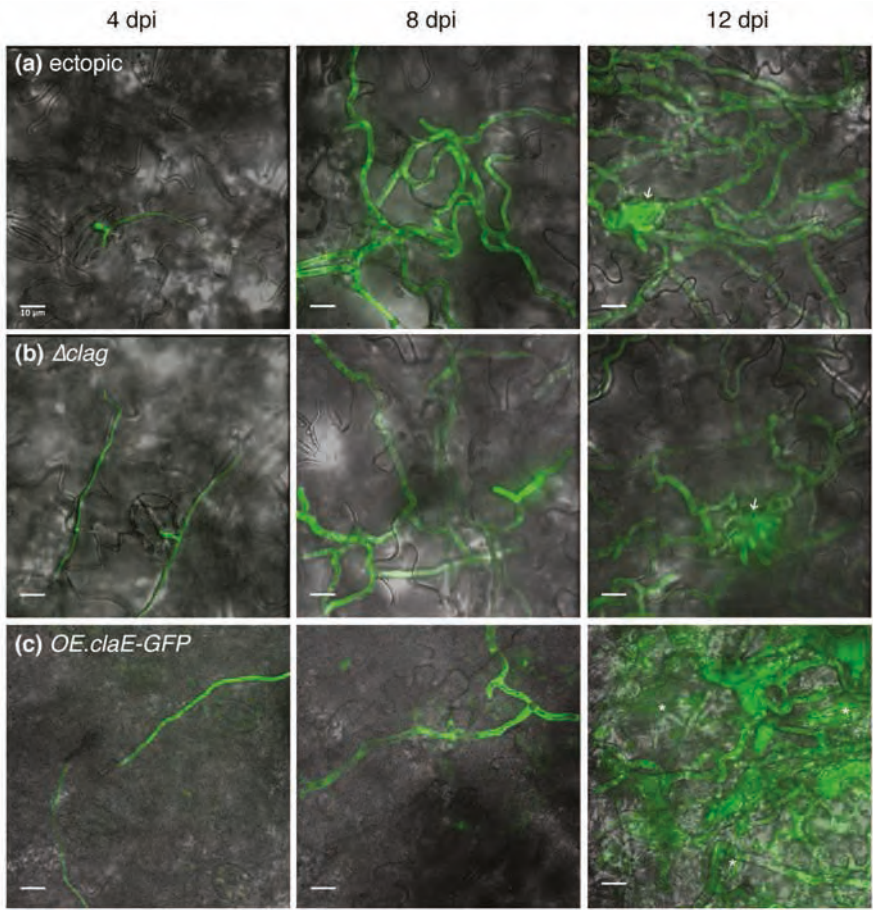


Figure 3. Microscopic examination of the infection process of *Cladosporium fulvum* mutants. Transformed *C. fulvum* strains express the Green Fluorescent Protein (eGFP) constitutively. Fluorescence was observed at 4, 8 and 12 days post-inoculation (dpi) of tomato leaves using a confocal microscope equipped with a spinning disc. Stromata in sub-stomatal chambers are indicated with arrows. **(a)** The ectopic insertion transformant showed typical stages of infection with penetration at 4 dpi, colonization at 8 dpi and start of conidiation at 12 dpi. **(b)** The Δ*clag* deletion mutant that does not produce cladofulvin showed similar infection to that of the ectopic insertion transformant control. **(c)** The cladofulvin over-producing *OE.claE::GFP* transformant showed normal penetration and colonization, however at 12 dpi, hyphae kept growing and no formation of stroma and conidia was observed. Asterisks indicate stomata with no visible stroma. Strong auto-fluorescence from plant cells was observed because of necrosis.

5.3.4 Cladofulvin plays a role in fitness and resistance to environmental stresses

When PDA plates were inoculated with an equal number of conidia of wild type, ectopic insertion transformants or Δ*clag* deletion mutants, and incubated for seven days, we observed about 50% reduction in the number of conidia produced by the Δ*clag* deletion mutants compared to the control strains (Fig. 5a). This may be a consequence of the significantly lower germination rate of the Δ*clag* deletion mutants (1.3-fold difference; Fig. 5b), which suggests that cladofulvin plays

a role in the fitness of *C. fulvum*, although this lower viability of mutant conidia did not alter their virulence.

Given that the cladofulvin gene cluster exhibits a strict down-regulation during colonization of tomato leaves and that cladofulvin is not a virulence factor, we hypothesized that the biological function of cladofulvin might be related to protection against abiotic and biotic stresses encountered by conidia outside of the plant. After growing *C. fulvum* for 18 days in still liquid cultures, mycelia had colonized the bottom of the well, but floating orange colonies had also grown on the surface (Fig. 5c). The submerged and floating colonies were separately recovered and both showed conidiation (Fig. 5c). However, HPLC analysis of ethyl acetate extracts revealed a 17-fold difference between the concentration of cladofulvin produced by the green-black submerged mycelium (3.1 ± 1.1 µg.mg^{-1} fungal biomass) and the floating orange colonies (51 ± 1.4 µg.mg^{-1} fungal biomass), suggesting that cladofulvin is mainly produced during conidiation at the water–air interface.

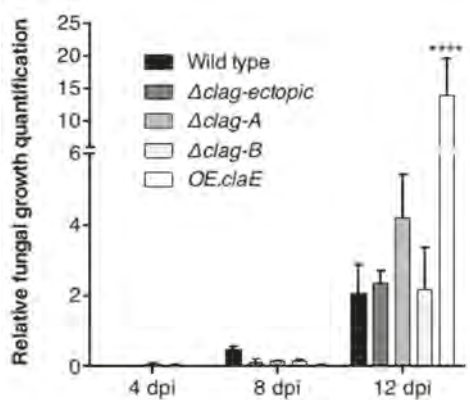


Figure 4. Quantification of fungal growth during infection of tomato by *Cladosporium fulvum* mutants. Total RNA was extracted at 4, 8 and 12 days post-inoculation (dpi). Fungal growth was estimated by RT-qrtPCR for Δ*clag* deletion mutants and *OE.claE* transformant. The expression of the fungal *actin* gene was normalised against the expression of the plant *RUBISCO* gene using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). The wild type and ectopic insertion transformants are shown as controls. Bars represent the mean and standard deviation of three biological replicates. A 2-way ANOVA with *post-hoc* Fisher's LSD multiple comparisons test was performed. Only significant differences are indicated (**** p-value < 0.0001).

Fungal conidia are protected from UV light through the synthesis of pigments. Indeed, phenolic compounds such as anthraquinones and melanins are photoprotective, strongly absorbing ultraviolet and visible light (Nguyen *et al.*, 2013). In order to measure the photoprotective effect of cladofulvin, the Δ*clag* mutant and control strains were irradiated for 180 seconds at 302 nm. The mutant conidia were clearly more sensitive to UV light, with significantly fewer germlings produced after 48 h compared to the controls (4.2-fold difference; Fig. 5b). This result suggests a role in UV protection for cladofulvin.

High and low temperatures are commonly encountered environmental stresses. To assess a protective role of cladofulvin against temperature stress, conidia from Δ*clag* deletion mutants, wild type and ectopic insertion transformant were subjected to three cycles of freezing (–20°C) and thawing. Again, mutant conidia were more sensitive with significantly fewer germlings produced after 48 hours compared to the controls (4.4-fold difference; Fig. 5b). To assess the effect of temperature on cladofulvin production, *C. fulvum* wild type was incubated for 12 days in the dark

at 22°C, by which time the fungus had started to conidiate. Plates were then incubated at 10°C, 22°C or 37°C for 72 h. HPLC examination of ethyl acetate extracts showed that relative to colonies incubated at the optimal growth temperature for *C. fulvum* (22°C), cladofulvin production was highly induced at 10°C, whilst its production was significantly repressed at 37°C (Fig. 5d). Taken together with the poor survival of Δ*clag* deletion mutants during freeze-thaw cycles, cladofulvin likely plays a role in protecting conidia of *C. fulvum* from low temperatures.

