Gene cluster conservation identifies melanin and perylenequinone biosynthesis pathways in multiple plant pathogenic fungi

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Summary

Perylenequinones are a family of structurally related polyketide fungal toxins with nearly universal toxicity. These photosensitizing compounds absorb light energy which enables them to generate reactive oxygen species that damage host cells. This potent mechanism serves as an effective weapon for plant pathogens in disease or niche establishment. The sugar beet pathogen Cercospora beticola secretes the perylenequinone cercosporin during infection. We have shown recently that the cercosporin toxin biosynthesis (CTB) gene cluster is present in several other phytopathogenic fungi, prompting the search for biosynthetic gene clusters (BGCs) of structurally similar perylenequinones in other fungi. Here, we report the identification of the elsinochrome and phleichrome BGCs of Elsinoë fawcettii and Cladosporium phlei, respectively, based on gene cluster conservation with the CTB and hypocrellin BGCs. Furthermore, we show that previously reported BGCs for elsinochrome and phleichrome are involved in melanin production. Phylogenetic analysis of the corresponding melanin polyketide synthases (PKSs) and alignment of melanin BGCs revealed high conservation between the established and newly identified C. beticola, E. fawcettii and C. phlei melanin BGCs. Mutagenesis of the identified perylenequinone and melanin PKSs in C. beticola and E. fawcettii coupled with mass spectrometric metabolite analyses confirmed their roles in toxin and melanin production.

Introduction

Fungi produce a plethora of secondary metabolites (SMs) that serve to enhance competitiveness in nature. Functional diversity of these compounds is high, including reported roles in virulence, biotic and abiotic stress protection and as metal transport agents (Williams et al., 1989; Demain and Fang, 2000; Rohlfis and Churchill, 2011; Stergiopoulos et al., 2012; Keller, 2015). For example, in some occasions SMs are involved in symbiotic relationships where microbial symbionts provide an antibiotic armoury against secondary infection to the symbiotically colonized plant in return for nutrients and protection (Rohlfis and Churchill, 2011; Stringlis et al., 2018). A major class of fungal SMs are the polyketides (Keller et al., 2005). For the biosynthesis of fungal aromatic polyketides, nonreducing polyketide synthases (NR-PKSs) play a central role as mediators of the first biosynthetic step (Keller et al., 2005; Crawford and Townsend, 2010; Brakhage, 2013; Gallo et al., 2013). Such PKS genes contain multiple domains that work conjointly, of which the β-ketoacyl synthase (Guedes and Eriksson, 2007), acyltransferase (AT) and acyl-carrier protein (ACP) domain are indispensable (Kroken et al., 2003; Keller et al., 2005; Crawford and Townsend, 2010; Gallo et al., 2013). By using the domains iteratively, a PKS generates a metabolite backbone which can be modified by other enzymes to yield the final metabolite (Keller et al., 2005; Bohnert et al., 2010; Crawford and Townsend, 2010).
The genes encoding these decorating enzymes are often found in direct proximity to the PKS gene to form a biosynthetic gene cluster (BGC) pathway (Keller and Hohn, 1997; Keller et al., 2005). In addition, BGCs often contain regulatory elements and transporters involved in shuttling the final secondary metabolite from the cell, and in the case of toxic metabolites, genes encoding autoresistance proteins (Keller, 2015; de Jonge et al., 2018).

A well-studied BGC is the cercosporin toxin biosynthesis (CTB) pathway. The CTB gene cluster was originally identified in Cercospora nicotianae, causal agent of leaf spot disease on tobacco, but is present in almost all Cercospora species (Assante et al., 1977; Choquer et al., 2005; de Jonge et al., 2018). The ubiquitous presence of the CTB gene cluster in the genus is likely explained by its role as a virulence facilitator (Callahan et al., 1999; Daub and Ehrenshaft, 2000; Choquer et al., 2005). Recently, de Jonge and colleagues (2018) used comparative genomics to show that the CTB gene cluster can also be found in several plant pathogenic fungal species outside the Cercospora genus, likely as a result of horizontal transfer of the entire CTB gene cluster (Bohnert et al., 2010; Crawford and Townsend, 2010; de Jonge et al., 2018). The majority of assessed species from the genus Colletotrichum, a large genus of crop and/or ornamental plant pathogens (Perfect et al., 1999), were shown to harbour full- to partial-length CTB gene clusters, of which the post-harvest apple fruit pathogen Co. fioriniae was shown to produce cercosporin (de Jonge et al., 2018). The core gene of the Cercospora CTB gene cluster is the NR-PKS gene CTB1 (Newman and Townsend, 2016), which is flanked by nine genes that encode decorating enzymes (CTB2, CTB3, CTB5, CTB6, CTB7, CTB9, CTB10, CTB11 and CTB12) (de Jonge et al., 2018). Besides those 10 genes essential for toxin formation, the cluster also encodes a zinc finger transcription factor (CTB8) for regulation of cluster gene expression, and two major facilitator superfamily (MFS) transporters; CTB4 that is necessary for toxin secretion and the cercosporin facilitator protein (CFP) involved in toxin autoresistance (Chen et al., 2007; Choquer et al., 2007; de Jonge et al., 2018). Upon activation, all CTB pathway enzymes work in a well-orchestrated manner to synthesise the metabolite from backbone formation to secretion of the toxin into the environment while providing the fungus with protection against cercosporin.