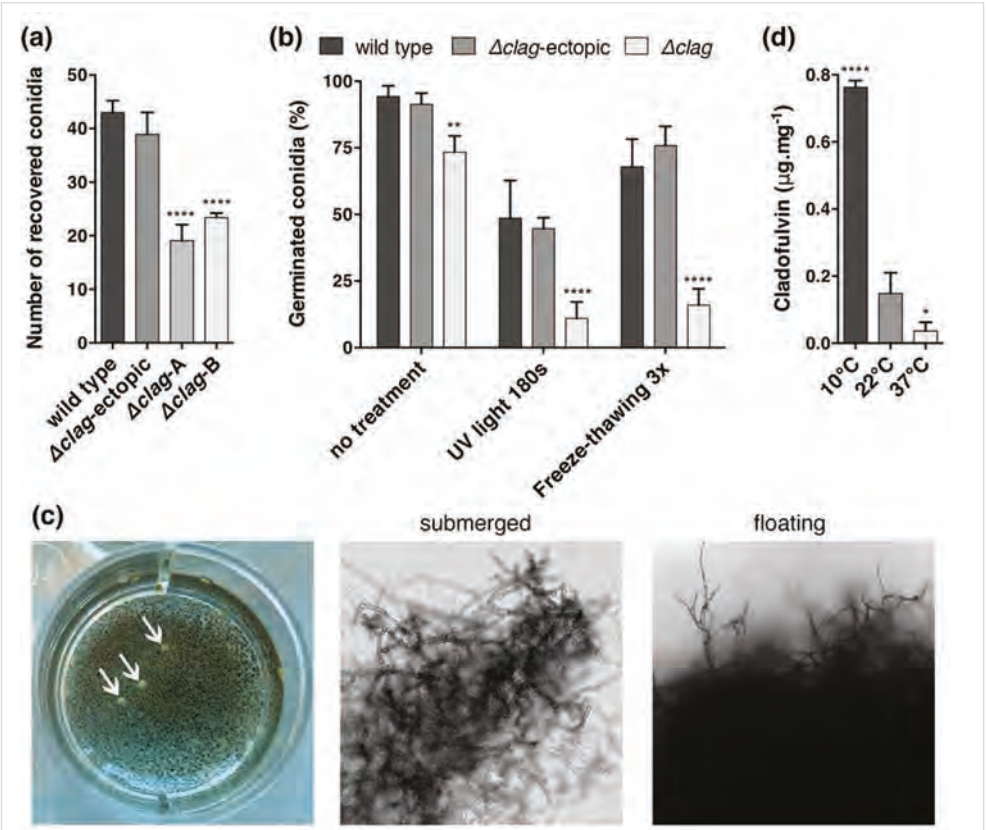


Figure 5. Characterization of the biological function of cladofulvin. (a) Conidiation rate of *Cladosporium fulvum* wild type, Δ*clag* deletion mutants and ectopic transformant was determined. **(b)** The percentage of germination was determined for 100 conidia of each strain 48 h after performing either no treatment, incubation under UV light (302 nm) for 180s, or three cycles of freeze-thawing. **(c)** *Cladosporium fulvum* grown in still liquid culture colonized the bottom of the well to form a dark green mycelium, but orange floating colonies (a few are indicated by arrows) also developed. Both submerged and floating colonies differentiated conidia. The shape of submerged conidia is rounder, which could be due to an anaerobic stress. The orange colour of floating colonies is likely due to the high production of cladofulvin. **(d)** Liquid chromatography quantification of cladofulvin produced by *C. fulvum* wild type after incubation for 72 h at 10, 22 or 37°C. Ethyl acetate extracts were analysed by HPLC and the concentration of cladofulvin was determined using the response factor (RF) of pure cladofulvin as reference. For (b) and (d), an ordinary one-way ANOVA with a *post-hoc* Dunnett's multiple comparisons test was performed to compare to wild type and 22°C conditions, respectively. For (c), a two-way ANOVA with a *post-hoc* Dunnett's multiple comparisons test was performed to compare to the wild-type condition for each treatment (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.0001).

5.4 Discussion

5.4.1 Down-regulation of SM gene clusters is a mechanism associated with biotrophic growth

Despite the potential of the biotrophic tomato pathogen *C. fulvum* to produce several SMs, cladofulvin is, so far, the only SM that has been isolated from this fungus (Agosti *et al.*, 1962; Collemare *et al.*, 2014; chapter 3). When the fungus colonizes tomato leaves, the majority of SM gene clusters are either silent or so lowly expressed that their relevance to pathogenesis was doubted (Collemare *et al.*, 2014). Previous transcriptional profiling indicated that *claG*, the core *PKS* gene that is responsible for cladofulvin production, is only expressed during early and late stages of infection but not during colonisation of the mesophyll tissue (Collemare *et al.*, 2014). It was hypothesized that repression of cladofulvin production was required to allow biotrophic growth of *C. fulvum* in the apoplast of tomato leaves (Collemare *et al.*, 2014). The cladofulvin gene cluster represented a unique opportunity to experimentally address this hypothesis. Selective induction of cladofulvin production inside tomato leaves resulted in accelerated accumulation of fungal biomass, likely causing desiccation of infected leaves. *C. fulvum* could not complete its infection cycle because leaf desiccation prevented conidiation under conditions of normal relative humidity. Thus, repression of cladofulvin production during tomato infection is a prerequisite to sustain the biotrophic growth of *C. fulvum*. This mechanism might be induced by *C. fulvum* sensing saccharose, the prevailing disaccharide present in the apoplast of tomato (Joosten *et al.*, 1990), because we previously found that saccharose strongly represses cladofulvin production (chapter 3).

In contrast to *C. fulvum*, genome analyses of other biotrophic fungi suggested that loss of SM biosynthetic pathways, among other genomic features, is associated with this lifestyle (Collemare & Lebrun, 2011). Extensive loss of biosynthetic pathways in fungal biotrophs such as *M. larici-populina*, *Puccinia* species and *T. melanosporum* might have contributed to their evolution towards obligate biotrophy and symbiosis. Down-regulation of SM genes as demonstrated here in *C. fulvum* is a mechanism that would allow a pathogenic fungus to become a facultative biotroph and, at the same time, to retain potentially useful biosynthetic genes for its life outside the host without violating the requirements for biotrophic parasitism.

5.4.2 Induction of the cladofulvin gene cluster compromises biotrophic growth of C. *fulvum* but increases its growth rate *in planta*.

The *OE.claE* transformant produced large amounts of cladofulvin, especially between 8 and 12 dpi. This is concomitant with drastic proliferation of *C. fulvum* hyphae and desiccation of tomato leaves. These observations are unlikely the direct result of cladofulvin potency because infiltration of pure cladofulvin into tomato leaves did not trigger any visible necrotic response, while the known phytotoxin dothistromin caused clearly visible necrosis (Collemare *et al.*, 2014).

Moreover, cladofulvin appears to be not secreted because cladofulvin has never been detected in apoplastic fluids. We envisage that leaf desiccation might be due to uncontrolled fungal growth that might have been stimulated by cladofulvin if colonizing hyphae would be better protected against the harsh conditions prevailing in the apoplast, including possible protection against reactive oxygen species (ROS) produced by tomato. Controlled and balanced fungal growth might be of great importance for fungal biotrophs and symbionts, preventing them from becoming a burden to their host. For example, deletion mutants in the grass symbiont *Epichloë festucae* of components of the NADPH oxidase complex (NoxA, NoxR and RacA; Tanaka *et al.*, 2006; Takemoto *et al.*, 2006; Tanaka *et al.*, 2008), of the stress-activated kinase SakA (Eaton *et al.*, 2010) and siderophore synthetase SidF (Johnson, 2008), all exhibited unrestricted growth, dramatically increasing the fungal biomass and breaking down mutualism, as shown by the altered development of infected plants. Further investigations are needed to confirm these hypotheses.

5.4.3 Cladofulvin is an *ex-planta* fitness enhancer and not a virulence factor for *C. fulvum*.

We demonstrated that cladofulvin production *in planta* is strictly controlled, with down-regulation of *claG* required to sustain *C. fulvum* biotrophic colonization of tomato leaves. However, *claG* is expressed during the early and late stages of the *C. fulvum* infection cycle, suggesting that cladofulvin might play an important role for growth of *C. fulvum* outside its host. In addition, we showed that both cladofulvin and *claG* are dispensable for virulence, but absence of cladofulvin appears to alter *C. fulvum* fitness. Thus, the biological function of cladofulvin is likely related to the *ex planta* survival of *C. fulvum*. No biological function nor activity against selected species of plants, bacteria, oomycetes and fungi, including *D. pulvinata* a mycoparasite of *C. fulvum*, could be determined so far (Collemare *et al.*, 2014; chapter 2), suggesting that cladofulvin is not involved in competition with other micro-organisms on the leaf surface or during saprotrophic growth. Recently, we showed that cladofulvin is extremely toxic towards animal tumour cell-lines (chapter 4), and it may therefore provide protection to *C. fulvum* conidia against pests (insects or fungivorous nematodes; Rohlfs *et al.*, 2007). However, this is unlikely because Colorado potato beetles (*Leptinotarsa decemlineata*) and cabbage loopers (*Trichoplusia ni*) were indifferent to *C. fulvum* conidia when presented with leaves inoculated with a high density of *C. fulvum* conidia. In a previous study, we suggested that cladofulvin production is linked to stress responses (chapter 3). In the present study, we did demonstrate that the germination of Δ*clag* conidia is impaired, and these conidia are more sensitive to UV irradiation and cycles of freezing and thawing. In addition, a cold treatment induced the production of cladofulvin in the wild type. Altogether, our results suggest that cladofulvin is involved in survival outside its host plant, which also explains why this SM is produced by all natural *C. fulvum* isolates tested so far. If the hypothesis that cladofulvin provided protection to fungal hyphae inside tomato leaves is correct, it is likely that this pigment is involved in protection against additional stress responses, such as ROS discussed above.

Altogether, we have provided the first experimental proof that repression of a fungal SM during tomato infection by *C. fulvum* is necessary for the biotrophic lifestyle of this fungus. This is the first demonstration of a mechanism that associates reduction of fungal chemical diversity with biotrophy. Studying the impact of SM gene loss on biotrophy is difficult considering that ancestral gene clusters are not known. However, *C. fulvum* appeared to be a good model to also study this alternative mechanism due to the numerous silent gene clusters present in its genome. The next challenge will be to activate *in planta* SM gene clusters that have been silent under all conditions tested so far (chapter 3).

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References

Agosti G, Birkinshaw JH, Chaplen P. 1962. Studies in the biochemistry of micro-organisms. 112. Anthraquinone pigments of strains of Cladosporium fulvum Cooke. *The Biochemical Journal* **85**: 528-30.

Bergmann S, Schümann J, Scherlach K, Lange C, Brakhage AA, Hertweck C. 2007. Genomics-driven discovery of PKS-NRPS hybrid metabolites from Aspergillus nidulans. *Nature Chemical Biology* **3**: 213-7.

Bölker M, Basse CW, Schirawski J. 2008. Ustilago maydis secondary metabolism-from genomics to biochemistry. *Fungal Genetics and Biology* **45**: S88-93.

Cary JW, Uka V, Han Z, Buyst D, Harris-Coward PY, Ehrlich KC, Wei Q, Bhatnagar D, Dowd PF, Martens SL, *et al.* 2015. An Aspergillus flavus secondary metabolic gene cluster containing a hybrid PKS–NRPS is necessary for synthesis of the 2-pyridones, leporins. *Fungal Genetics and Biology* **81**: 88-97.

Chiang Y, Szewczyk E, Davidson AD, Entwistle R, Keller NP, Wang CCC, Oakley BR. 2010. Characterization of the Aspergillus nidulans monodictyphenone gene cluster. *Applied and Environmental Microbiology* **76**: 2067-74.

Choquer M, Dekkers KL, Chen H, Cao L, Ueng PP, Daub ME, Chung K. 2005. The CTB1 gene encoding a fungal polyketide synthase is required for cercosporin biosynthesis and fungal virulence of Cercospora nicotianae. *Molecular plant-microbe interactions : Molecular Plant-Microbial Interactions* **18**: 468-76.

Collemare J, Lebrun M. 2011. *Fungal Secondary Metabolites: Ancient Toxins and Novel Effectors in Plant-Microbe Interactions*. Wiley-Blackwell.

Collemare J, Billard A, Böhnert HU, Lebrun M. 2008. Biosynthesis of secondary metabolites in the rice blast fungus Magnaporthe grisea: the role of hybrid PKS-NRPS in pathogenicity. *Mycological Research* **112**: 207-15.

Collemare J, Griffiths S, Iida Y, Jashni MK, Battaglia E, Cox RJ, de Wit PJGM. 2014. Secondary Metabolism and Biotrophic Lifestyle in the Tomato Pathogen Cladosporium fulvum. *PloS One* **9**: e85877.

Daub ME, Hangarter RP. 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. *Plant Physiology* ***73***, 855-7.

Demain AL, Fang A. 2000. The natural functions of secondary metabolites. *Advances in Biochemical Engineering/ Biotechnology* **69**: 1-39.

Duplessis S, Cuomo CA, Lin Y, Aerts A, Tisserant E, Veneault-Fourrey C, Joly DL, Hacquard S, Amselem J, Cantarel BL, *et al.* 2011. Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proceedings of the National Academy of Sciences* **108**: 9166-71.

Eaton CJ, Cox MP, Ambrose B, Becker M, Hesse U, Schardl CL, Scott B. 2010. Disruption of signaling in a fungal-grass symbiosis leads to pathogenesis. *Plant Physiology* **153**: 1780-94.

Inglis DO, Binkley J, Skrzypek MS, Arnaud MB, Cerqueira GC, Shah P, Wymore F, Wortman JR, Sherlock G. 2013. Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of Aspergillus nidulans, A. fumigatus, A. niger and A. oryzae. *BMC Microbiology* **13**: 91.

Johnson L. 2008. Iron and siderophores in fungal-host interactions. *Mycological Research* **112**: 170-83.

Joosten MHAJ, Hendrickx LJM, Wit PJGM. 1990. Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of Cladosporium fulvum (syn. Fulvia fulva). *Netherlands Journal of Plant Pathology* **96**: 103-112.

Liao H, Chung K. 2008. Genetic Dissection Defines the Roles of Elsinochrome Phytotoxin for Fungal Pathogenesis and Conidiation of the Citrus Pathogen Elsinoë fawcettii. *Molecular Plant-Microbe Interactions* **21**: 469-479.

Livak KJ, Schmittgen TD. 2002. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-8.

Lousberg RJJC, Salemink CA, Weiss U, Batterham TJ. 1969. Pigments of Elsinoe species. Part II. Structure of elsinochromes A, B, and C. *Journal* of the *Chemical Society*: 1219-1227.

Martin F, Aerts A, Ahrén D, Brun A, Danchin EGJ, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V, *et al.* 2008. The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. *Nature* **452**: 88-92.

Martin F, Kohler A, Murat C, Balestrini R, Coutinho PM, Jaillon O, Montanini B, Morin E, Noel B, Percudani R, *et al.* 2010. Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. *Nature* **464**: 1033-38.

Mehrabi R, Gohari AM, da Silva GF, Steinberg G, Kema GHJ, de Wit PJGM. 2015. Flexible gateway constructs for functional analyses of genes in plant pathogenic fungi. *Fungal Genetics and Biology* **79**: 186-92.

Mesarich CH, Griffiths SA, van der Burgt A, Ökmen B, Beenen HG, Etalo DW, Joosten MHAJ, de Wit PJGM. 2014. Transcriptome Sequencing Uncovers the Avr5 Avirulence Gene of the Tomato Leaf Mold Pathogen Cladosporium fulvum. *Molecular Plant-Microbe Interactions* **27**: 846-57.

Nguyen K, Chollet-Krugler M, Gouault N, Tomasi S. 2013. UV-protectant metabolites from lichens and their symbiotic partners. *Natural Product Reports.* **30**: 1490-508.

Ohm RA, Feau N, Henrissat B, Schoch CL, Horwitz BA, Barry KW, Condon BJ, Copeland AC, Dhillon B, Glaser F, *et al.* 2012. Diverse lifestyles and strategies of plant pathogenesis encoded in the genomes of eighteen Dothideomycetes fungi. *PLoS Pathogens* **8**: e1003037.

Osbourn A. 2010. Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. *Trends in Genetics : TIG* **26**: 449-57.

Spanu P, Abbott JC, Amselem J, Burgis TA, Soanes DM, Stüber K, Ver Loren van Themaat E, Brown JKM, Butcher SA, Gurr SJ, *et al.* 2010. Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* **330**: 1543-6.

Rohlfs M, Albert M, Keller NP, Kempken F. 2007. Secondary chemicals protect mould from fungivory. *Biology Letters* **3**: 523-5.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, *et al.* 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**: 676-82.

Stergiopoulos I, Collemare J, Mehrabi R, de Wit PJGM. 2012. Phytotoxic secondary metabolites and peptides produced by plant pathogenic Dothideomycete fungi. *FEMS Microbiology Reviews* **37**: 67-93.

Takemoto D, Tanaka A, Scott B. 2006. A p67Phox-like regulator is recruited to control hyphal branching in a fungal-grass mutualistic symbiosis. *The Plant Cell* **18**: 2807-21.