Cercosporin is a member of the perylenequinone family that, upon photo-activation, displays almost universal toxicity to a wide spectrum of organisms (Zhenjun and Lown, 1990; Daub and Ehrenshaft, 2000; Ahonsi et al., 2005; Guedes and Eriksson, 2007; Daub et al., 2013). Exposure to visible and near-UV light energetically activates perylenequinones to an excited triplet state that reacts with oxygen to form reactive oxygen species (Foote, 1976; Guedes and Eriksson, 2007). This photodynamic activity can be attributed to the 3,10-dihydroxy-4,9-perylenequinone chromophore backbone that is shared among perylenequinones (Hudson et al., 1997). Structural differences between perylenequinone family members are mostly due to divergent side chains attached to the mutual backbone structure (Daub et al., 2005) (Fig. 1). For example, the methylenedioxy bridge is a unique feature of cercosporin and is absent in other perylenequinones such as hypocrellin, elsinochrome and pleiochrome (Fig. 1) (Weiss et al., 1987; de Jonge et al., 2018).

Previous studies have implicated PKS genes in the production of perylenequinones in other plant pathogenic fungi. For example, transcriptome analysis and a CRISPR-Cas9 gene editing approach in the bamboo pathogen Shiraiia bambusicola gave compelling evidence that SbaPKS encodes the PKS orchestrating hypocrellin biosynthesis (Zhao et al., 2016; Deng et al., 2017). Similarly, targeted disruption of EIPKS1 in the citrus scab pathogen Elsinoë fawcettii abrogated elsinochrome production (Liao and Chung, 2008). Likewise, Cppks1 was found to be responsible for PKS activity for pleiochrome production in the purple eyespot pathogen Cladosporium phelei based on homology to EIPKS1 (So et al., 2015). Our previous phylogenetic analysis of PKSs revealed that EIPKS1 clusters with melanin biosynthesis PKSs and is relatively distant from CTB1 (de Jonge et al., 2018), corroborating previous findings by Liao and Chung (2008). Correspondingly, annotated EIPKS1 flanking genes showed high similarity to established melanin biosynthesis genes, whereas SbaPKS flanking genes for hypocrellin biosynthesis resembled those found in the CTB cluster. Melanin is an integral component of the cell wall that has proposed functions in protection from environmental factors, appressorial penetration of host plants and pathogenesis (Wheeler and Bell, 1988; Langfelder et al., 2003; Liu and Nizet, 2009). In Mycosphaerella fijensis, research suggested that secreted fungal DHN-melanin acts as a virulence factor through the photogeneration of singlet molecular oxygen in a similar manner to the perylenequinones (Beltrán-García et al., 2014). DHN-melanin biosynthesis has been characterized extensively in many fungi, including Magnaporthe oryzae, Colletotrichum lagenarium, Alternaria alternata, Botrytis cinerea, Verticillium dahliae and Aspergillus spp. In the rice blast fungus M. oryzae for instance, DHN-melanin production is known to be mediated by a four-gene cluster which is regulated in hyphae by the transcription factor Pig1 (Supporting Information Fig. 1) (Thompson et al., 2000; Tsuji et al., 2000; Talbot, 2003; Oh et al., 2008). However, fungal DHN-melanin pathways may vary in the biosynthesis of the first common intermediate 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN or T4HN). For example, the PKS ALB1 (for ‘albino’) is
responsible for the first biosynthetic step in *Aspergillus fumigatus*, resulting in the biosynthesis of the heptaketide naphthopyrone YWA1, which is subsequently hydrolyzed by Ayg1 to produce T4HN (Fujii et al., 2004; Pihet et al., 2009). Two alternative routes can be found in the necrotrophic grey mould fungus *B. cinerea*. In this case, the

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PKSs Bcpks12 and Bcpks13 synthesize different precursors for the joint DHN-melanin pathway (Schumacher, 2016). While Bcpks12 produces the pentaketide T4HN directly, Bcpks13 synthesizes the hexaketide 2-acetyl-1,3,6,8-tetrahydroxynaphthalene (AT4HN) that is subsequently converted to yield T4HN (Schumacher, 2016). In either case, the resulting T4HN will serve as substrate for a hydroxynaphthalene (HN) reductase leading to scytalone formation. In the next step, scytalone will be dehydrated by a scytalone dehydratase resulting in the formation of 1,3,8-trihydroxynaphthalene (1,3,8-THN or T3HN). Subsequent reduction by a HN reductase yields vermoleone which is subsequently dehydrated to form 1,8-DHN; an immediate reduction by a HN reductase yields vermelone which serves as substrate for biosynthesis. In the next step, scytalone will be dehydrated by a scytalone dehydratase resulting in the formation of 1,3,8-trihydroxynaphthalene (1,3,8-THN or T3HN). Subsequent reduction by a HN reductase yields vermoleone which is subsequently dehydrated to form 1,8-DHN; an immediate precursor of melanin (Tsai et al., 1999; Thompson et al., 2000; Tsuji et al., 2000; Talbot, 2003).

Here, we set out to prepare draft genome sequences for E. fawcettii and C. phlei to unravel the evolutionary trajectories of both melanin and perylenequinone BGCs. We show that the gene clusters hosting Cppks1 and EIPKS1 have high similarity to established gene clusters involved in DHN-melanin biosynthesis and have only limited similarity to the perylenequinone biosynthesis clusters to which they were previously attributed. Consequently, we also sought to establish the BGCs involved in production of elsinochrome in E. fawcettii and phleochrome in C. phlei using targeted gene replacement of both perylenequinone and melanin PKS genes in E. fawcettii and C. beticola to provide proof for their involvement in toxin and DHN-melanin production.

Results

E. fawcettii and C. phlei genomics

To initiate our investigation on perylenequinone and melanin BGCs of E. fawcettii and C. phlei, we developed draft genome sequences of both species. Nuclear and mitochondrial DNA of E. fawcettii strain CBS 139.25 and C. phlei strain CBS 358.69 were sequenced to approximately 138-fold and 110-fold coverage, respectively, on the Illumina HiSeq 4000 platform (paired-end, 100-bp reads). Reads were de novo assembled by SPAdes yielding draft genome assemblies of 25.3 Mb on 398 scaffolds for E. fawcettii and 31.9 Mb on 794 scaffolds for C. phlei. The, respective, scaffold N50 values and L50 folds for E. fawcettii and C. phlei CLAPHL08786-RA (this study)] from E. fawcettii and Cppks1 (So et al., 2015) [CLAPH08786-RA (this study)] from C. phlei that were previously implicated in perylenequinone biosynthesis did not cluster phylogenetically with the established perylenequinone cercosporin PKSs CbCTB1 and CnCTB1 of C. beticola and C. nicotianae respectively. Instead, EFPS1 and Cppks1 formed a clade with confirmed melanin PKSs, including Bcpks12 and Bcpks13 of the grey mould fungus B. cinerea (Schumacher, 2016), Wdpks1 of the zoopathogenic black yeast Wangiella (Exophiala) dermatisidis (Feng et al., 2001), GIPKS1 of the filamentous fungus Glarea lozoyensis (Zhang et al., 2003), NodPKS1 of an endophytic Nodulisporium strain (Fulton et al., 1999), COGPKS1 of the cucumber anthracnose causal agent Co. lagenarium (Fuji et al., 1999), the predicted C. beticola melanin biosynthesis PKS CbPKS1 (CBET3_09638) and the S. bambusicola melanin PKS SHIR08477. The finding that E. fawcettii ELSFAW09157-RA, C. phlei CLAPHL08786-RA, S. bambusicola SHIR08477 and CbPKS1 reside in a cluster (Fig. 2) with extensive collinearity to established DHN-melanin clusters (Fig. 3) suggests a role in melanin production and hints that EIPKS1 and Cppks1 were previously misannotated as perylenequinone biosynthesis genes (Liao and Chung, 2008; So et al., 2015).

The cercosporin PKSs in C. beticola (CbCTB1), C. nicotianae (CnCTB1) and C. oriniae (EXF84093) form a perylenequinone clade with the previously confirmed hypocrellin PKS (SbaPKS) (Zhao et al., 2016; Deng et al., 2017), ELSFAW08003 from E. fawcettii, CLAPH05460 from C. phlei as well as with the putative elsinochrome PKS in P. nodorum (EAT83782.2) (Chooi et al., 2017), the putative perylenequinone PKS M. oryzae (MGG_00428) and the C. beticola CbCTB1 paralog CBET3_10910 (Fig. 2). As phylogenetic conservation can be an indication of related metabolite production (de Jong et al., 2018), this clustering suggests that PKSs of this clade are involved in biosynthesis of the perylenequinones. Therefore, we suggest renaming ELSFAW08003 to EIETB1 for elsinochrome toxin.
Fig. 2. Phylogeny of PKSs of related Ascomycetes revealing distinct DHN-melanin and perylenequinone clades. Maximum likelihood phylogenetic tree illustrating the phylogenetic relationship of all predicted nonreducing polyketide synthases (PKSs) from the selected species set (Supporting Information Table 1) plus those derived from the set of PKSs used by Collemare and colleagues (2014). The tree was constructed from the aligned full-length β-ketoacyl synthase (Guedes and Eriksson, 2007) domains. The right panel indicates domain architecture of each PKS determined by Pfam domain annotation. Protein accessions are coloured depending on the taxonomic class of the producing species, and the species identifier can be found in the taxa labels. Established biosynthetic end products for a subset of the listed PKSs is indicated by the background colour, highlighting two DHN-melanin sub-groups, naphthoquinones, anthraquinones, perylenequinones, aflatoxin-like compounds and resorcylic acid lactones. [Figure can be viewed at wileyonlinelibrary.com] © 2018 The Authors. *Environmental Microbiology* published by Society for Applied Microbiology and John Wiley & Sons Ltd., *Environmental Microbiology*, 21, 913–927
biosynthesis 1, and C. phlei CLAPHL05460 to CpPTB1 for phlechrome toxin biosynthesis 1.