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B. 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *The Plant Cell* **18**: 1052-66.

Tanaka A, Takemoto D, Hyon G, Park P, Scott B. 2008. NoxA activation by the small GTPase RacA is required to maintain a mutualistic symbiotic association between Epichloë festucae and perennial ryegrass. *Molecular Microbiology* **68**: 1165-78.

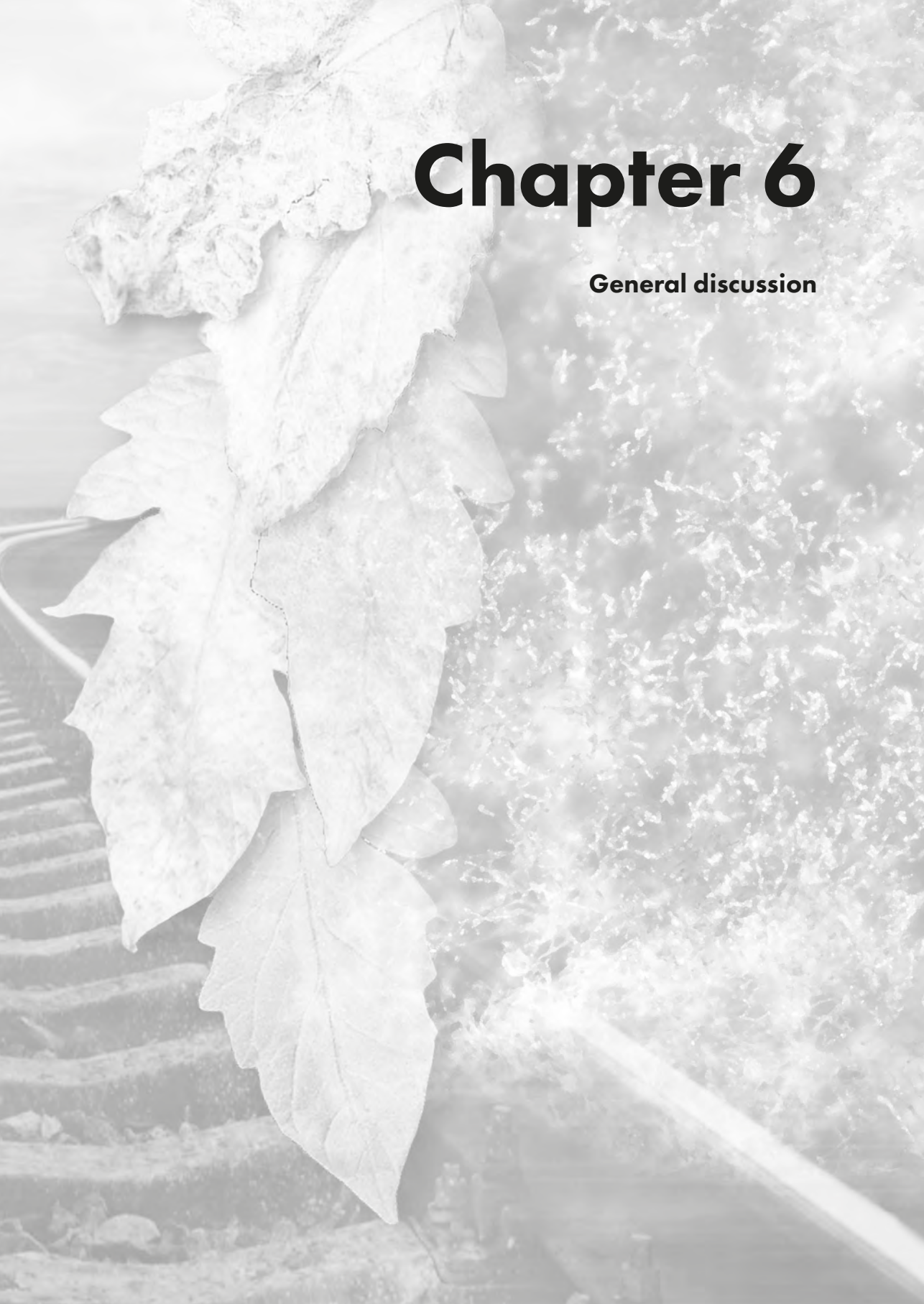
Van den Ackerveken GF, Dunn RM, Cozijnsen AJ, Vossen JP, Van den Broek HW, De Wit PJ. 1994. Nitrogen limitation induces expression of the avirulence gene avr9 in the tomato pathogen Cladosporium fulvum. *Molecular & General Genetics : MGG* **243**: 277-85.

Winterberg B, Uhlmann S, Linne U, Lessing F, Marahiel MA, Eichhorn H, Kahmann R, Schirawski J. 2009. Elucidation of the complete ferrichrome A biosynthetic pathway in Ustilago maydis. *Molecular Microbiology* **75**: 1260-71.

Wolpert TJ, Dunkle LD, Ciuffetti LM. 2002. Host-selective toxins and avirulence determinants: what's in a name? *Annual Review of Phytopathology* **40**: 251-85.

de Wit PJGM, van der Burgt A, Ökmen B, Stergiopoulos I, Abd-Elsalam KA, Aerts AL, Bahkali AH, Beenen HG, Chettri P, Cox MP, *et al.* 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.

Ökmen B, Etalo DW, Joosten MHAJ, Bouwmeester HJ, de Vos RCH, Collemare J, de Wit PJGM. 2013. Detoxification of α-tomatine by Cladosporium fulvum is required for full virulence on tomato. *New Phytologist* **198**: 1203-14.



Chapter 6

General discussion

Cladosporium fulvum is a biotrophic, non-obligate, plant pathogenic fungus that infects tomato. Analysis of the *C. fulvum* genome revealed many genes related to the production of secondary metabolites (SM): 23 predicted core SM genes; 10 PKSs, 10 NPSs, 2 PKS-NRPS hybrids and one DMATS (de Wit *et al.*, 2012). Two were truncated (*Pks4* and *Nps1*) and five were pseudogenized (*Pks9*, *Hps2*, *Nps5*, *Nps7* and *Nps10*), suggesting that *C. fulvum* can produce at least 15 SMs. Expression of these SM genes was determined RT-qrtPCR analysis of the fungus grown *in planta* and *in vitro*. The vast majority were silent or poorly expressed under all conditions tested (chapter 2). Only the core SM gene involved in cladofulvin biosynthesis, *Pks6/claG*, was clearly active *in vitro*. The gene was also active *in planta*, during the pre-penetration and conidiation phases, but not during colonisation of the apoplast. Our aim to increase the chemical diversity of *C. fulvum* using growth conditions and targeted mutagenesis of known global regulators did not yield new compounds (chapter 3). The dimeric anthraquinone, cladofulvin, remained the sole metabolite produced by the fungus. We elucidated the cladofulvin biosynthetic pathway and identified the gene responsible for nataloe-emodin dimerization (chapter 4). We also established the relevance of cladofulvin to the fungus and to its growth on tomato (chapters 3, 4 and 5).

6.1 ClaM will help discovering similar anthraquinone-dimerizing cytochrome P450s

Aflatoxin, sterigmatocystin and dothistromin are structurally related SMs produced by *Aspergillus flavus*, *Aspergillus parasiticus* and *Dothistroma septosporum*, respectively. Comparative genomics approaches have shown that a conserved biosynthetic gene cluster produces these compounds. Changes to the gene complement of an ancestral cluster resulted in diverged biosynthetic pathways and increased SM diversity. Using a similar approach we showed that the cladofulvin gene cluster in *C. fulvum* and the monodictyphenone biosynthetic gene cluster in *Aspergillus nidulans* are highly syntenic (chapter 2). Several genes involved in monodictyphenone biosynthesis were absent in *C. fulvum*, explaining the absence of this compound in SM extracts (Collemare *et al.*, 2014; chapter 2). Conversely, the cladofulvin gene cluster contained two extra genes that were absent in *A. nidulans*, a predicted oxidoreductase, *claN*, and a cytochrome P450, *claM*. Targeted deletion of *claM* resulted in mutants that could not produce cladofulvin, but accumulated emodin and nataloe-emodin instead, which showed that the cytochrome P450 ClaM was required for the dimerization of nataloe-emodin in *C. fulvum* (chapter 4). This was expected, as the dimerization of naphthoquinones and perylenequinones in *Streptomyces* species is also catalyzed by cytochrome P450s (Zhao *et al.*, 2005; Funa *et al.*, 2005). Cytochrome P450s are a large class of enzymes containing members that are distinct in their substrate specificity and the chemical reactions that they catalyze (Isin & Guengerich, 2007). High numbers of these genes in a given genome would make it challenging to functionally characterize them all. The sequence of ClaM should be useful for narrowing the search for similar enzymes in other fungal species. Then, candidate *claM* homologues could be targeted for gene deletion analysis in their

respective organisms. By inspecting the SM profile of each mutant for the loss of dimers, positive hits would be easy to identify. Alterporriols (alterporriol A – W) are a large family of dimeric anthraquinones produced by *Alternaria* species. Remarkably, the marine *Alternaria* species (isolate ZJ-2008003) studied by Zheng *et al* (2012) produces 16 SMs during growth *in vitro*, including four alterporriols (N-R) and seven highly related compounds. One would expect that P450 enzymes are involved in the dimerization of all these compounds. By enlarging the pool of validated cytochrome P450s, conserved domains might be better defined and the determinants of substrate specificity might be approached.