**Perylenequinone and DHN-melanin biosynthesis gene cluster alignments**

While PKS genes are indispensable for polyketide formation, it is the full complement of genes in a BGC that is responsible for the biosynthesis of the end product. Therefore, synten of the predicted BGCs of orthologous PKS genes was assessed. Using the established C. beticola CTB gene cluster and S. bambusicola hypocrelin gene cluster as references, putative perylenequinone-orthologous toxin clusters secreted by C. phlei and hypocteinin A was shown to be one of the perylenequinones secreted by S. bambusicola. The phylogenetic tree of Ascomycetes used in this study was constructed based on mash protein-level kmer hash overlaps. Alignment plots were prepared with MultiGeneBlast. For all species, the indicated identifiers are transcript IDs and the corresponding sequences can be retrieved from Ensemble Fungi and/or NCBI GenBank. CTB orthologs are coloured relative to the C. beticola CTB cluster genes while DHN-melanin BGC genes are colour coded relative to M. oryzae DHN-melanin. Colour key and annotated functions are explained in the legend next to the alignment plots. E. fawcetii and S. bambusicola genes, unrelated to previously annotated genes in the cercosporin BGC remain in white but are labelled by gene identity. [Color figure can be viewed at wileyonlinelibrary.com]
hypocrellin and the predicted BGCs for elsinochrome and phleichrome (Fig. 3A). Overall, eight genes are shared between the cercosporin, hypocrellin and predicted elsinochrome and phleichrome BGCs (Fig. 3A). When compared to these perylenequinone pathways, the CTB gene cluster encodes two additional proteins; a putative α-keto-glutarate-dependent dioxygenase (CTB9) and a candidate dehydratase (CTB10) that have been shown to be essential for the formation of the methylenedioxy bridge in cercosporin (de Jonge et al., 2018). The predicted C. phlei phleichrome BGC contains all orthologous C. beticola CTB genes except for the above-mentioned CTB9 and CTB10, in agreement with the lack of the methylenedioxy bridge in phleichrome. Likewise, the predicted E. fawcettii elsinochrome BGC lacks CTB9 and CTB10 as well as the cercosporin MFS transporter (CTB4) and the NADPH-dependent oxidoreductase (CTB6). Interestingly, the E. fawcettii BGC contains ELSFAW08009, which only has an ortholog in the hypocrellin gene cluster (SHIR08482) and in no other of the aligned BGCs (Fig. 3A). ELSFAW08009 and SHIR08482 encode a putative salicylate hydroxylase based on sequence similarity to the conserved protein domain family TIGR03219 (E-value 2.98e-18), members of which are salicylate 1-monoxygenases. Besides sharing this gene with the elsinochrome pathway and lacking orthologs to CTB9 and CTB10, the hypocrellin cluster also lacks CTB homologues CTB4, CTB6 and CTB7 compared to the cercosporin pathway (Fig. 3A). The elsinochrome BGC in E. fawcettii contains one additional gene encoding a MFS transporter with predicted association to perylenequinone biosynthesis, and comparably, the hypocrellin BGC in Shiraia sp. contains two additional genes that encode a MFS transporter and a short-chain dehydrogenase respectively. These genes were previously reported as HYP11 and HYP12 (KM434884.1) and were...
differentially expressed in a hypocreellin-producing strain as compared to a nonproducer (Zhao et al., 2016).

Similarly, predicted DHN-melanin clusters of C. beticola, C. phlei, E. fawcettii, S. bambusicola sp. slf14 and Co. floriniae were aligned to the established DHN-melanin cluster of M. oryzae, A. fumigatus, A. alternata, Bipolaris maydis (Cochliobolus heterostrophus) and both alternative clusters of B. cinerea (Fig. 4B). All BGCs share homologous PKS genes, a THN-reductase and a prefoldin-encoding gene. Prefolds are frequently associated with DHN-melanin BGCs, but a functional role in DHN-melanin biosynthesis has not been established to date. Furthermore, the putative melanin clusters of C. beticola, C. phlei, E. fawcettii, S. bambusicola sp. slf14 and Co. floriniae encode a transcription factor with homology to M. oryzae Pig1 and Co. lagenarium CMR1, which are frequently observed in other established melanin clusters (Tsuij et al., 2000).

Targeted replacement and characterization of perylenequinone and melanin PKS genes

The predicted perylenequinone and melanin PKS genes for C. beticola (CbCTB1 and CbPKS1 respectively) and E. fawcettii (EfETB1 and EfpKS1 respectively) were targeted for split marker gene replacement. At least two unique site-directed transformants were assessed for involvement in metabolite production. The wild type and all knockout mutant strains were grown under conditions to induce perylenequinone production. The presence or absence of cercosporin (C. beticola) and elsinochrome (E. fawcettii) in culture extracts was determined via UPLC-MS (Fig. 4A and B). For both fungal species, perylenequinone production was abrogated in the perylenequinone PKS mutants (ΔCbCTB1 and ΔEfETB1 mutants for C. beticola and E. fawcettii respectively) but not in the melanin PKS mutants (Fig. 4A and B). There were no obvious differences in growth rate for either of the C. beticola or E. fawcettii mutants versus the corresponding wild type strains. Additionally, ΔCbPKS1 and ΔEfpKS1 melanin mutants had a pale buff colour as opposed to the dark grey pigmentation observed in wild type and perylenequinone-deficient mutant (ΔCbCTB1 and ΔEfETB1 for C. beticola and E. fawcettii respectively) strains (Fig. 5A and B). The amount of melanin present in the C. beticola and E. fawcettii cultures was determined spectrophotometrically. In both species, the ΔPKS1 mutant had a significantly lower melanin content than either the wild type or ΔCbCTB1/ΔEfETB1 (Fig. 5C).

Discussion

Phylogenetic analysis based on PKS KS domain conservation can help to predict SM structure and gene evolution (Keller et al., 2005; Gallo et al., 2013). In this study, we used KS domain sequence alignments and phylogenetic analysis of selected plant pathogenic fungi to separate PKSs into distinct clades. One of the clades hosted PKSs involved with perylenequinone biosynthesis including CbCTB1, the well-studied C. beticola PKS essential for cercosporin biosynthesis, and the PKS of the hypocreellin pathway in S. bambusicola sp. slf14. We also observed clustering of PKSs involved in DHN-melanin formation such as Bcpks12 and Bcpks13 of B. cinerea and COGPKS1 of Co. lagenarium (Fig. 2). As previously reported (Liao and Chung, 2008), phylogenetic analyses of KS and AT domain sequences indicated a closer relationship of EfpKS1 to melanin PKSs than to perylenequinone PKSs. Furthermore, high similarity of the full length amino acid sequence to the annotated EfpKS1 led So and colleagues (2015) to hypothesize that Cppks1 was involved in phleomycin production. Our KS domain alignment confirms the phylogenetic analysis by Liao and Chung (2008) where EfpKS1 and Cppks1 form a cluster with established DHN melanin biosynthesis PKSs of other Ascomycetes (Fig. 2). Consequently, we used comparisons to well-characterized melanin BGCs in various Ascomycetes to show that PKSs belonging to the DHN-melanin clade are putatively involved with melanin biosynthesis in C. beticola, E. fawcettii, C. phlei and S. bambusicola sp. slf14 (Fig. 3B). Whole-cluster homology of predicted cognate clusters to various well-established DHN-melanin clusters strengthened our hypothesis that CbPKS1, EfpKS1 and Cppks1 are involved with melanin production.

To gain further support, we generated PKS mutants in our candidate melanin biosynthesis PKS genes in C. beticola and E. fawcettii. As predicted, the melanin null mutants ΔEfpKS1 and ΔCbPKS1 displayed pale phenotypes characteristic of previously described melanin-deficient mutant fungal strains (Chumley and Valenty, 1990) (Fig. 5A and B). Additional quantitative determination of melanin in ΔCbPKS1 of C. beticola and ΔEfpKS1 of E. fawcettii indicated reduced melanin content of the culture compared to wild type and ΔCbCTB1 or ΔEfETB1 perylenequinone knockout mutant lines respectively (Fig. 5C and D). Interestingly, the C. beticola ΔCbPKS1 and E. fawcettii ΔEfpKS1 mutants were still able to produce cercosporin and elsinochrome, respectively (Fig. 4A and B), unlike the observation by Liao and Chung (2008) where ΔEfpKS1 mutants in E. fawcettii were reported to be elsinochrome-deficient. Therefore, we suspect that the phenotype they observed may be due to in vitro conditions that were insufficient for perylenequinone induction. Previous functional analysis of the EfpKS1 cluster raised questions when overexpression of predicted cluster genes under different conditions did not correlate with elsinochrome production and