6.1.1 Determining and modifying the substrate specificity of ClaM

The absence of emodin dimers in the SM profile of *C. fulvum* wild type (chapter 4) suggests that emodin, a precursor of nataloe-emodin, is unlikely a substrate of ClaM. This suggests that ClaM is sensitive to very subtle changes in substrate, as emodin and nataloe-emodin are isomers that differ only in the position of a single hydroxyl group. Obtaining the tertiary (3D) structure of ClaM could assist the prediction of amino acid residues involved in substrate docking and could help determine the exact mechanism of nataloe-emodin dimerization. It would also be of great interest to test the ability of ClaM to dimerize other isomers of nataloe-emodin, such as helminthosporin or islandicin, before testing more structurally diverse anthraquinones. This could be tested by developing an *in vitro* assay using purified ClaM. Alternatively, *claM* could be expressed in the natural producers of these compounds to detect any resulting dimers. A realistic proof of concept could be the modification of ClaM to accept emodin as a substrate. The emodin producing *A. oryzae-claF*::*claG*::*claH* transformant would be an excellent choice of host (chapter 4).

6.2 How can the knowledge of fungal SM genes and biosynthetic pathways benefit the production of medicines?

Although the majority of our medicinal SMs are produced by *Actinobacteria*, mostly the *Streptomyces* genus, it should not be forgotten that the first anti-bacterial was derived from a filamentous fungus. Commercially successful SMs derived from fungi include the cholesterol lowering statins and the immunosuppressive cyclosporine. Sequencing efforts have revealed that Ascomycota fungi have a particularly high potential for SM production. Current large scale sequencing efforts, such as the 1000 fungal genome project, will reveal much about the distribution and diversity of SM genes within the fungal kingdom. In addition to the discovery of SMs with useful activities, the study of biosynthetic genes and pathways are very relevant to the chemicals industry.

6.2.1 Replacing synthetic organic chemistry with biocatalysis and metabolic pathways

Complex multi-step chemical synthesis can suffer from poor efficiency, particularly when intermediates require purification after each reaction step. Industrial scale organic chemistry can involve large volumes of hazardous reagents that react under high temperatures and pressures. It is now accepted that biological solutions are crucial for bringing sustainability to the chemical industry (Murphy, 2011). Biocatalysis concerns the production of chemicals by purified enzymes, crude cell-free extracts, or inside living organisms. Replacing purely synthetic chemistry with biocatalytic steps, or if possible, a total biosynthetic pathway can yield considerable advantages. Enzymes may be stereo- (chiral), regio- (positional) and chemo- (functional group specific) selective. This selectivity reduces or negates the need for protecting groups, minimizes side reactions, and creates fewer environmental problems than chemical synthesis. Enzymes also catalyze reactions under mild operating conditions and thus require far less energy. Full biosynthesis is the production of desirable compounds *via* complete metabolic pathways, thereby negating the need for synthetic and semi-synthetic chemistry steps altogether.

Cephalexin is an anti-infective that was traditionally semi-synthesised by DSM (The Netherlands) using penicillin G obtained by fermenting *Penicillium chrysogenum*. By modifying penicillin G pathway and adding two genes, DSM engineered a total biosynthetic approach that replaced 13 chemical steps. This halved the cost of production by reducing energy and materials by 65% (DSM, investor relations). BASF (Germany) replaced a six-step chemical process for the production of vitamin B2 by a single fermentation method using the fungus *Ashbya gossypii*, thereby improving their environmental credentials and reducing their production costs by 40% (Frazzetto, 2003). Another example is the genetic engineering of *Streptomyces peucetius* to produce the anti-cancer SM daunorubicin with epirubicin, a commercial derivative that was traditionally semi-synthesised by a low efficiency process (Madduri *et al.*, 1998). Reducing operating costs and complying with environmental legislation are both cornerstones of the new bio-based economy that allow profit maximization.

6.3 The relevance of SMs to the lifestyle of *C. fulvum*

The classification of *C. fulvum* as a biotroph or hemi-biotroph is contentious and often contradictory. Some report that this fungus is indeed a biotroph because it causes little overt damage to infected tomato leaves during the first 10 days of infection, when extensive colonization of the mesophyll cells occurs (Wit, 1977; Oliver *et al.*, 2000). Sometimes infected tomato leaves can be perfectly healthy in appearance, despite being densely colonised and bearing many conidiophores on the abaxial side. However, severe infections of tomato by this fungus have been described that resulted in wilting, partial defoliation or even death of the host (see refs within Thomma *et al.*, (2005)). For this reason the fungus has also been called a hemi-biotroph.

This damage is assumed to be linked to asphyxiation or desiccation caused by the formation of too many stromata and emergence of too many conidiophores from stomata (see refs within Thomma *et al.*, (2005)). When any necrosis or desiccation is observed, it is indeed confined to the later stages of infection, after conidiation. The disease phenotype is consistently symptomless during the pre-conidiation phases of growth. It is reasonable to assume that infections which result in obvious damage to tomato might be determined by external conditions, such as temperature fluctuations, water availability, or a fall in relative humidity. Tolerance to this form of stress would likely be impaired by dysfunctional stomata that cannot close due to the presence of emerging fungal conidiophores.

Hemi-biotrophs, such as *Elsino*ë *spp.* and *Cercospora spp.*, and necrotrophs, such as *Alternaria alternata*, *Botrytis cinerea* and *Cochliobolus spp.* are fungi that damage host tissue during colonization of their respective hosts and before conidiation. Hemi-biotrophs initially grow as biotrophs before becoming more aggressive later on, whereas necrotrophs begin colonization with the death of host cells. Carbohydrate-active enzymes (CAZymes) such as cell-wall degrading enzymes (CWDEs) are also secreted by hemi-biotrophs and necrotrophs to damage plant cells and liberate nutrients, whereas biotrophic fungi tend to have far fewer (Zhao *et al.*, 2013). The perylenequinones are a family of photosensitizing SMs produced by *Elsinoë spp.* and *Cercospora spp.* during the respective infections (Daub & Hangarter, 1983; Liao & Chung, 2008). Upon exposure to sunlight, perylenequinones generate reactive oxygen species (ROS) that damage host cells and release nutrients.

In contrast, *C. fulvum* feeds in a subtle and intimate manner. Carbon acquisition is thought to occur at the physical interface between the invading hyphae and the extracellular matrix surrounding host mesophyll cells (Wit, 1977; Thomma *et al.*, 2005). The fungus is thought to convert plant-derived saccharose into glucose and fructose using an invertase, followed by uptake of the monosaccharides (Joosten *et al.*, 1990). SMs and CAZymes are not synonymous with such biotrophic feeding strategies. The genome of *C. fulvum* indeed carries a large number of genes encoding predicted CAZymes, SM biosynthetic enzymes and proteases (de Wit *et al.*, 2012). Many are pseudogenes that cannot yield functional enzymes (de Wit *et al.*, 2012; van der Burgt *et al.*, 2013). RNA-seq and expressed sequence tag (EST) data showed that the majority of the predicted-functional CAZyme and SM genes are silent or poorly expressed during growth on tomato (de Wit *et al.*, 2012). From the remaining core SM gene catalogue, only *PKS6*/*claG* and *NPS9*, were active (Collemare *et al.*, 2014; chapter 2). Crucially, these genes were active during the early and late phases of *in planta* growth, but not during the biotrophic phase. Indeed, cladofulvin was not produced by *C. fulvum in vitro* with saccharose as the carbon source (chapter 3), suggesting that the repression of cladofulvin biosynthetic genes *in planta* might be triggered by plant-derived saccharose. As the cladofulvin non-producer *C. fulvum* Δ*clag* mutants produced normal disease symptoms on tomato, cladofulvin is not a pathogenicity factor. Altogether, SMs do not feature prominently in the *C. fulvum* tomato interaction, which appears to be a hallmark of biotrophic fungi.