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complementation of the transcription factor-like gene EFtSF1 null mutant (with EFtSF1) was unable to restore elsinochrome production to wild type level (Chung and Liao, 2008). Here, putative ∆EfTSF1 transformants were identified on the basis of elsinochrome deficiency, potentially dismissing any true site-directed mutants that were still able to produce elsinochrome (Chung and Liao, 2008). Therefore, it could be that their elsinochrome-deficient phenotype was evoked by off target T-DNA insertion. The reduction in virulence observed for their ∆EfPKS1 mutant is not surprising as melanin has been reported to be a virulence factor for many filamentous fungi (Wheeler and Bell, 1988; Langfelder et al., 2003; Liu and Nizet, 2009). Besides contribution to fungal virulence, melanin has also been reported to play an important role in protection against environmental stresses. Recently, studies of the causal agent of Septoria tritici blotch on wheat, Zymoseptoria tritici, have indicated a correlation between fungicide resistance and melanization level of the producing fungus which led to the identification of the putative Z. tritici melanin PKS (Lendenmann et al., 2014; Lendenmann et al., 2015). Similarly, CbPKS1 and CBET3_09636, encoding a predicted tetrahydroxynaphthalene (T4HN) reductase (now renamed to Cb4HNR as it is homologous to 4HNR of M. oryzae), that we propose to belong to the melanin BGC have been recently reported to be more highly expressed in fungicide-resistant C. beticola strains compared to fungicide-sensitive strains (Bolton et al., 2016). Consequently, we propose that melanin production in C. beticola is mediated by CbPKS1 which forms T4HN in the first biosynthetic step. Subsequently, T4HN will serve as substrate for Cb4HNR which reduces it to yield scytalone. Taken together, these results strongly suggest that EfpKS1 and Cppks1 are involved in DHN-melanin biosynthesis, in contrast to earlier reports (Liao and Chung, 2008; So et al., 2015).

To identify the legitimate elsinochrome and phleicrome PKS genes in E. fawcettii and C. phlei, respectively, we went back to our KS domain alignment where predicted PKSs CpPTB1 of C. phlei and EfETB1 of E. fawcettii clustered together with established cercosporin biosynthesis PKSs CTB1 in C. beticola and C. nicotianae (Fig. 2). In line with these initial functional predictions, alignments of the corresponding predicted gene clusters display high similarity and gene conservation within each clade (Fig. 3A). Also, structural differences between perylenequinones can be explained by comparing the predicted metabolite clusters on a gene level. For example, cercosporin and phleicrome only differ in the additional methylenedioxy bridge that is found in the cercosporin molecule (Fig. 1). Accordingly, the
predicted phlechrome biosynthesis pathway lacks CTB9 and CTB10 that have been shown to be responsible for methylenedioxy bridge formation (de Jonge et al., 2018). Site-directed gene replacement of EIETB1 in E. fawcettii and CbCTB1 in C. beticola led to the successful generation of perylenequinone mutants that are deficient in toxin production under perylenequinone-inducing conditions (Fig. 4A and B). Since SM production relies on different environmental conditions, not every medium is suitable to activate SM production (Calvo et al., 2002; VanderMolen et al., 2013). For C. beticola, previous research has shown that growth on ‘thin’ PDA plates under natural light stimulates cercosporin production (Frandsen, 1955; Fajola, 1978; Jenns et al., 1989), which was shown here to stimulate elsinochrome production.

In conclusion, we have identified BGCs of structurally related SM compounds based on the phylogenetic relationship of their encompassing PKSs and overall conservation level of the associated cluster genes. By using an established CTB gene cluster as reference, it was possible to identify gene clusters responsible for the synthesis of related perylenequinone compounds in different fungal species. Likewise, we successfully identified clusters associated with DHN-melanin production in C. beticola, E. fawcettii, C. phlei, and S. bambusicola using the same approach and the confirmed DHN-melanin cluster as input. Future research using this methodology will be useful for the identification of other perylenequinones and their corresponding BGCs in other fungi.

Experimental procedures

Elsinoë fawcettii and Cladosporium phlei genome sequencing

For high-quality genomic DNA extraction of Elsinoë fawcettii strain CBS 139.25 and Cladosporium phlei strain CBS 358.69, mycelia was scraped from the surface of PDA agar petri dishes and extracted using the CTAB method (Bolton et al., 2016). Library preparation (500 bp) and subsequent paired-end (PE) sequencing on the Illumina HiSeq 4000 platform was done by BGI Americas (BGI Americas, Cambridge NA). Approximately 34 million high-quality sequence reads with an average length of 100 bp were generated for both samples, representing 134- and 111-fold coverage for E. fawcettii and C. phlei respectively. Draft genomes were assembled using SPAdes (version 3.9.0), with default parameters and k-mers 21, 33, 55, 77 and 99. Prediction of protein-coding gene models was performed ab initio using the previously prepared Cercospora beticola training parameters (de Jonge et al., 2018) in Augustus (version 3.2.1). Protein function and subcellular localization was predicted by Interpro (Finn et al., 2017). Genome sequences and annotations are available under Bioproject PRJNA475685 and permanently linked on figshare under doi https://doi.org/10.6084/m9.figshare.6173834.

Secondary metabolite phylogenetic analyses

Phylogenetic analysis of the type I PKS genes and phylogenetic tree analyses were largely performed as described in de Jonge and colleagues (2018). In short, we used Pfam domain scanning analyses by HMMER3 (Mistry et al., 2013) with hmm profiles for domains PF00109.25 (Beta-ketoacyl synthase, N-terminal domain) and PF02801.21 (Beta-ketoacyl synthase, C-terminal domain) to identify all PKSs in the predicted proteomes of C. beticola (09-40), C. phlei (CBS 358.69), E. fawcettii (CBS 139.25), S. bambusicola (SfI14), P. nodorum (SN15), C. heterostrophus (C5), A. alternata (SRC11R21), A. fumigatus (Af293), B. cinerea (B05.10), Co. fibrillosa (PJ7) and M. oryzae (70-15) that were obtained from NCBI GenBank or Ensembl Fungi. In total we identified 240 proteins across these 11 proteomes. In addition, we added 70 PKSs from Colle-mare and colleagues (2014) and Cppks1 (AFP89389.1) from So and colleagues (2015) to the set of PKSs for further analyses. All 311 PKS proteins were subsequently aligned by Maft (v7.271) using default parameters, after which we extracted the KS domain proportion as previously defined by Pfam scanning. This resulted in an alignment with 311 proteins across 832 positions that was used to prepare a maximum likelihood phylogenetic tree using RAxML (version 8.2.11), incorporating 100 rapid bootstraps and subsequent automatic, thorough ML search. We then selected the subclass of 94 nonreducing PKSs for further analysis, as defined previously by Kroken and colleagues (2003). The final phylogenetic tree and figure was prepared in EvoView (Zhang et al., 2012). In this tree, we collapsed the outgroup clade with 20 members containing PKSs involved with citrinin biosynthesis, as indicated in Fig. 2. Inclusion in the final set of 74 noncollapsed, nonreducing PKSs is indicated in Supporting Information Table 1. All 311 PKS proteins were subsequently aligned by Maft (v7.271) using default parameters, after which we extracted the KS domain proportion as previously defined by Pfam scanning. This resulted in an alignment with 311 proteins across 832 positions that was used to prepare a maximum likelihood phylogenetic tree using RAxML (version 8.2.11), incorporating 100 rapid bootstraps and subsequent automatic, thorough ML search. We then selected the subclass of 94 nonreducing PKSs for further analysis, as defined previously by Kroken and colleagues (2003). The final phylogenetic tree and figure was prepared in EvoView (Zhang et al., 2012). In this tree, we collapsed the outgroup clade with 20 members containing PKSs involved with citrinin biosynthesis, as indicated in Fig. 2. Inclusion in the final set of 74 noncollapsed, nonreducing PKSs is indicated in Supporting Information Table 1.

Secondary metabolite cluster alignment visualization

For comparative analyses of the secondary metabolite clusters across multiple genome sequences we initially identified orthologous protein families across the aforementioned proteomes using orthoFinder (Emms and Kelly, 2015). Subsequently, we used the MultiGeneBlast algorithm (multigeneblast.sourceforge.net), integral part of antiSMASH (Weber et al., 2015), to prepare gene-by-gene cluster alignments across all species and we then re-coloured individual genes within each gene cluster according to the protein family analysis.

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Deletion mutant generation

Site-directed gene replacements of CTB1 and CbPKS1 in C. beticola strain 1–90 and of EITBot1 and EIPKS1 in E. fawcettii strain CBS 139.25 were generated using the split-marker approach as described in Bolton and colleagues (2016). Primers are listed in Supporting Information Table 2. Regardless of phenotype, all putative knock-out mutants were screened for site-directed gene replacement. Successful gene deletion was confirmed by the presence of a PCR product using a forward primer upstream of the 5’ flanking region of the target gene design and hygromycin reverse primer MDB-1145 (Supporting Information Fig. 2). Additionally, absence of an amplicon using target gene-specific primers confirmed deletion of the target gene (Supporting Information Table 2).

Perylenequinone production assay

Mycelial plugs of 5 mm in diameter from PKS mutant and wild-type C. beticola and E. fawcettii strains were grown on thin potato dextrose agar (PDA, Difco™, BD Diagnostic Systems, Sparks) plates (3.0 ml PDA in a 50 mm Petri plate, amended with 150 μg/ml hygromycin B (Roche, Mannheim, Germany; for mutant strains) under a 16 h light-8 h dark cycle at 21°C for 72 h. Mycelial plugs of each E. fawcettii strain were ground with a micro-pestle in 1 ml potato dextrose broth (PDB, Difco™, BD Diagnostic Systems, Sparks), spread onto thin PDA plates and were grown under 24 h light at 28°C for 7 days.

Total mycelial tissue was excised from the agar plate, blended at high speed for 20 s and extracted with ethyl acetate whilst stirring for 5 min in the dark. Single plate extracts were filtered using two layers of miracloth and dried under a stream of nitrogen (21°C) for 24 h. The reddish-brown residues were resuspended in 200 μl methanol. Cercosporin concentration was calculated by measuring absorbance at 255 nm using an Agilent Cary 8454 UV–Visible spectrophotometer (Agilent Technologies, Inc., Santa Clara) and 21, 500 as the molar extinction coefficient (Mialet and Blein, 1995). Extracts were diluted to ~100 pg μl⁻¹ with methanol and centrifuged at 3000 x g for 5 min. At a minimum, duplicate plate extracts were submitted for mass spectrometric analyses of each fungal strain.