6.4 Cladofulvin biosynthesis prevented biotrophic growth

By linking the expression of the pathway-specific activator of cladofulvin biosynthesis, *claE*, to the expression of the early acting effector gene *Avr9*, we induced cladofulvin biosynthesis during the biotrophic phase of infection (chapter 5). Growth of the *OE.claE* transformant was stronger than the wild type fungus and infected leaves rapidly became desiccated. Given that tomato leaves do not become necrotic when infiltrated with pure cladofulvin (chapter 2), we hypothesised that the accelerated growth of *OE.claE* caused the observed disease symptoms and that tomato leaves cannot sustain the burden of too much fungal growth. The link between a high concentration of cladofulvin and increased production of fungal biomass is difficult to explain. Cladofulvin could protect hyphae against ROS species or other plant defence mechanisms. Faster growth could destabilise biotrophy by increasing pressure on the host tissue for resources. More extensive colonisation might prevent the host tissue from functioning normally. Given that *C. fulvum* secreted many proteases, chitinases, and effectors proteins to establish biotrophy, accelerated fungal growth might not give sufficient time for these proteins to accumulate and act. Plant cells might die as part of a hypersensitive response. The observation that a *C. fulvum* strain that overproduces cladofulvin during biotrophic growth triggered early desiccation of tomato leaves and was not able to differentiate conidia unless relative humidity was artificially increased, suggests that *C. fulvum* is a real biotroph that needs a living host to complete its infection cycle (chapter 4).

6.4.1 Cladofulvin protects conidia against abiotic stresses

Integration of all results from chapter 3 already suggested that cladofulvin production might be linked to stress responses. Accordingly, although the virulence of *C. fulvum* Δ*clag* was not compromised by the absence of cladofulvin, a number of *in vitro* experiments showed that this strain was impaired in a number of ways (chapter 5). Mutant conidia took longer to germinate, and when exposed to freezing temperatures, many more conidia than wild type were not viable anymore. Indeed, this phenotype fits with the stimulation of cladofulvin production in the cold. Consistent with the use of anthraquinone-based pigments for photoprotection in other organisms (Nguyen *et al.*, 2013), Δ*clag* conidia were also more sensitive to UV radiation. Additional stresses would provide useful information about the natural role of cladofulvin, including susceptibility to oxidative or osmotic stress, fungicides or hyperparasites such as *Dicyma pulvinata*. Indeed, we found that cladofulvin exhibits high toxicity against animal cells (chapter 4). Although cladofulvin was not effective against *D. pulvinata* (chapter 2) and at deterring incidental insect grazer (cabbage looper and Colorado potato beetle, chapter 5), future tests should include fungivores.

6.5 Why are so many SM genes still encoded in the *C. fulvum* genome?

The comparison of the *C. fulvum* and *D. septosporum* genomes revealed that *C. fulvum* is bloated by repetitive elements, pseudogenes, and many genes that are predicted-functional but not expressed during parasitism (de Wit *et al.*, 2012; van der Burgt *et al.*, 2013). It is reasonable to believe that such a high load of repetitive elements contributes to a strong selection pressure. Why have the silent SM genes not been lost? It remains possible that these SM genes are indeed active but produce SMs at sub-detectable concentrations. Single and multiple gene deletions followed by phenotypic screening is the only way to properly assess the role of these genes to the life of fungus. Pathogenicity assays using tomato as a host would determine their relevance to parasitism. As *C. fulvum* is a capable saprophyte, it is possible that the majority of SM genes are invoked during growth of the fungus *ex planta.* Little is currently known about the lifestyle of *C. fulvum* outside its interaction with tomato. SM genes might be induced only by very specific stimuli, such as the presence of other organisms (Seto *et al.*, 2005; Chagas *et al.*, 2013; Bertrand *et al.*, 2014; Netzker *et al.*, 2015). Such knowledge would be useful for designing strategies to activate the remaining silent gene clusters. Future work could include the co-cultivation of *C. fulvum* and other microorganisms that the fungus might naturally encounter. These could include endophytes and foliage-dwellers of tomato, free-living soil microbes, or a mycoparasitic fungivore that predates *C. fulvum* such as *D. pulvinata* (Tirilly *et al.*, 1983). It would be interesting to sequence the genomes of additional *C. fulvum* isolates to know whether or not the sequenced strain is representative. Comparative genomics would reveal much about the evolutionary trajectory of this fungus. Comparing gene loss and pseudogenization across a natural population would help determine which genes are under positive selection.

6.6 Concluding remarks

Starting with genomic data, we functionally interrogated the secondary metabolome of *Cladosporium fulvum*. As part of this effort we elucidated the biosynthetic route to cladofulvin production, a dimeric anthraquinone that is structurally related to several families of chemotherapy drugs and anti-infectives. During this work an enzyme capable of dimerizing anthraquinone monomers was identified, a type of cytochrome P450 that was expected but had not yet been found. To achieve our aims a multidisciplinary approach was pursued, combining many powerful *in silico*, biomolecular and analytical techniques such as comparative genomics, phylogenetics, transcriptional profiling, targeted mutagenesis, heterologous gene expression, mass-directed liquid chromatography and nuclear magnetic resonance (NMR) spectroscopy. Heterologous expression of biosynthetic gene clusters in *Aspergillus oryzae* M-2-3 was a particularly effective method that yielded data swiftly. This system is especially suited to obtaining the products of silent genes or purely *in silico* genetic data.

From food to pharmacy, the mastery of microbes, enzymes, and metabolic pathways is essential for the relentless transition towards a sustainable bio-based economy. For this reason the vast genetic potential held by fungal collections and centres of research, such as the CBS-KNAW Fungal Biodiversity Centre, Utrecht (www.cbs.knaw.nl) and the Fungal Genetics Stock Center, USA, have much to offer corporations invested in diverse biotechnological solutions.

References

Bertrand S, Bohni N, Schnee S, Schumpp O, Gindro K, Wolfender J. 2014. Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. *Biotechnology Advances* **32**: 1180-204.

Chagas FO, Dias LG, Pupo MT. 2013. A mixed culture of endophytic fungi increases production of antifungal polyketides. *Journal of Chemical Ecology* **39**: 1335-42.

Collemare J, Griffiths S, Iida Y, Jashni MK, Battaglia E, Cox RJ, de Wit PJGM. 2014. Secondary Metabolism and Biotrophic Lifestyle in the Tomato Pathogen Cladosporium fulvum. *PloS One* **9**: e85877.

Daub ME, Hangarter RP. 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. *Plant Physiology 73*: 855-7.

Frazzetto G. 2003. White biotechnology. *EMBO reports* **4**: 835-7.

Funa N, Funabashi M, Ohnishi Y, Horinouchi S. 2005. Biosynthesis of hexahydroxyperylenequinone melanin via oxidative aryl coupling by cytochrome P-450 in Streptomyces griseus. *Journal of Bacteriology* **187**: 8149-55.

Isin EM, Guengerich FP. 2007. Complex reactions catalyzed by cytochrome P450 enzymes. *Biochimica et Biophysica Acta* **1770**: 314-29.

Joosten MHAJ, Hendrickx LJM, Wit PJGM. 1990. Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of Cladosporium fulvum (syn. Fulvia fulva). *Netherlands Journal of Plant Pathology* **96**: 103-112.

Liao H, Chung K. 2008. Cellular toxicity of elsinochrome phytotoxins produced by the pathogenic fungus, Elsinoë fawcettii causing citrus scab. *New Phytologist* **177**: 239-50.

Madduri K, Kennedy J, Rivola G, Inventi-Solari A, Filippini S, Zanuso G, Colombo AL, Gewain KM, Occi JL, MacNeil DJ, *et al.* 1998. Production of the antitumor drug epirubicin (4'-epidoxorubicin) and its precursor by a genetically engineered strain of Streptomyces peucetius. *Nature Biotechnology* **16**: 69-74.

Murphy AC. 2011. Metabolic engineering is key to a sustainable chemical industry. *Natural Product Reports* **28**: 1406-25.

Netzker T, Fischer J, Weber J, Mattern DJ, König CC, Valiante V, Schroeckh V, Brakhage AA. 2015. Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Frontiers in Microbiology* **6**: 299.