Mass spectrometric analyses

Positive mode electrospray ionization settings were optimized for cercosporin by infusing a methanolic cercosporin standard (5 ng/μl) (Sigma; St. Louis) into a Waters (Milford, MA) Acquity triple quadrupole mass spectrometer. The precursor ion, product ions, optimum collision energies and cone voltage were determined by the AutoTune Wizard within the MassLynx 4.1 software (Waters; Milford, MA). Ion transitions used for cercosporin detection were m/z 535 → 415 and m/z 535 → 485 using a cone voltage of 60 and collision energies of 25 and 20 V respectively.

Elsinochrome standard was not available, therefore, an extract from wild type E. fawcettii was infused into the mass spectrometer and fragmentation of ions appearing at m/z 547 (the molecular mass of elsinochromes B & D) were optimized using the AutoTune Wizard within the MassLynx 4.1. Presumptive elsinochrome ion transitions used were m/z 547 → 487 and m/z 547 → 457 using a cone voltage of 60 and collision energies of 20 and 35 V respectively. In some elsinochrome analyses, the mass spectrometer was used as a single sector instrument to collect molecular ions at m/z 547 (elsinochromes B & D), m/z 545 elsinochrome A and m/z 549 (elsinochrome C). For both cercosporin and elsinochrome MS/MS experiments, the desolvation temperature was set at 500°C, and the source temperature was set at 150°C. Cone gas (N2) flow was set at 50 l/h and desolvation gas flow was set at 800 l/h, whereas the collision gas (Ar) flow was 0.16 ml/min.

Cercosporin and elsinochrome (isomers B and D) were analysed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a Waters (Milford, MA) Acquity UPLC and Acquity triple-quadrupole mass spectrometer. Data were acquired, processed and quantified using MassLynx 4.1 with Target Lynx systems. Aliquots of sample extracts (10 μl) were injected onto a 2.1 × 30 mm (1.7 μm) Acquity CSH C18 column protected by a 5 mm CHS guard column (Waters; Milford, MA). Cercosporin and elsinochrome were eluted with a binary gradient consisting of solvent A (0.1% formic acid in pure water) and solvent B (0.1% formic acid in acetonitrile) flowing at 1 ml/min. The gradient program was started at 95% A and transitioned to 25% A over 2 min, 5% A at 2.1 min and held at 5% A until 2.5 min when solvent A was ramped back to 95% A at 3 min. Solvent composition was held constant until the end of the run time at 4 min. The column temperature was 30°C.

Melanin production assay

Three mycelial plugs of 5 mm diameter from each of wild type and mutant C. beticola strains were ground with a micropestle in 1 ml V8 broth (10% (v/v) clarified V8 juice (Campbell’s Soup Co., Camden), 0.5% (w/v) CaCO3) and spread onto single Nylon membranes (Nytran® SuPer-Charge Nylon transfer membrane, Schleicher and Schuell, Keene, USA) overlaying V8 agar (as broth but with 1.5% (w/v) agar (BD, Franklin Lakes, USA)) plates (6 ml in a 50 mm Petri plate). E. fawcettii strains were
grown in the same way as *C. beticola* except for larger plate sizes (15 ml in a 90 mm Petri plate). *C. beticola* was grown under 24 h light at 21°C for 7 days and *E. fawcettii* for 10 days. Total mycelial tissue was excised and weighed before extracting melanin according to Gadd (1982). The tissue was boiled for 5 min in 10 ml distilled water, centrifuged and the pigment extracted from the supernatant by autoclaving with 3 ml of 1 M NaOH (20 min, 120°C). The extract was then acidified to pH 2 with concentrated HCl to precipitate melanin. The precipitate was washed three times with distilled water and dried under a stream of nitrogen (21°C).

Melanin extracts were solubilized in 2 ml of 2 M sodium hydroxide at 50°C. A spectrophotometric assay was used as described by Kauser and colleagues (2003) to measure melanin absorbance at 475 nm with a standard curve of synthetic melanin (Sigma-Aldrich, Milwaukee) from 1 to 100 μg per ml to determine melanin content. The mean melanin content was determined as micrograms of melanin per gram of mycelial tissue for three replicates (individual cultures) and the standard error of the mean calculated. One-way ANOVA was performed using a *P*-value of 0.05 as the significance threshold with a post hoc Tukey HSD test to determine differences between the mean melanin contents of wild type strains and each of the three mutants for *C. beticola* and *E. fawcettii*.

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MKE-179 and -180, MKE-177 and -178, respectively, from left to right. All PCR products were separated alongside the Invitrogen 1 Kb Plus DNA ladder (Thermo Fisher Scientific, Inc., Waltham) as a size reference.

Suppl. Table 1. List of the polyketide synthase (PKS) accession codes used in this study.
Suppl. Table 2. Primers used in this study.
Appendix S1: Supplementary Material.