Nguyen K, Chollet-Krugler M, Gouault N, Tomasi S. 2013. UV-protectant metabolites from lichens and their symbiotic partners. *Natural Product Reports* **30**: 1490-508.

Oliver RP, Henricot B, Segers G. 2000. *Cladosporium fulvum, Cause of Leaf Mould of Tomato*. Springer Netherlands. 65-91.

Seto Y, Kogami Y, Shimanuki T, Takahashi K, Matsuura H, Yoshihara T. 2005. Production of phleichrome by Cladosporium phlei as stimulated by diketopiperadines of Epichloe typhina. *Bioscience, Biotechnology, and Biochemistry* **69**: 1515-9.

Tirilly Y, Kloosterman J, Sipma G, Bosch den JK. 1983. A fungitoxic sesquiterpene from Hansfordia pulvinata. *Phytochemistry* **22**: 2082-2083.

Thomma BPHJ, van Esse HP, Crous PW, Wit PJGM. 2005. Cladosporium fulvum (syn. Passalora fulva), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Molecular Plant Pathology* **6**: 379-93.

Wit PJGM. 1977. A light and scanning-electron microscopic study of infection of tomato plants by virulent and avirulent races of Cladosporium fulvum. *Netherlands Journal of Plant Pathology* **83**: 109-122.

Zhao B, Guengerich FP, Bellamine A, Lamb DC, Izumikawa M, Lei L, Podust LM, Sundaramoorthy M, Kalaitzis JA, Reddy LM, *et al.* 2005. Binding of two flaviolin substrate molecules, oxidative coupling, and crystal structure of Streptomyces coelicolor A3(2) cytochrome P450 158A2. *Journal of Biological Chemistry* **280**: 11599-607.

Zhao Z, Liu H, Wang C, Xu J. 2013. Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics* **14**: 274.

de Wit PJGM, van der Burgt A, Ökmen B, Stergiopoulos I, Abd-Elsalam KA, Aerts AL, Bahkali AH, Beenen HG, Chettri P, Cox MP, *et al.* 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.

van der Burgt A, Jashni MK, Bahkali AH, de Wit PJGM. 2013. Pseudogenization in pathogenic fungi with different host plants and lifestyles might reflect their evolutionary past. *Molecular Plant Pathology* **15**: 133-44.

Summary

Secondary metabolites (SMs) are biologically active organic compounds that are biosynthesised by many plants and microbes. Many SMs that affect the growth, behaviour or survival of other organsism have been re-purposed for use as medicinal drugs, agricultural biocides and animal growth promoters. The majority of our anti-infective and anti-cancer drugs are currently derived from *Streptomyces*, bacteria that are free living, filamentous, and ubiquitous in terrestrial habitats. Genome sequencing and mature *in silico* approaches to genome mining has revealed that filamentous fungi contain very large numbers of genes related to SM production. Yet these genes are typically silent under laboratory conditions. There are now many tools and strategies available to activate or clone silent SM genes. This thesis details our efforts to apply various methods to define and then manipulate SM genes in *Cladosporium fulvum*, a biotrophic pathogenic fungus of tomato containing many silent SM genes and gene clusters.

In **chapter 1,** the relevance of SMs to medicine and agriculture is considered. Filamentous fungi are presented as untapped sources of potential useful SMs, as their genomes are often rich in SM biosynthetic genes that are silent under most conditions. Methods to activate these silent genes and increase the chemical diversity of fungi are detailed. These include the deletion or over-expression of genes encoding regulatory proteins, the use of chemical inhibitors, and the manipulation of growth conditions. Heterologous expression of silent SM genes in a production host is also discussed as a tool for bypassing host regulatory mechanisms altogether. *C. fulvum* is introduced as an organism that has been intensively studied as a biotrophic plant pathogen. Genomic analysis showed that this fungus has twenty-three core SM genes, a large catalogue composed of 10 polyketide synthases (PKSs), 10 non-ribosomal peptide synthases (NPS), one PKS-NPS hybrid and one dimethylallyl tryptophan synthase (DMATS). Transcriptional profiling showed that the majority was silent during growth on tomato and *in vitro*. Cladofulvin is introduced as the sole detectable SM produced by *C. fulvum* during growth *in vitro*. This presented an opportunity to apply the aforementioned strategies to induce these silent genes and obtain new compounds. The importance of cladofulvin and structurally related anthraquinones are briefly discussed as potential medicines. The value of the cladofulvin biosynthetic gene cluster is also emphasised as a potential source of novel biosynthetic enzymes.

In **chapter 2** the SM gene catalogue identified during the analysis of the *C. fulvum* genome was analysed in further detail. Each locus containing a core SM gene was inspected for other biosynthetic genes linked to SM production, such as those encoding decorating enzymes and regulators. Products of these SM genes or gene clusters were speculated, based on their similarity to those characterized in other fungi. Six gene clusters were located in the genome of *C. fulvum* that are conserved in other fungal species. Remarkably, two predicted functional gene clusters were linked to the production of elsinochrome (*PKS1*) and cercosporin (*PKS7*), toxic perylenequinones that generate reactive oxygen species (ROS). We profiled the expression of core SM genes during the growth of *C. fulvum* under several *in vitro* conditions. Expression of each core SM gene was measured by RT-qrtPCR

and the resulting SM profile was determined by LC-MS and NMR analyses. Confirming previous findings, the majority of SM genes remained silent and only cladofulvin was detected. During growth on tomato only two core genes, *PKS6* and *NPS9*, were clearly expressed, but both were significantly down-regulated during colonization of the mesophyll tissue of tomato leaves. We confirmed that cladofulvin does not cause necrosis on solanaceous plants when infiltrated into their leaves. In contrast to other biotrophic fungi that have a reduced SM production capacity, our studies of *C. fulvum* suggest that down-regulation of SM biosynthetic pathways might represent another mechanism associated with a biotrophic lifestyle.

In **chapter 3** our efforts to activate cryptic pathways in *C. fulvum* are described, with the aim of discovering new compounds. Many Ascomycete-specific global regulators of SM production and morphological development in other fungi were identified in *C. fulvum*. We investigated three intensively studied regulators, VeA, LaeA and HdaA. Deleting or over-expressing the genes encoding these regulators in *C. fulvum* yielded no new detectable SMs. Cladofulvin biosynthesis was strongly affected by each regulator; HdaA is an activator while VeA and LaeA are repressors of cladofulvin production. Attempts were made to stimulate SM production in the mutants and wild type strains by growing them on different carbon sources, but only cladofulvin biosynthesis was affected. Interestingly, cladofulvin production was stimulated by carbon limitation and strongly repressed in the presence of saccharose. Similar to observations made in other fungi, the deletion of *VeA* or *LaeA* did not affect viability, but maturation and conidiation were affected. Sporulation was not overtly affected by the loss of *HdaA*, but Δ*hdaa* deletion mutants did not produce cladofulvin. This suggests that cladofulvin production is not required for asexual reproduction. The main finding of this chapter is that global regulator manipulation can not considered to be a universal tool to discover new fungal natural products.

In **chapter 4**, anthraquinones and closely related compounds such as anthrones, anthracyclines and xanthones are considered. Emodin is perhaps the most well characterised anthraquinone that is produced by many fungi and plants. Once synonymous only with constipation, this former laxative has since been investigated for its useful anti-cancer, anti-diabetic, anti-infective and anti-inflammatory properties. Cladofulvin is a homodimeric anthraquinone composed of nataloe-emodin joined in a remarkably asymmetrical configuration. Dimeric anthraquinones and xanthones are also bioactive, most commonly tested for anti-infective and anti-cancer activities. Despite the ubiquity and medicinal qualities of anthraquinones and related compounds, very few of their biosynthetic pathways are known. No enzymes capable of dimerizing anthraquinones had yet been identified. In this chapter we demonstrated that cladofulvin was very cytotoxic towards human cancer cell-lines, crucially, up-to 300-fold more than its monomeric precursor nataloe-emodin against certain cell-lines. This became an added incentive to elucidate the cladofulvin pathway and identify the enyzme responsible for dimerizing nataloe-emodin. We confirmed earlier predictions that *PKS6*/*claG* is the core gene which starts cladofulvin biosythesis. Deletion of *claG* abolished cladofulvin production and no related metabolites were observed. A route to cladofulvin biosynthesis was proposed, guided by the work performed on the monodictyphenone biosynthetic pathway in *Aspergillus nidulans*.

We predicted early acting cladofulvin genes and cloned them for heterologous expression in *A. oryzae* strain M-2-3. Using this approach we were able to confirm the first five genes in cladofulvin biosynthesis, *claBCFGH*, which yielded a reduced and dehydrated form of emodin. This is the point at which the pathways to cladofulvin and monodictyphenone production diverge. It was speculated that this emodin-related intermediate might be converted into nataloe-emodin by *claK* and/or *claN*. Finally, it was confirmed that the final step in the cladofulvin pathway is encoded by *claM*. Targeted deletion of *claM* yielded a mutant that accumulated nataloe-emodin and emodin but no cladofulvin. We discuss how the sequence of *claM* and ClaM will accelerate the discovery of functionally similar genes and enzymes, providing a template to engineer enzymes capable of forming novel dimers from existing monomers.

In **chapter 5** the natural role of cladofulvin was considered. This SM is consistently produced by *C. fulvum* and global regulator mutants *in vitro*. The respective biosynthetic genes appear most active during early and late stages of infection of tomato, but are down-regulated during biotrophic growth phase (chapter 2). The Δ*clag* mutants (chapter 3) were not overtly different from the wild type during growth *in vitro*. We inoculated tomato plants with this mutant in order to test whether or not cladofulvin was required for normal infection. Simultaneously, we inoculated a *C. fulvum* transformant carrying an extra copy of the cladofulvin pathway-specific relulator, *OE.claE*, fused to the promoter region of the *Avr9* effector gene. The strain was expected to produce cladofulvin once the fungal hyphae penetrate host stomata and begin to colonise the apoplastic space. In this way, we aimed to test the effect of cladofulvin over-production on disease symptom development. The growth of each strain on tomato plants was monitored by RT-qrtPCR at 4, 8 and 12 days post inoculation (dpi). At each time point the infections were inspected microscopically to detect any phenotypic abnormalities. We report that the loss of *claG* did not result an abnormal infection. Both wild type and Δ*claG* mutants sporulated without causing necrosis or dessication of host leaves. In distinct contrast, brown spots appeared on leaves infected by the *OE.claE* transformant between 8 – 12 dpi. This was accompanied by much stronger fungal growth and significant accumulation of cladofulvin. The leaves became desiccated and brittle before the fungus conidiated. Possible reasons for this phenotype are discussed. A small suite of *in vitro* experiments was performed on the Δ*clag* and wild type strains in order to test the role of cladofulvin in survival. Consistent with the absence of a photoprotective pigment, Δ*clag* spores were considerably more sensitive to ultraviolet (UV) radiation. Suggesting a role in protection against low temperatures, Δ*clag* spores were less resistant to repeated cycles of freezing and thawing. Cladofulvin biosynthesis was stimulated and repressed by cold and heat shocking mature *C. fulvum* colonies, respectively. Altogether, these results suggested that cladofulvin confers resistance to abiotic stress.

In **chapter 6** the results obtained in this thesis are discussed in a broader context. Particularly, the discovery of the cytochrome P450 that is involved in dimerization of anthraquinones might enable discovery of homologous genes encoding enzymes with different specificities. Combining bioinformatic and functional analyses should prove to be a powerful strategy for discovering compounds with new biological activies, or enzymes relevant to metabolic engineering.

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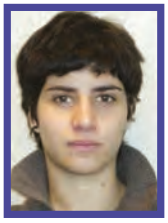

Curriculum vitae

Scott Andrew Griffiths was born on October 25, 1982, in Swansea, Wales, United Kingdom. For the next 18 years he lived, studied and worked in the neighbouring town of Llanelli (St Elli's Parish). After obtaining pre-university qualifications in Biology, Business Studies, Computer Science and Economics, he left Llanelli to obtain a B.Sc. in Medical Genetics from Swansea University. During this degree he became versed in many aspects of biochemistry, microbiology and molecular genetics. His final year laboratory project involved the regulation of secondary metabolism in *Streptomyces coelicolor*, a genus of filamentous bacteria that produce antibiotics and other essential medicines. Experience with this bacterium led directly to a Ph.D. from the Wellington group at Warwick University, a research lab with a strong track record in the study of microbial ecology, gene transfer and population dynamics in terrestrial systems. He explored the complex dynamics resulting from parasite-host interactions using bacteriophage ϕC31 and *S. coelicolor* as a model system. He was then employed at Warwick in order to create and analyse regulatory mutants of *S. coelicolor* perturbed in phosphate metabolism. This work was part of a large systems biology consortium tasked with mastering the switch from primary to secondary metabolism in *Streptomyces*. He left Warwick with an enduring affinity for systems biology, microbial metabolism and a love of multidisciplinary research. In January 2012 he joined the group of Professor Pierre de Wit in Wageningen University and began to study *Cladosporium fulvum*, a fungal plant pathogen of tomato. His appointment coincided with the fruition of a very special project: obtaining and analysing the *C. fulvum* genome. Many silent gene clusters were identified that related to secondary metabolite production, transforming this fungus from a pathogen into a potential source of useful compounds. Together with colleagues and collaborators, he employed many parallel strategies to mine this fungus for useful compounds and biosynthetic enzymes. His project considered the natural relevance of secondary metabolism to the fungus itself, as a pathogen and free living saprophyte. He also contributed to several other projects that resulted in peer-reviewed publications. He hopes to apply his experiences to the field of metabolic engineering and synthetic biology.

Publications

First authorships

Griffiths, S., Saccomanno, B., de Wit, P.J.G.M., Collemare, J. (2015) Regulation of secondary metabolite production in the fungal tomato pathogen *Cladosporium fulvum*. Fungal Genetics and Biology *84*, 52-61.

Griffiths, S., Mesarich, C., Saccomanno, B., Vaisberg, A., Lazarus, C., de Wit, P.J.G.M., Cox, R., Collemare, J. Elucidation of the cladofulvin biosynthetic pathway in *Cladosporium fulvum* reveals a cytochrome P450 monooxygenase required for dimerization of a monomeric anthraquinone. Under review, PNAS.

Griffiths, S., Mesarich, C., Saccomanno, B., Overdijk, E., Cox, R., de Wit, P.J.G.M., Collemare, J. Activation of a repressed secondary metabolite gene cluster in the fungus *Cladosporium fulvum* prevents biotrophic parasitism of tomato leaves. In preparation, Molecular Plant Pathology.

Griffiths, S., Cox, R., Saccomanno, B., de Wit, P.J.G.M., Collemare, J. Divergence of perylenequinone and melanin biosynthetic gene clusters. In preparation, PloS Genetics.

Co-authorships

Mesarich, C.H., **Griffiths, S.A.**, van der Burgt, A., Ökmen, B., Beenen, H.G., Etalo, D.W., Joosten, M.H.A.J., and de Wit, P.J.G.M. (2014). Transcriptome Sequencing Uncovers the Avr5 Avirulence Gene of the Tomato Leaf Mold Pathogen *Cladosporium fulvum*. Molecular Plant-Microbe Interactions *27*, 846-57.

Ökmen, B., Collemare, J., **Griffiths, S**., van der Burgt, A., Cox, R., and de Wit, P.J.G.M. (2014). Functional analysis of the conserved transcriptional regulator CfWor1 in *Cladosporium fulvum* reveals diverse roles in the virulence of plant pathogenic fungi. Molecular Microbiology *92*, 10-27.

Collemare, J., **Griffiths, S**., Iida, Y., Jashni, M.K., Battaglia, E., Cox, R.J., and de Wit, P.J.G.M. (2014). Secondary Metabolism and Biotrophic Lifestyle in the Tomato Pathogen *Cladosporium fulvum*. Plos One *9*, e85877.

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., and Bradshaw, R. E. 2012. (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.

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., and Bradshaw, R.E. (2012). Dothistromin genes at multiple separate loci are regulated by AflR. Fungal Genetics and Biology. *51*, 12-20.

Mesarich, C.H., **Griffiths, S**., Ökmen, B., Wang, C., Rovenich, H., Collemare, J., Beenen, H.G., Deng, C.H., Mihajlovski, A. and de Wit, P.J.G.M. Novel effectors of *Cladosporium fulvum* identified by transcriptome sequencing elicit cell death in wild species of tomato. In preparation, Molecular Plant-Microbe Interactions or Molecular Plant Pathology.

Collemare, J., **Griffiths, S.**, Cox, R., and de Wit, P.J.G.M. The evolutionary history of the 1,8-dihydroxynaphthalene melanin biosynthetic pathways in fungi reveals a conserved role for the Ayg1 hydrolase. In preparation, PloS One.

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